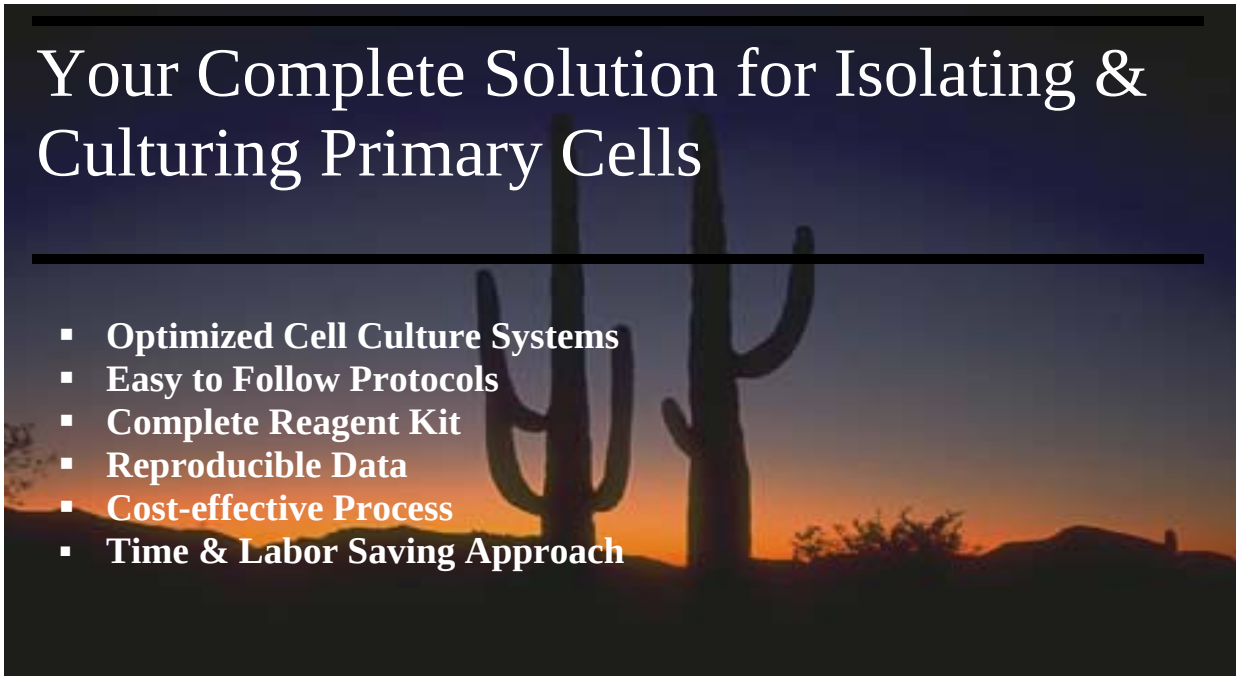
CHI SCIENTIFIC

Handbook of Primary Cell Culture

A Practical Manual to the Labtoratory Standard

❖ Bench-proven Protocols ❖ Consistent Quality ❖ Standard Performance



☎1.800.986.6008 Web www.chiscientific.com

CHI Scientific is committed providing world-class technical support for all categories of products. Our technical service representatives rely on years of laboratory and research experience to assist you with product selection and help you maximize product performance.

U. S. and Canada Customers
Toll Free: (800)986-6008
☎(978)897-5460

Facsimile
Fax: (978)897-5462

Internet
Web www.chiscientific.com
E-mail: help@chiscientific.com

Mail
CHI Scientific, Inc.
Customer Service Department
63 Great Road
Maynard, MA 01754
USA

International Customers
☎(978)897-5461

Facsimile
Fax: (978)897-5462

Internet
Web www.chiscientific.com
E-mail: help@chiscientific.com

Mail
CHI Scientific, Inc.
Customer Service Department
63 Great Road
Maynard, MA 01754
USA

Preface

Dear Researchers,

Welcome to CHI Scientific!
Our foremost goal is to make your experience with primary cell culture both positive and stimulating, so that you can design the most cutting edge experiments that address important biological questions. Using primary cells for experimentation has always been a high priority among the scientific community; however, the difficulties of defining the optimal culture conditions and the process of standardizing protocols have dissuaded even the most ambitious scientists.

We are pleased to bring you the most complete solution to your primary cell culture needs. Our PrimaCell™ systems and the related products are designed to enable you to culture primary cells with ease, dependability and reproducibility. Our efforts have accomplished two important objectives: first, we have streamlined the process of culturing primary cells and second, we have standardized the conditions and reagents that yield similar results regardless of the lab and its personnel.

To accompany our product line we have developed the following Primary Cell Culture Handbook. Currently it is a collection of protocols that can be used to isolate and culture primary cells from various species. The protocols give you a realistic idea of what is involved to isolate your cell type of choice, before you embark on the process. We plan to provide you with future editions of this Handbook that will contain in depth information about each cell type including morphology, behavior, microenvironment, and pathology among others. In addition, we will include several comprehensive chapters that will elaborate on the current issues of primary cell culture, their implementation in basic science and drug discovery, their relevance in investigating physiological systems and novel approaches to their manipulation using siRNA.

We believe that you will enjoy browsing through the Handbook and invite you to return to our website for future editions.

Maria Karasarides, PhD
Chief Scientific Officer

Alfred L. Chi, PhD
Chief Executive Officer

Table of Contents

CHI SCIENTIFIC

Chapter 1 Standardization of Primary Cell Culture Process

Chapter 1: Standardization of Primary Cell Culture Process

1.1 Primary Cell Culture System - PrimaCell™

The need to isolate individual primary cells from whole tissues arises from our desire to understand the contribution of each cell type on the function of whole tissues and organs. Over the last three decades, protocols for primary cell culture have been refined and have now become an essential tool in biomedical research. Intense interest in this field has resulted in numerous published protocols for the isolation of various cell types, their culture conditions, and evaluation of their differentiation state. Thus, primary cells isolated directly from human or animal tissues can be maintained in the differentiated state for a relatively short period (days to weeks) under standard culture conditions. Functionally, differentiated primary cell cultures have a limited life span, and although maintenance of the differentiated properties have been improved by various culture conditions and culture media additives, such as growth factors and extracellular matrix components, cell specific functions usually degrade rapidly and the cultures are not suited for experimentation. The culture systems used in most labs today are based on mechanical and/or enzymatic dis-aggregation of animal tissue into single cells. While most procedures require sacrificing the animals, biopsy specimens or samples from surgically removed material can also be used, however, their use is limited due to sample size and variations in genotype, strain/breed, age and other factors.

Culture conditions and tissue sample handling are two major factors that cause the variations that usually render primary cell culture both frustrating and unsuccessful for most laboratories. The available literature and published protocols are often time consuming and expensive since researchers have to prepare and purchase small batches of specialty products to even attempt the protocols. In addition, the variation between laboratories and individual scientists is a major obstacle that most often leads to difficulty and failure in isolating primary cells from published protocols.

It is widely accepted that data obtained from primary cells is not only desired, but, most relevant when trying to study physiological interactions. While most researchers understand this concept, they are limited to the use of cell lines that contain an undefined set of mutations and chromosomal abnormalities. Nevertheless, the laboratories that have made the leap to primary cell cultures face additional limitations and obstacles when it comes to comparing data within experiments and between laboratories. These difficulties arise due to the variability among the reagents used and the procedures implemented by individual laboratories to isolate and culture primary cells. It is simply impossible to avoid such variability because, until recently, a streamlined approach for standardized primary cell culture was not available.

The end-result from such variability leads to unreliable, non-reproducible results that are very difficult to reconcile and compare between laboratories. Even within the same laboratory variation between different preparations of primary cells is often an issue due to the variability of materials used in the media and serum preparation, forcing researchers to spend hours standardizing protocols. The ultimate solution to many of these problems is the creation of a standardized cell culture system that includes all the reagents and protocols leaving only the origin of the tissue as the major source of variability.

It is with these thoughts that scientists at CHI Scientific have set out to develop the first standardized system to isolate and culture primary cells from mammalian tissues. The system is comprised of a kit called PrimaCell™ that comes complete with the materials and the protocols to allow scientists to isolate primary cells from a given tissue type. The flexibility, ease and standardization of species-specific, tissue-specific and cell-specific primary cultures systems are extremely important in biomedical research and drug discovery. Experimentation in primary cells allows a deeper and more physiologically relevant view into the cellular function of proteins and enzymes that will allow the design of more specific drugs with meaningful and specific targets. Perhaps the most useful feature of standard primary cell cultures is the possibility to modulate and compare the metabolic and regulatory pathways of interest and to delineate the physiological effects of various compounds and drugs. The refinement and standardization of these experimental tools can be expected to accelerate cellular and genetic research. Furthermore, a reliable cell culture system can be available as a screening tool in drug discovery, thereby reducing the need for live animals.

The PrimaCell™ System comes in a kit that consists of seven components described below in Figure 1: (1) Tissue-specific tissue dissociation systems; (2) Tissue-specific fibroblast or non-target cell growth inhibitory cocktails; (3) Basal culture and growth media; (4) Complete growth media (growth factors and cytokines) supplements; (5) Highly purified and special treated serum or plant additives; (6) Buffers for tissue preparation; and (7) Detailed instruction manual and complete protocol of isolation and culture of primary cells.

We have developed a PrimaCell™ kit specific for the isolation of cells from specific tissues from various species. Please see our complete listing at www.chiscientific.com

Figure 1: Primary Cell Culture System - PrimaCell™

Description: PrimaCell™ kit is complete primary cell culture systems to guide you step-by-step culturing various tissue primary cells of avian, human, mouse and rat. Each PrimaCell™ system is customized for different types of Avian, Mouse, Rat and Human cells and consists of seven components.

PrimaCell™ Kit Components:

(1) Tissue-specific tissue dissociation systems;
(2) Tissue-specific fibroblast growth inhibitory cocktails, FibrOut™
(3) Complete growth media;
(4) Growth supplements(growth factors and cytokines);
(5) Highly purified and special treated serum;
(6) Buffers for tissue preparation;
(7) Bench-proven instruction and protocols.

(1) OptiTDS™ (tissue-specific tissue dissociation systems), is a mixture of several tissue dissociation enzymes with appropriate distribution ratios resolved in an optimal buffer. Each tissue dissociation system is customized to be tissue-specific, cell-specific, and proven to work effectively in accordance with the corresponding PrimaCell™ system to bring the best results for each type of the primary cultured cells. Each tissue dissociation system is supplied with the optimal working buffers.

(2) FibrOut™ (Tissue-specific fibroblast growth inhibitory cocktails), is a complete system that consists of several biochemical compounds and reagents that prevent fibroblast or non-target cell overgrowth. This ensures that the target cells are allowed to grow at optimal concentrations producing nearly pure cell populations. Each FibrOut™ is customized to be tissue-specific and cell-specific, and proven to work effectively in accordance with the corresponding PrimaCell™ system to ensure optimal growth and proliferation of the target cells. Each FibrOut™ is supplied with optimal working buffers.

(3) Basal culture and growth media: Specially formulated medium for target cells cultured and grown at optimized conditions. Each set of culture and growth media is customized to be tissue-specific, cell-specific, and proven to work efficiently with the corresponding PrimaCell™ system.

Figure 2: A representative illustration of PrimaCell™ Kit. All components of one kit for culture mouse Keratinocytes are shown here.

(4) **Complete growth media Supplements (growth factors and cytokines)**: Quality controlled batches of growth factors, cytokines and other growth supplements that compose conditioned growth medium for optimal culture of the target cells. Each set of growth media supplements is customized to be tissue-specific, cell-specific, and proven to work effectively with the corresponding PrimaCell™ system.

(5) **Highly purified and specially treated serum or plant additives**: Quality controlled batches of animal, human serum, and/or plant additives required in the conditioned growth medium. Each set of purified and specially treated serum or plant additives is customized to be tissue-specific, cell-specific, and proven to be working effectively in with the corresponding PrimaCell™ system to bring the best results for each type of primary cells.

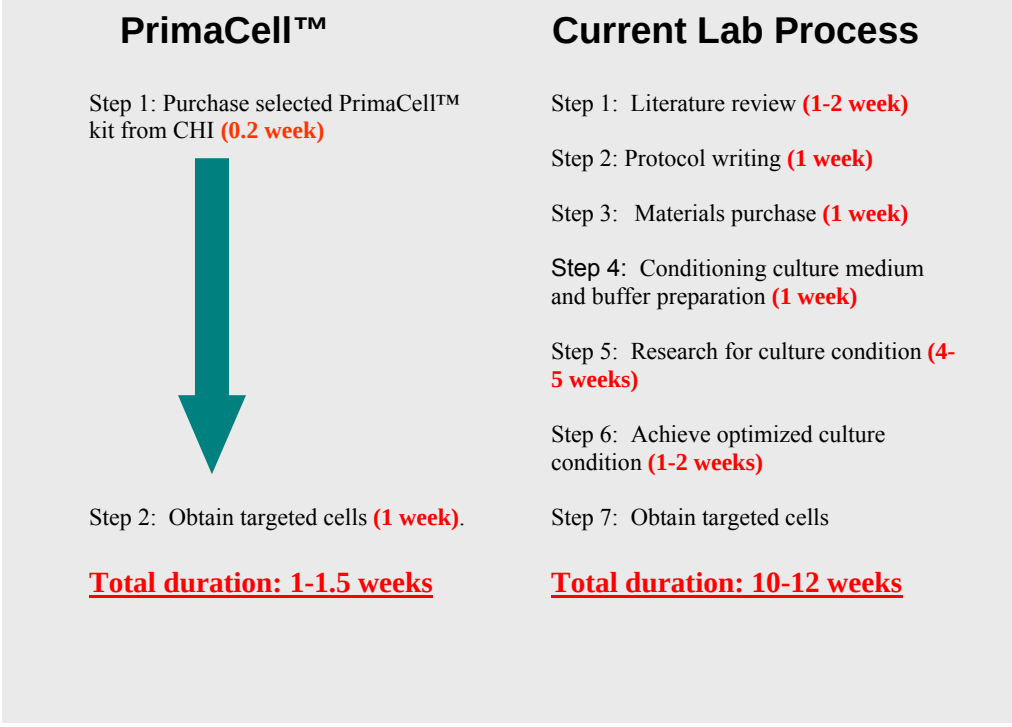
(6) **Buffers for tissue preparation**: Sterilized and purified buffers and solutions for treating and preparing tissues prior to the tissue dissociation processes. Each set of buffers for tissue preparation is

customized to be tissue-specific and cell-specific, and proven working effectively in accordance with the corresponding PrimaCell™ system.

(7) **Detailed instruction manual**: Tissue-specific and cell-specific protocols provide standardized laboratory-proven procedures and conditions with step-by-step guidance for the easy isolation and culture of primary cells from tissues.

PrimaCell™, the most innovative primary cell culture system, has several unique advantages over the standard practice of isolating primary cells. Theses include (1) An optimized culture system for the desired primary cells from individual organ types; (2) Easy-to-follow protocols without the need for additional optimization; (3) Complete reagent kits to minimize batch and vendor variations; (4) Reproducible data to set a compatible platform for comparison within experiments and across laboratories; (5) Cost-effective processes; and (6) Time- and labor-saving approach to culturing quality primary cells.

Figure 3: PrimaCell™ Simplifies Primary Cell Culture Processes



1.2 Tissue Type Considerations

The successful culture of primary cells requires that one obtain a maximum yield of functionally viable single cells from whole tissues. The primary factors that affect successful dissociation of whole tissues have to do with tissue origin, species, age of the animal, the dissociation medium and enzyme(s) used, the amount of impurities in any crude enzyme preparation, the concentration(s) of enzyme(s), and the temperature and incubation times. While factors dealing with type of tissue and species cannot be controlled, conditions associated with tissue dissociation are manageable. Below are some special considerations that factor into successful primary cell preparations.

Epithelial Tissue: The epithelium is a layer of cells that constitute the lining for all external (skin)

and internal body surfaces and cavities, including a specialized type of epithelial tissue known as endothelium that lines the interior surface of blood vessels. Epithelial tissues function to protect organs by regulating the secretion, absorption, excretion, filtration and diffusion of all substances as well as serve to regulate the responses to sensory and mechanical stimuli. Epithelial cells are packed tightly and in such organized structures that there is very little intercellular material between adjacent cells. Typically epithelial layers have an apical surface, the one surface that is free and not in contact with other cells. Directly opposite to the apical surface is the basal surface that is attached to the basement membrane. The basement membrane is the anchor between the epithelial layer and the underlying connective tissue and is made up of a mixture of carbohydrates and proteins such as collagens, laminins and integrins. The tight junctions between individual epithelial cells and the attachment to the basement membrane make tissue dissociation difficult. In fact, incomplete or inefficient tissue dissociation is one of the major hurdles encountered in primary cell preparations that often lead to low yields or poor cellular quality.

Connective Tissue: As discussed previously the basement membrane anchors the epithelial tissue to a layer of specialized tissue called connective tissue. In general connective tissue serves to provide structural support and bind or "connect" all types of tissue. Connective tissue exists in many different forms and is characterized by large amounts of extracellular matrix with few cells scattered throughout this matrix. The connective tissue matrix is made up of collagenous, elastic and reticular fibers and cells that include fibroblasts, adipocytes, histiocytes, lymphocytes, monocytes, eosinophils. neutrophils, macrophages, mast cells, and mesenchymal cells.

Extracellular Matrix: The extracellular matrix is the non-living viscous substance in which the cells and fibers of connective tissues are embedded. The extracellular matrix is primary composed of glycoproteins (collagens, laminins, fibronectin and elastins) and proteoglycans (chondroitin, heparan, keratan sulfates and hyaluronic acid). Most vertebrate cells can survive only if they are anchored to the extracellular matrix through specialized integrin proteins.

Collagen fibers: The collagen fibers are present in varying concentrations in virtually all connective tissues. Measuring 1-10 μm in thickness, they are unbranched, wavy, and contain repeating transverse bands at regular intervals. Biochemically, native collagen is a major fibrous component of extracellular connective tissue (skin, tendon, blood vessels, bone). Fibroblasts and possibly other mesenchymal cells synthesize the collagen subunits and release them into the extracellular matrix where they undergo enzymatic processing and aggregate into collagen fibers. The collegen interchains are often cross-linked via the hydroxyprolyl residues that serve to stabilize the collagen complex and make the fibers insoluble and resistant to hydrolytic attack by most proteases. The abundance of collagen fibers and the degree of cross-linking tend to increase with advancing age, making cell isolation more difficult from adult tissues.

1.3 Tissue Dissociation Enzyme Specificities

While many enzyme systems have been applied for a particular type of cell isolation, the enzymes discussed here have traditionally been used for a wide variety of tissues from many different species of various ages.

Collagenase: The most common collagenase which is commercially available is derived from *Clostridium histolyticum.* Clostridial collagenase degrades the helical regions in native collagen preferentially at the X-Gly bond in the sequence Pro-X-Gly-Pro where X is most frequently a neutral amino acid. Purified clostridiopeptidase A alone is usually inefficient in dissociating tissues due to incomplete hydrolysis of all collagenous polypeptides and its limited activity against the high

concentrations of non-collagen proteins and other macromolecules found in the extracellular matrix. The collagenase most commonly used for tissue dissociation is a crude preparation containing clostridiopeptidase A in addition to a number of other proteases, polysaccharidases and lipases. Crude collagenase is well suited for tissue dissociation since it contains the enzyme required to attack native collagen and reticular fibers in addition to the enzymes that hydrolyze the other proteins, polysaccharides and lipids in the extracelluar matrix of connective and epithelial tissues. There are four basic subtypes of collagenases with different enzymatic activity have been identified and commercially available on the market (Table 1):

Table 1	Collagenases Subtypes
Type 1	Containing average amounts of assayed activities (collagenase, caseinase, clostripain, and tryptic activities). It is generally recommended for epithelial, liver, lung, fat, and adrenal tissue cell preparations.
Type 2	Containing greater clostripain activity. It is generally used for heart, bone, muscle, thyroid and cartilage.
Type 3	Selected because of low proteolytic activity. It is usually used for mammary cells.
Type 4	Selected because of low tryptic activity.. It is commonly used for islets and other applications where receptor integrity is crucial.

Trypsin: Trypsin is a pancreatic serine protease with specificity for peptide bonds involving the carboxyl group of the basic amino acids, arginine and lysine. Trypsin is one of the most highly specific proteases known, although it also exhibits some esterase and amidase activity. Purified trypsin alone is usually ineffective for tissue dissociation since it shows little selectivity for extracellular proteins. Combinations of purified trypsin and other enzymes such as elastase and/or collagenase have proven effective for dissociation.

Elastase: Pancreatic elastase is a serine protease with specificity for peptide bonds adjacent to neutral amino acids. It also exhibits esterase and amidase activity. While elastase will hydrolyze a wide variety of protein substrates, it is unique among proteases in its ability to hydrolyze native elastin, a substrate not attacked by trypsin, chymotrypsin or pepsin. It is produced in the pancreas as an inactive zymogen, proelastase, and activated in the duodenum by trypsin. Elastin mostly appears in the elastic fibers of connective tissues, thus elastase is frequently used with other enzymes like trypsin or collagenase to dissociate tissues which contain extensive intercellular fiber networks.

Hyaluronidase: Hyaluronidase is a polysaccharidase specifically cleavage of endo-N-acetylhexosaminic bonds between 2-acetoamido-2-deoxy-beta-D-glucose and D-glucuronate. These bonds are common in hyaluronic acid and chondroitin sulfate A and C. Because these substances are found in high concentrations in the ground substance of virtually all connective tissues, hyaluronidase is often used in combination with a crude protease such as collagenase, for the dissociation of connective tissues.

Papain: Papain is a sulfhydryl protease from *Carica papaya* latex. Papain has wide specificity and degrades most protein substrates more extensively than the pancreatic proteases. It also exhibits esterase activity. With some tissues papain has proved less damaging and more effective than other proteases. Papain has been widely used for obtaining high yields of viable, morphologically intact cortical neurons.

Chymotrypsin: Chymotrypsin is a protease that preferentially catalyzes the hydrolysis of peptide bonds involving the aromatic amino acids tyrosine, phenylalanine, and tryptophan. In addition it acts upon the peptide bonds of leucyl, methionyl, asparagenyl and glutamyl residues, and the amides and esters of susceptible amino acids. Chymotrypsin is used to a limited extent in tissue dissociation, usually in combination with trypsin and elastase.

Deoxyribonuclease I: Often as a result of cell damage, deoxyribonucleic acid leaks into the dissociation medium increasing viscosity and causing handling problems. Purified deoxyribonuclease is sometimes included in cell isolation procedures to digest the nucleic acids without damaging the intact cells.

Neutral Protease (Dispase): Neutral Protease (Dispase) is a bacterial enzyme produced by *Bacillus polymyxa* that hydrolyses N-terminal peptide bonds of non-polar amino acid residues and is classified as an amino-endopeptidase. The unique feature of this enzyme is that it possesses a mild proteolytic dissociation activity while preserving the integrity of the cell membrane. It makes the enzyme especially useful for the isolation of primary cells and the subsequent sub-culture. Neutral Protease (Dispase) is also frequently used as a secondary enzyme in conjunction with collagenase and/or other proteases in many primary cell isolation and tissue dissociation applications. Neutral Protease (Dispase) dissociates fibroblast-like cells more efficiently than epithelial-like cells. Thus it has been used for differential isolation and culture applications.

Trypsin Inhibitor (soybean): The trypsin inhibitor from soybean inactivates trypsin in equimolar ratios, however, it exhibits no effects on the esterolytic, proteolytic or elastolytic activities of porcine elastase. It inhibits trypsin enzymatic activity during tissue dissociation procedure to increase yield of a particular cell type.

1.4 Yield and Viability

To obtain a maximum yield of functionally viable, single cells, the choice of the most appropriate enzymes in the correct ratios are crucial including the appropriate incubation times with the tissues. Figure 3 illustrates the relationship between cell viability and yield *vs.* selection of dissociation enzymes and incubation time. Aggressive dissociation or poor dissociation of tissues will result in low cell viability. Outlined below are some possible explanations for sub-optimal cell isolation and the solutions to obtain the most optimal results.

1) Low yield and low viability: Most likely tissues are dissociated too aggressively which results in cellular damage. Change the type of enzyme and/or decrease the working concentration. (e.g. from trypsin to collagenase/ from Type 2 collagenase to Type 1).

2) Low yield and high viability: The tissues are digested gently using enzymes that are too mild or the incubation time is too short. Increase the enzyme concentration and/or the incubation time and monitor both the yield and the viability response. If the yield remains poor, evaluate a stronger digestive type of enzyme and/or the addition of secondary enzyme(s).

3) High yield and low viability: The tissues are probably digested with the appropriate enzymes but the enzyme composition is too strong and/or at a high working concentration that causes cellular damage. Reduce the concentration and/or the incubation time and monitor the yield and the viability response. Alternatively, dilute the proteolytic action by adding bovine serum albumin (BSA) (0.1 - 0.5% w/v) or soybean trypsin inhibitor (0.01 - 0.1% w/v) to the enzyme mixture.

4) High yield and high viability: These conditions strike the optimal balance for the selected tissues.

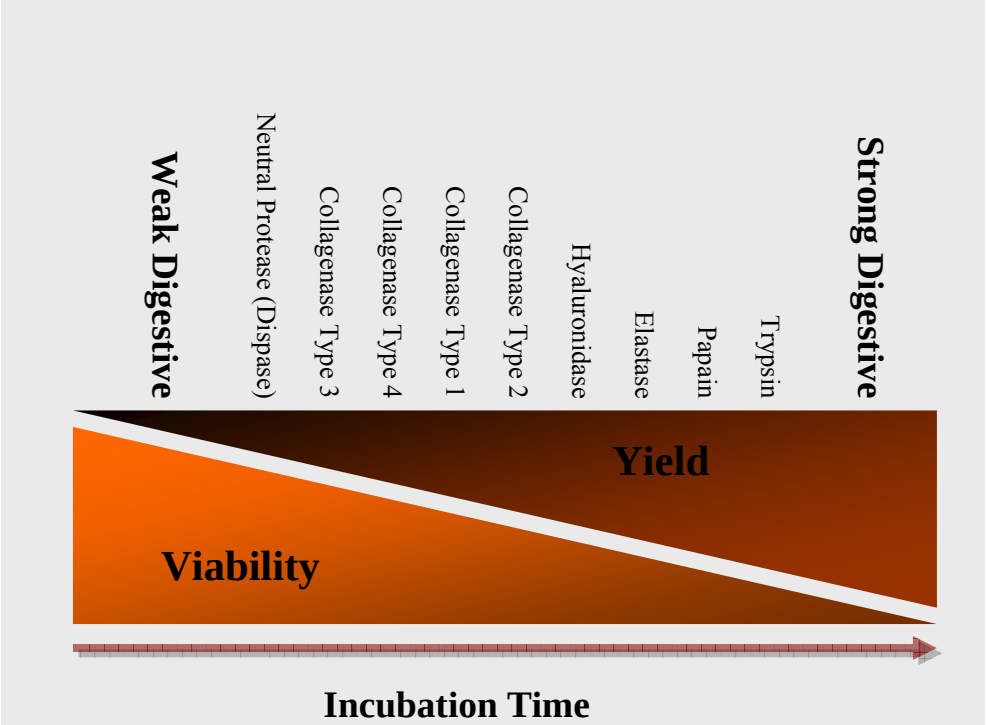


Figure 4: Schematic illustration of relationship between cell viability and yield *vs.* selection of dissociation enzymes and incubation time.

In addition to the dissociation procedures mentioned above, trituration can be a crucial part of primary cell isolation. This action of repeatedly pipetting the mixture allows the tissues to break up into fragments, after the tissues have been incubated in the dissociation enzymes. If done too vigorously, cells will be destroyed lowering viability; if done too weakly, tissue fragments will be left intact lowering yield. The correct method is to use gentle trituration, using a 10 ml pipette by filling and emptying the barrel at a rate of about 3.0 ml per sec. The best method to determine a suitable trituration rate for the tissue of choice is through trial and error cautious to avoid any bubbles in the cell suspension.

1.5 OptiTDS™, Tissue dissociation optimization

Although optimization of cell isolation procedures for a particular cell type is complicated by several factors, some principal guidelines regarding selected tissue types of species are established. The information in this handbook regarding cell isolation and the enzymes used, when combined with logic and suitable experimental design, should lead to the development of a satisfactory cell isolation outcome. In general there is an area of optimal cell recovery balanced between yield and viability; working near the middle of this range will reduce variability in the results of the cell isolation procedure.

CHI Scientific has invested in R & D efforts to establish the optimum tissue dissociation system, OptiTDS™ for mouse, rat, human and avian tissues. The OptiTDS™ kits are developed based on the enzyme(s) composition, working concentrations and the buffer or media systems published in the

reference(s) in a searchable database. A detailed description of this kit is shown in Figure 4

Figure 5: Tissue dissociation system - OptiTDS™

Description: Tissue dissociation system, OptiTDS™, is a proprietary mixture of several tissue dissociation enzymes/reagents with appropriate distribution ratios resolved in an optimum buffer, to produce a maximum number of single viable cells. Each tissue dissociation system is customized to be tissue-specific and cell-specific, and proven to work effectively in accordance with the corresponding PrimaCell™ system to bring you the best results for the primary cell type of your choice.

OptiTDS™ Kit Components:

Each OptiTDS™ kit contains 20 ml of tissue dissociation enzyme working solution sufficient for 3-5 g of tissue.

(1) Concentrated tissue dissociation enzymes/reagents (2 x 1.0 ml)

(2) Tissue-specific optimum tissue dissociation digestion buffer system (2 x 9.0 ml).

1.6 Fibroblast Inhibitory System - FibrOut™

Very often primary cell cultures are confounded by the outgrowth of contaminating fibroblasts. The population of the target primary cells is directly affected since fibroblasts usually grow at much faster rates than other cell types. Experimentation is also confounded because often the biochemical or biological experiments represent a mixture of the reactions in the target cells and the fibroblasts. A search of the literature reveals that there are numerous published efforts to inhibit fibroblast outgrowth and this process appears to be tissue specific. For instance, the addition of melatonin to melanocytes (target cells) preparations potently inhibits the proliferation of fibroblasts (contaminating cells) that were passed along in the isolation procedure from whole human skin. Another example is that of adenosine and its inhibition of collagen and protein synthesis in cardiac fibroblasts. Specific antibodies can act as inhibitors of fibroblasts growth and proliferation. A DNA topoisomerase inhibitor (camptothecin) can be included in culture medium to eliminate non-neuronal cells proliferation in neuron-type primary cell isolation. In addition, it has been reported that D-valine is a selective agent for rat epididymal epithelial cells, but not for rabbit epithelial cells, and that cytosine arabinoside is a simple and effective means to control the proliferation of fibroblast-like cells in both rat and rabbit epididymal cell cultures. Together, a tissue-specific optimal fibroblast inhibitory system can be developed to improve the performance of primary cell isolation.

The fibroblast growth control system, FibrOut™ developed by CHI Scientific is a proprietary combination of several biochemical compounds, antibodies, or specialty reagents, which prevent overgrowth of fibroblastic or contaminating cells, effectively increasing the yield of target cells. Each fibroblast growth inhibitory system is customized to be tissue-specific and cell-specific, and proven to work most effectively with our corresponding PrimaCell™ system. Each fibroblast growth inhibitory system is supplied with optimal working buffers.

Figure 6: Fibroblast Inhibitory System - FibrOut™

Description: The fibroblast growth control system, FibrOut™ is a combination of several biochemical compounds and reagents, which prevent overgrowth of fibroblastic and contaminating cells and increase targeted cell growth during primary cell culture. Each fibroblast growth inhibitory system is customized to be tissue-specific and cell-specific, and proven to work effectively with our corresponding PrimaCell™ system. Each fibroblast growth inhibitory system is supplied with optimal working buffers.

FibrOut™ Kit Components:

Each FibrOut™ system may contain one or more of the following chemicals:

(1) Trypsin;
(2) Collagenase;
(3) D-valine;
(4) Cis-OH-proline;
(5) Ethylmercurithiosalicylate;
(6) Phenobarbitone,
(7) Formulated serum substitutes.
(8) Antimesodermal antibodies.

1.7 Primary Cell Characterization System - Primarker™

When dealing with primary cell cultures, defining the population of target cells and confirming the identification of the cell type before pursuing experiments is crucial.

Figure 7: Primary Cell Characterization kit - Primarker™

Description: Primary Cell Characterization kit, Primarker™ is a convenient primary cell characterization system developed for individual type of primary cells based on unique markers that exist on cells. This fluorescence-based system consists of marker-specific primary antibody, fluorescence-labeled secondary antibody and all necessary buffers and reagents for live-cell identification of cell-specific primary cultured cells.

Primarker™ Kit Components:

Each Primarker™ system contain the following items:

(1) marker-specific primary antibody;
(2) fluorescence-labeled secondary antibody;
(3) Cell Fixation reagent;
(4) Cell permeablization reagent;
(5) Nuclei staining reagent;
(6) blocking buffers and reagents

Numerous unique cell markers have been reported in the literature and many selections can be chosen to characterize the isolated primary cells. The Primary Cell Characterization kit, Primarker™ developed by CHI Scientific is a convenient primary cell characterization system to assay cell specific markers. This fluorescence-based system consists of a marker-specific primary antibody, a fluorescence-labeled secondary antibody and all the necessary buffers and reagents for live-cell identification of cell-specific primary cultured cells.

References:

1. Carossino AM, Lombardi A, Matucci-Cerinic M, Pignone A, Cagnoni M. *Effect of melatonin on normal and sclerodermic skin fibroblast proliferation.* Clin Exp Rheumatol. 1996 Sep-Oct; 14(5):493-8.
2. Dubey RK, Gillespie DG, Jackson EK. *Adenosine inhibits collagen and protein synthesis in cardiac fibroblasts: role of A2B receptors.* Hypertension. 1998 Apr; 31(4):943-8.
3. Brewer GJ. *Isolation and culture of adult rat hippocampal neurons.* J Neurosci Methods. 1997 Feb;71(2):143-55.
4. Andersen PL, Doucette JR, Nazarali AJ. *A novel method of eliminating non-neuronal proliferating cells from cultures of mouse dorsal root ganglia.* Cell Mol Neurobiol. 2003 Apr;23(2):205-10.
5. Sköld CM, Liu XD, Umino T, Zhu YK, Ertl RF, Romberger DJ, Rennard SI. Blood monocytes attenuate lung fibroblast contraction of three-dimensional collagen gels in coculture. Am J Physiol Lung Cell Mol Physiol. 2000 Oct; 279(4):L667-74.
6. Worthington Biochemical Corporation, "Cell isolation theory", Book: *Tissue Dissociation Guide*, 1-5, 2000.

Chapter 2 Mouse Primary Cell Culture System – Mouse PrimaCell™

Mouse Airway PrimaCell™: Bronchial and Tracheal Epithelium
(Cat No. 2-82001)

I. General Description:

This protocol is developed for attachment and growth of normal Mouse bronchial epithelial (NHBE) cells from newborn or adult Mouse Airway with Mouse Airway PrimaCell™ system (Cat No. 2-82001). This system provides an optimal condition of tissue dissociation system, Airway OptiTDS™ that yields 4-7 times of single cells more than most of the tissue dissociation protocols published in the literature. In addition, this system ensures a high viability of the target cells with improved gradient contained in the culture medium. With CHI's proprietary fibroblast inhibitory system, FibrOut™, cells are growing with contamination of minimized amount of the non-epithelial cells.

This procedure involves explanting fragments of large airway tissue in a serum-free modified medium (LHC-9) in order to initiate and subsequently propagate fibroblast-free outgrowths of NHBE cells; four subculturings and 30 population doublings are routine.

Mouse Airway PrimaCell™ system applies to all type tissue samples from Mouse at all age though younger tissue samples are recommended for yielding maximum amount of viable target cells. However, tissue samples contain pathological organism (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Mouse Airway PrimaCell™ System

- **Mouse Airway Tissue Washing Medium**, (1 × 100 ml) --- *A mixture of L-15, BSA, penicillin, streptomycin, gentamycin*.
- **Mouse Airway Tissue Healing Medium (HB Medium),** (5 × 100 ml) --- *A modified formulation based on CMRL 166 medium*.
- **Mouse Airway Fibroblast Growth Inhibitors, Airway FibrOut™** --- Airway FibrOut™ (5 × 200 µl) --- *A mixture of collagenase, D-valine, and gentamycin.*
- **Mouse Airway PrimaCell™ Growth Medium,** (5 × 100 ml) --- *A modified LHC-9 medium*.
- **Mouse Airway PrimaCell™ Growth Medium Supplements with Serum** (5 × 1 ml) --- *Highly purified and special-treated EGF, Epinephrine, Hydrocortisone sodium succinate, 3,3'5-triiodo-L-thyronine, insulin, Adenine SO4, Thymidine, Lipoic acid, Phosphoethanolamine.*
- **Mouse Airway Tissue Healing Medium Supplements with Serum** (5 × 1 ml) --- *A mixture of insulin, hydrocortisone, β-retinyl acetate, glutamine, penicillin, stretomycin, gentamucin, fungison, FBS.*
- **Mouse Airway PrimaCell™ Culture Dish Coating Solution** --- *A mixture of FN/V/BSA, mouse fibronectin, collagen, BSA, and LHC basal medium*

1.2 Required Materials but not provided

- Plastic tissue culture dishes (60 and 100 mm)
- Surgical scissors

- Half-curved microdissecting forceps
- Gloves sterilized with autoclave (Mouse tissue can be contaminated with biologically hazardous agents)
- Controlled atmosphere chamber
- Rocker platform
- Phase-contrast inverted microscope

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, culture plate (Corning, NY) must be coated with the provided coating solution.

2.2 Tissue Preparation and Healing

Replicative cultures of NHBE cells can be established from several sources of donated airway specimens, including surgeries and autopsies. Of these, tissue recovered by surgery from noncancerous patients or donors undergoing ‘‘immediate’’ autopsies yield the greatest quantity of culturable cells and are least likely to harbor malignant cells. Cells can also be obtained by biopsy or brushing of airways during bronchoscopy.

2.2.1 Complete Healing Medium Prepration

Thaw out the Mouse Airway Tissue Healing Medium Supplements with Serum on ice. To every 100 ml Mouse Airway Tissue Healing Medium, add 1 ml Mouse Airway Tissue Healing Medium Supplements with Serum, mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use.

2.2.2 Procedures for tissue dissociation and healing

1. Coat a culture dish with 1 ml of the Mouse Airway PrimaCell™ II Culture Dish Coating Solution per 60-mm dish, and incubate the dish in a humidified CO_2 incubator at 37℃ for at least 2 h (not to exceed 48 h). Vacuum aspirate the mixture and fill the dish with 5 ml of Mouse Airway Tissue Washing Medium.
2. Aseptically dissected lung tissue from health mice within 4-6 h is placed into ice-cold Mouse Airway Tissue Washing Medium for transport to the laboratory, where the bronchus is further dissected from the peripheral lung tissues.
3. Before culturing, scratch an area of one square centimeter at one edge of the surface of the 60-mm culture dishes with a scalpel blade.
4. Open the airways (submerged in the Mouse Airway Tissue Washing Medium) with surgical scissors, and cut (slice, do not saw) the tissue with a scalpel into two pieces, 20 × 30 mm.
5. Using a scooping motion to prevent damage to the epithelium, pick up the moist fragments and place them epithelium side up onto the scratched area of the 60-mm dish. Remove the Mouse Airway Tissue Washing Medium, and incubate the fragments at room temperature for 3 to 5 min to allow time for them to adhere to the scratched areas of the dishes.
6. Add 3 ml of Mouse Complete Airway Tissue Healing Medium/FibrOut to each dish and place them in a controlled-atmosphere chamber. Flush the chamber with a high-O_2 gas mixture and place it on a rocker platform. Rock the chamber at 10 cycles per minute, causing the medium to

flow intermittently over the epithelial surface. Incubate rocking tissue fragments at 37℃, changing the medium and atmospheric pressure after Day 1 and again after Day 2 - intervals for 6-8 d. This step improves subsequent explant cultures by reversing any ischemic damage to the epithelium that occurred from time of death of the donor until the tissue was placed in the ice-cold Mouse Airway Tissue Washing Medium.

2.2.3 Storage:

Reconstituted tissue dissociation systems should occur before use and can only be stored for 2-4 days at 4 ^{o}C. For long-term use, it should be aliquoted and stored at -20 ^{o}C. Avoid repeated freeze-thaw cycles.

2.3 Explant Outgrowth Cultures and Subculture

2.3.1 Medium Preparation.

Thaw out the Mouse Airway PrimaCell™ Growth Medium Supplements with Serum, Mouse Airway Fibroblast Growth Inhibitors, and Airway FibrOut™ on ice.

Mouse Complete Airway PrimaCell™ Growth Medium: To every 100 ml Mouse Airway PrimaCell™ Growth Medium, add 1 ml Mouse Airway PrimaCell™ Growth Medium Supplements with Serum and 200 µl Mouse Airway Fibroblast Growth Inhibitors, Airway FibrOut™, mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use. ***Important:*** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*.

2.3.2 Procedures For Tissue Explant

7. Before explanting, scratch seven areas of the surface of each 100-mm culture dish with a scalpel. Coat the surfaces of the scratched culture dishes with the Mouse Airway PrimaCell™ Culture Dish Coating Solution, and aspirate the surplus solution as before.
8. Cut the moist ischemia-reversed fragments into 7 × 7-mm pieces, and explant the pieces epithelium side up on the scratched areas. Incubate the pieces at room temperature without medium for 3-5 min, as before.
9. Add 10 ml of Mouse Complete Airway PrimaCell™ Growth Medium/FibrOut to each dish, and incubate explants at 37℃ in a humidified 5% air/CO_2 incubator. Replace spent medium with fresh medium every 3 to 4 d.
10. After 8 to 11 d of incubation, when epithelial cell outgrowths radiate from the tissue explants more than 0.5 cm, transfer the explants to new culture dishes scratched and coated with Mouse Airway PrimaCell™ Culture Dish Coating Solution to produce new outgrowths of epithelial cells. This step can be repeated up to seven times with high yields of NHBE cells.

2.3.3 Procedures for Dissociation and Subculture of Bronchial Epithelium

11. Incubate the postexplant outgrowth cultures in Mouse Complete Airway PrimaCell™ Growth Medium for an additional 2 to 4 d before trypsinizing (with the trypsin/EGTA/PVP solution) for subculture or for experimental use.
12. Aspirate the medium and bathe the culture two times with Mouse Airway Tissue Washing Medium.

13. Remove the Mouse Airway Tissue Washing Medium and incubate the culture at room temperature in 0.5 M urea for 5 min.
14. Remove the urea solution by aspiration and bathe the cells in a minimal volume of 0.05mg/mL Tripsin and 0.5mM/ml EGTA, and incubate at room temperature until the cells float frees (usually 5–10 min).
15. Resuspend the cells with Mouse Airway Tissue Washing Medium and pellet by centrifugation (125 g for 5 min).
16. Resuspend the cells with Mouse Complete Airway PrimaCell™ Growth Medium, enumerate the cells and reinoculate at the desired cell density into culture dishes that have been coated with Mouse Airway PrimaCell™ Culture Dish Coating Solution.

III Cryopreservation

Both dissociated NHBE cells and bronchial tissue fragments can be cryopreserved with good viability using relatively routine procedures, as follows.

17. Suspend the tissue (0.5-cm^3 fragments) or pelleted normal mouse bronchial epithelial (NHBE) cells ($2–5 \times 10^6$) in 0.5 ml of cold 2 × Mouse Complete Airway PrimaCell™ Growth Medium in a freezing vial.
18. Add 0.5 ml of DMSO freezing medium to the vial, swirl the mixture, and close the vial.
19. Transfer vial(s) to a controlled-rate freezer and freeze the cells/tissues at 1℃/min according to the manufacturer's directions.
20. Transfer the frozen vials to liquid N2 for storage.
21. Resurrect the cells/tissues by rapidly warming the vial to 37℃.
22. Swab the vial with 70% alcohol, open it, and transfer the cells to 10 ml of Mouse Complete Airway PrimaCell™ Growth Medium.
23. Pellet the cells, resuspend in Mouse Complete Airway PrimaCell™ Growth Medium, and inoculate into Mouse Airway PrimaCell™ Culture Dish Coating Solution-coated culture dishes containing Mouse Complete Airway PrimaCell™ Growth Medium.

IV Characterization

NHBE are identified by several criteria based on the characteristic structure and function of normal epithelium. In explant outgrowth cultures, polygon-shaped epithelial cells grow out from the periphery of the explant onto the culture dish before the fusiform fibroblast cells. Cytochemical stains can further distinguish epithelial cells and fibroblasts in primary cultures. Squamous epithelial cells stain positively with the immunoperoxidase method for prekeratin and keratin, whereas fibroblasts stain negatively. In some cultures, epithelial cells will stain positively with alcian blue-PAS before and after treatment with diastase, indicating the production of acidic and neutral mucopolysaccharides, two components of mucus. Most cultures, however, will not exhibit positive staining for mucous substances. Epithelial cells in first-passage cultures are similar in appearance to those in explant outgrowth cultures, and they continue to react positively with the keratin antibodies. Scanning electron microscopy of subcultures shows colonies composed of prolate spherical cells covered with varying numbers of microvilli and apposed cell borders. In addition, mouse bronchial epithelial cells do not form colonies in soft agar when plated at a density of 100,000 per 1 ml, and chromosome analysis shows that the cells retain the normal mouse karyotype (2N = 46) throughout the replicative phase. Additionally, these cells are metabolically active and capable of converting xenobiotics to DNA adducts.

V References:

4. Yim HW, Slebos RJ, Randell SH, Umbach DM, Parsons AM, Rivera MP, Detterbeck FC, Taylor JA. *Smoking is associated with increased telomerase activity in short-term cultures of Mouse bronchial epithelial cells.* Cancer Lett. 2006 Mar 3;
5. Doherty GM, Christie SN, Skibinski G, Puddicombe SM, Warke TJ, de Courcey F, Cross AL, Lyons JD, Ennis M, Shields MD, Heaney LG. *Non-bronchoscopic sampling and culture of bronchial epithelial cells in children.* Clin Exp Allergy. 2003 Sep; 33(9):1221-5.
6. Mattinger C, Nyugen T, Schafer D, Hormann K. *Evaluation of serum-free culture conditions for primary Mouse nasal epithelial cells.* Int J Hyg Environ Health. 2002 Apr; 205(3):235-8.
7. de Jong PM, van Sterkenburg MA, Kempenaar JA, Dijkman JH, Ponec M. *Serial culturing of Mouse bronchial epithelial cells derived from biopsies.* In Vitro Cell Dev Biol Anim. 1993 May; 29A (5):379-87.
8. Robbins RA, Koyama S, Spurzem JR, Rickard KA, Nelson KJ, Gossman GL, Thiele GM, Rennard SI. *Modulation of neutrophil and mononuclear cell adherence to bronchial epithelial cells.* Am J Respir Cell Mol Biol. 1992 Jul;7(1):19-29.
9. Lechner JF, Wang Y, Siddiq F, Fugaro JM, Wali A, Lonardo F, Willey JC, Harris CC, Pass HI. *Mouse lung cancer cells and tissues partially recapitulate the homeobox gene expression profile of embryonic lung.* Lung Cancer. 2002 Jul; 37(1):41-7.
10. Lechnewr JF, & LeVeck MA. *A serum free method for culturing normal Mouse bronchial epithelial cells at clonal density.* J. Tissue Cult. Methods 9: 43-48.

Mouse Airway PrimaCell™: Bronchial and Tracheal Epithelium

Mouse Airway Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82001	**Mouse Airway PrimaCell™ System**	**kit**	**$499**
7-62011	Bronchial and Tracheal Fibroblast Growth Inhibitors (for 500 ml medium), Airway FibrOut™, Mouse	1 ml	$146
9-42001	Bronchial and Tracheal PrimaCell™ Basal Medium, Mouse	500 ml	$61
9-32011	Bronchial and Tracheal PrimaCell™ Growth Medium Supplements (for 500 ml medium), Mouse	set	$140
4-22011	Mouse Bronchial and Tracheal Tissue Disassociation system	ea	$128
9-92001	Mouse Airway Tissue Preparation Buffer Set	ea	$75

Mouse Airway Primary Cell Characterization

6-12811	**Mouse Bronchial and Tracheal Epithelium Primarker™ Kit**	**kit**	**$220**
6-12812	Mouse Bronchial and Tracheal Epithelium Primarker™ antibody set	set	$180
6-12813	Mouse Bronchial and Tracheal Epithelium Primarker™ buffer system	set	$90

Mouse Bone Marrow PrimaCell™: Hematopoietic Cells
(Cat No. 2-82003)

I. General Description:

This protocol is developed for Hematopoietic Cells from newborn or adult Mouse Bone marrow with Mouse Bone marrow PrimaCell™ system (Cat No. 2-82003). This system provides an optimal condition of tissue dissociation system, Bone Marrow OptiTDS™ that yields 3-4 times of single cells more than most of the tissue dissociation protocols published in the literature. In addition, this system ensures a high viability of the target cells with improved gradient contained in the culture medium. With CHI's proprietary fibroblast inhibitory system, FibrOut™, cells are growing with contamination of minimized amount of the non-Hematopoietic Cells.

Marrow is aspirated into growth medium and maintained as an adherent cell multilayer for at least 12, and up to 30, weeks. Stem cells and maturing myeloid cells are released from the adherent layer into the growth medium. Granulocyte/macrophage progenitor cells can be assayed in soft gels.

Mouse Bone marrow PrimaCell™ system applies to all type tissue samples from mouse at all age though younger tissue samples are recommended for yielding maximum amount of viable target cells. However, tissue samples contain pathological organism (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Mouse Bone marrow PrimaCell™ System

- **Mouse Bone marrow PrimaCell™ system, Bone marrow OptiTDS™,** (2 x 1 ml) --- *A mixture of collagenase, collagenase I, collagenase I and Mouse bone marrow OptiTDS™ Reconstitution Buffer.*
- **Mouse bone marrow OptiTDS™ Digestion Buffer**, (2 × 9 ml).
- **Mouse Bone Marrow Fibroblast Growth Inhibitors, Bone Marrow FibrOut™** (5 × 200 µl) --- *A mixture collagenase and gentamycin*.
- **Mouse Bone Marrow Tissue Washing Medium**, (1 × 100 ml) --- *HBSS with 5% BSA, 200 U/ml of penicillin, 200 µg/ml of streptomycin, and 50 µg/ml of gentamycin*.
- **Mouse Bone Marrow PrimaCell™ Basal Culture Medium,** (5 × 100 ml) --- *A modified Fischer's Medium.*
- **Mouse Bone Marrow PrimaCell™ Medium Supplements**, (5 × 1.0 ml) --- *A mixture of Hydrocortisone, Sodium Succinate, penicillin, streptomycin, and NaHCO3*.
- **Mouse Bone Marrow PrimaCell™ Serum**, (10 × 10 ml) --- *Highly purified special-treated Horse Serum.*

1.2 Required Materials but not provided

- 70% sterile ethanol
- Plastic tissue culture dishes (60 and 100 mm)
- Surgical scissors
- Half-curved microdissecting forceps

- Pipettes (10 and 25 ml)
- Gloves sterilized with autoclave (mouse tissue can be contaminated with biologically hazardous agents)
- Rocker platform
- Phase-contrast inverted microscope

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, collagen-coated culture plate (Corning, NY) must be used in this experiment.

2.2 Surgical specimens

Bone Marrow: Hematopoietic cells are from mouse bone marrow in femur. One femur contains 1.5-2.0 × 10^7 nucleated cells.

1. Sacrify the mouse by cervical dislocation or institutional procedures.
2. Wet the fur with 70% alcohol and remove both femurs. Collect femurs in a Petri dish on ice containing Mouse Bone Marrow Tissue Washing Medium.
3. In a laminar flow hood:
   (a) Clean off any remaining muscle tissue using gauze swabs.
   (b) Hold the femur with forceps and cut off the knee end. The 21G needle should fit snugly into the bone cavity.
   (c) Cut off the other end of the femur as close to the end as possible.
   (d) Keep the Mouse Bone Marrow Tissue Washing Medium soaked femurs in ice till tissue dissociation procedure.

2.3 Tissue Preparation and Dissociation

2.3.1 Mouse Bone Marrow OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Mouse Bone Marrow Tissue Dissociation System, OptiTDS™, is suited for optimal dissociation of normal adult and newborn Bone marrow samples to yield maximum number of single primary cells of colonic tissues.

2.3.2 Enzyme Compositions

- Collagenase: from *Clostridium Histolyticum*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase II: from *Clostridium Histolyticum*

2.3.3 System Components

- Mouse Bone Marrow Tissue Dissociation System, OptiTDS™, 2 x 1.0 ml.
- Mouse Bone Marrow OptiTDS™ Digestion Buffer, 2 × 9 ml.

2.3.4 Procedures For Tissue Preparation and Dissociation

4. Add 1.0 ml of Mouse Bone Marrow Tissue Dissociation System, Bone OptiTDS™ to one vial of Mouse Bone OptiTDS™ Digestion Buffer (9 ml). Warm the diluted enzyme working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 5-7 g tissue samples per 10 ml diluted enzyme working solutions.
5. Insert the tip of the bone into a bottle of 10 ml diluted enzyme working solutions, and aspirate and depress the syringe plunger several times until all the bone marrow is flushed out of the femur.
6. Repeat steps 5 with the other bones.

2.3.5 Storage:

Reconstituted tissue dissociation systems should occur before use and can only be stored for 2-4 days at 4 ^{o}C. For long-term use, it should be aliquoted and stored at -20 ^{o}C. Avoid repeated freeze-thaw cycles.

2.4 Culture of Primary Mouse Keratinocytes

2.4.1 Medium Preparation.

Thaw out the Mouse Bone marrow PrimaCell™ Medium Supplements and Mouse Bone marrow PrimaCell™ I Serum on ice. To prepare complete Mouse Bone Marrow Medium, add one vial of Mouse Bone marrow PrimaCell™ I Medium Supplements and 10 ml Mouse Bone marrow PrimaCell™ I Serum to every 100 ml Mouse Bone Marrow PrimaCell™ Basal Culture Medium; adding one vial of Mouse Bone marrow Fibroblast Growth Inhibitors, Bone Marrow FibrOut™ to Complete Mouse Bone Marrow Medium makes Complete Mouse Bone Marrow Culture Medium. Mix thoroughly and warm the complete medium at a 37 ^{o}C water bath for 10 min prior use.
Important: *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination.*

2.6.1 Primary Cell Culture

7. Disperse the marrow to a suspension by pipetting the large marrow cores through a 10-ml pipette. There is no need to disaggregate small clumps of cells.
8. Centrifuge cells for 2 min at 580 g at room temperature and remove the supernatant.
9. Dispense 10-ml aliquots of the cell suspension into 25-cm^2 tissue culture flasks by using Complete Mouse Bone Marrow Medium/FibrOut, swirling the suspension often to ensure an even distribution of the cells in the 10 cultures.
10. Gas the flasks with 5% CO_2 in air and tighten the caps.
11. Incubate the cultures horizontally at 33℃.
12. Feed the cultures weekly:
    a) Agitate the flasks gently to suspend the loosely adherent cells.
    b) Remove 5 ml of growth medium, including the suspension cells; take care not to touch the layer of adherent cells with the pipette.
    c) Add 5 ml of fresh Complete Mouse Bone Marrow Medium containing FibrOut™ to each flask; to avoid damage; do not dispense the medium directly onto the adherent layer.

d) Gas the cultures and replace them in the incubator.

2.4.3 Subculture and Propagation

13. Gently rinse the culture dish twice with 0.02% (0.7 mM) EDTA.
14. Add 3 ml of 0.25% trypsin/0.1% (2.5 mM) EDTA, and incubate at 37℃. Examine the dish under phase microscopy every 5 min to detect cell detachment.
15. When most cells have detached, add 12 ml Complete Mouse Bone Marrow Medium to inactive the trypsin activity.
16. Pipette the contents of the dish to ensure complete hematopoietic cells detachment.
17. Aspirate and centrifuge the cells for 5 min at 350 g.
18. Aspirate the supernatant, resuspend the cells in a Complete Mouse Bone Marrow Medium, and replate at 2-4 × 10^4 cells per 100-mm dish.
19. Refeed the culture twice a week with Complete Mouse Bone Marrow Medium.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample; the best results are reported when cells from preconfluent primary cultures are used.

20. Detach cells as for the subculture, and centrifuge at 100 g for 10 min.
21. Resuspend cells in Complete Mouse Bone Marrow Medium and count.
22. Dispense aliquots of 2 × 10^6 cells/ml in Complete Mouse Bone Marrow Medium with 10% glycerol into cryopreservation tubes.
23. Equilibrate at 4℃ for 1-2 h.
24. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
25. To recover cells:
    a) Thaw cryotubes quickly in a 37℃ water bath.
    b) Dilute cells tenfold with medium.
    c) Centrifuge cells and resuspend them at an appropriate concentration in the desired Complete Mouse Bone Marrow Medium, and seed culture vessel.

Mouse cells can be grown in all media for 4-7 weeks and can be subcultured only 4-5 times.

IV Fibroblast Contamination

There are several techniques have been published in the literature to deal with fibroblast contamination during colorectal primary cell culture. These include: (1) Physically remove a well-isolated fibroblast colony by scraping it with a sterile blunt instrument (e.g., a cell scraper). Care has to be taken to wash the culture up to six times to remove any fibroblasts that have detached in order to prevent them from reseeding and reattaching to the flask. (2) Differential trypsinization can be attempted with the carcinomas. (3) Dispase preferentially (but not exclusively) removes the epithelium during passaging and leaves behind most of the fibroblastic cells attached to the culture vessel. During subculture, cells that have been removed with dispase can be preincubated in plastic petri dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the hematopoietic cells. (4) Reduce the concentration of serum to about 2.5-5% if there are heavy concentrations of fibroblastic cells. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and that using any or all of the preceding techniques will eventually push the cells through so many divisions that any

fibroblasts will senesce.

Mouse Bone marrow PrimaCell™ system includes a fibroblast elimination system, the Mouse Bone marrow Fibroblast Growth Inhibitors, Bone marrow FibrOut™. It contains a mixture of D-valine, proline, collagenase and gentamycin. This system can effectively eliminate Bone marrow fibroblast contamination while has no affect on the behavior of hematopoietic cells.

V Characterization of Hematopoietic Cells

Because Hematopoietic Cells are very heterogeneous, containing many cell types, many assays have been developed in order to characterize the cell populations that are present in a sample. Upon culture of these cells, the ratio of different populations may change significantly, such that the total cell number generated is not an adequate measure of outcome. Therefore, the use of appropriate assays is critical to determine the success of particular culture technique. Histology is the first method to assay Hematopoietic Cells. These methods utilize spreads of Wright-Giemsa stained cells under oil-immersion microscopy, or automated instruments that have been developed tocarry out these differentials (counting of different cell types). It is most useful for assessing mature cell populations with large numbers and distinctive morphological features. Flow cytometry has been used extensively in the study of Hematopoietic Cells. Antibodies detecting different cell types have been developed. Because of the close relation of many cell types, combinations of antigens are often required to definitively identify a particular cell. In order to deal with rarity of stem and progenitor cells, many in vitro biological function assays have been developed, such as colony-forming unit assay (CFU) and long-term culture- initiating cell assay (LTC-IC), . Most of these assays are performed by culturing cells under defined conditions and examining their progeny, both in number and type.

VI References:

1. Koller, M.R.P., B. O.; Masters, J. R. W., *Human Cell Culture: Primary Hematopoietic Cells*. 1999: Springer.
2. Ivanovic Z, Belloc F, Faucher JL, Cipolleschi MG, Praloran V, Dello Sbarba P. *Hypoxia maintains and interleukin-3 reduces the pre-colony-forming cell potential of dividing CD34(+) murine bone marrow cells.* Exp Hematol. 2002 Jan; 30(1):67-73.
3. Rosler ES, Brandt JE, Chute J, Hoffman R. *An in vivo competitive repopulation assay for various sources of human hematopoietic stem cells.* Blood. 2000 Nov 15; 96(10):3414-21.
4. Okubo T, Matsui N, Yanai N, Obinata M. *Stroma-dependent maintenance of cytokine responsive hematopoietic progenitor cells derived from long-term bone marrow culture.* Cell Struct Funct. 2000 Apr; 25(2):133-9.
5. Ploemacher RE, Engels LJ, Mayer AE, Thies S, Neben S. *Bone morphogenetic protein 9 is a potent synergistic factor for murine hemopoietic progenitor cell generation and colony formation in serum-free cultures.* Leukemia. 1999 Mar; 13(3):428-37.

Mouse Bone Marrow PrimaCell™: Bone Marrow Hematopoietic Cell

Mouse Bone Marrow Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82003	**Mouse Bone Marrow PrimaCell™ System**	**kit**	**$499**
7-62021	Mouse Bone Marrow Fibroblast Growth Inhibitors (for 500 ml medium), Bone Marrow FibrOut™	1 ml	$146
9-42002	Mouse Bone Marrowl PrimaCell™ Basal Medium	500 ml	$61
9-32021	Mouse Bone Marrow Hematopoietic cells PrimaCell™ Growth Medium Supplements and serum (for 500 ml medium)	set	$140
4-22021	Mouse Bone Marrow Tissue Disassociation system	ea	$128
9-92002	Mouse Bone Marrow Tissue Preparation Buffer Set	ea	$75

Mouse Bone Marrow Primary Cell Characterization

6-13011	**Mouse Bone Marrow Hematopoietic Cell Primarker™ Kit**	**kit**	**$220**
6-13012	Mouse Bone Marrow Hematopoietic Cell Primarker™ antibody set	set	$180
6-13013	Mouse Bone Marrow Hematopoietic Cell Primarker™ buffer system	set	$90

Mouse Bone PrimaCell™: Osteoblasts
(Cat No. 2-82002)

I. General Description

An osteoblast (from the Greek words for "bone" and "germ" or embryonic) is a mononucleate cell that is responsible for bone formation. Osteoblasts produce osteoid, which is composed mainly of Type I collagen. Osteoblasts are also responsible for mineralization of the osteoid matrix. Primary cultures of osteoblasts are advantageous for studies of bone cell metabolism and differentiation because they retain a normal genotype.

Although bone is mechanically difficult to handle, thin slices treated extensively with Tissue Preparation Buffer subsequently digested in bone tissue dissociation system provide in the kit, give rise to cultures of osteoblasts that have some functional characteristics of the tissue. This protocol is developed for attachment and growth of normal Mouse Bone cells from newborn or adult Mouse Bone with Mouse Bone PrimaCell™ system (Cat No. 2-82002). This system provides an optimal condition of tissue dissociation system, Bone OptiTDS™ that yields 5-7 times of single cells more than most of the tissue dissociation protocols published in the literature. In addition, this system ensures a high viability of the target cells with improved gradient contained in the culture medium. With CHI's proprietary fibroblast inhibitory system, FibrOut™, cells are growing with contamination of minimized amount of the non-epithelial cells.

The Bone PrimaCell™ system is suited for culturing Osteoblasts from bone tissues of Mouse.

1.1 The Bone PrimaCell™ system include:

- **Mouse Bone Tissue Dissociation System, Bone OptiTDS™** (2 × 1.0 ml) --- *collagenase I with a modified reconstitution buffer*.
- **Mouse Bone OptiTDS™ Digestion Buffer,** (2 × 9 ml)
- **Bone Fibroblast Growth Inhibitors, Bone FibrOut™** (5 × 1.0 ml) --- *A mixture of cis-OH-proline, collagenase, Gentamycin.*
- **Mouse Bone Tissue Washing Medium**, (1 × 100 ml) --- *A modified HBSS with 5% BSA, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Mouse Bone Osteoblasts PrimaCell™ Medium Supplements With Serum** (5 × 1.0 ml): *A mixture of highly purified and special treated serum and proprietary osteoblast supplements.*
- **Basal Bone Osteoblasts PrimaCell™ Basal Culture Medium (**5 × 100 ml) --- A modified Ham's F12 culture medium.
- **Mouse Bone Tissue Preparation Buffer** (1 × 100 ml) --- A mixture of EDTA/EGTA solution.

1.2 Required Materials but not provided

- Centrifuge tube, 50 ml
- Nylon gauze cell strainer (BD Bioscience)
- Petri dishes, collagenase -I coated, 100 mm (Corning, NY)

- Scalpels, curved forceps
- 70% ethanol, sterile
- PBSA (PBS containing 10% BSA), sterile.
- 9-cm Petri dishes
- 25- or 75-cm2 flasks (Corning, Falcon, Nunc)

II. Procedures

2.1 Material Preparation

Bone culture suffers from the inherent problem that the hard nature of the tissue makes manipulation difficult. However, conventional primary explant culture or digestion in collagenase I release cells that may be passaged in the usual way. All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, culture plate (Corning, NY) must be coated with collagen-I.

2.2 Surgical specimens

Mouse bone: Mouse primary osteoblasts should be isolated from bone samples from new born mice (about 2-4-week old) under sterile conditions. Each mouse bone yield large number of cells (5-10 × 10^6/1 g tissue specimen), with a 30-40% plating efficiency.

1. The bone specimens for culture should be immediately placed in a 100-mm tissue culture dish containing 5-10 ml of Mouse Bone Tissue Washing Medium.
2. Wash trabecular bone specimens repeatedly with Mouse Bone Tissue Washing Medium to remove the fat and blood cells. The trabeculae are excised with scalpel and forceps under sterile conditions.
3. After collecting as much bone as possible, wash the remaining blood and fat cells away by rinsing the specimens three times with Mouse Bone Tissue Washing Medium and cut into 2-5 mm fragments.
4. Wash the cut trabeculae with Mouse Bone Tissue Washing Medium.
5. Discard the Mouse Bone Tissue Washing Medium and incubate tissue specimens with 5-10 ml 70% ethanol for 1 min, followed by inoculating with 5-10 ml fresh Mouse Bone Tissue Preparation Buffer for 5 min, and finally wash the bone samples in 5-10 fresh Tissue Washing Medium.

2.3 Tissue Preparation and Dissociation

2.3.1 Mouse Bone OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Mouse Bone Tissue Dissociation System, Bone OptiTDS™, is suited for optimal dissociation of normal adult and newborn Bone biopsies samples to yield maximum number of single primary cells of colonic tissues.

2.3.2 Enzyme Compositions

- Collagenase I: from *Clostridium Histolyticum*

2.3.3 System Components

- Mouse Bone Tissue Dissociation System, Bone OptiTDS™ (2 × 1ml)
- Mouse Bone OptiTDS™ Digestion Buffer, (2 × 9 ml)

2.3.4 Procedures For Tissue Dissociation

6. Prepare fresh enzyme working solutions: to each vial of Mouse Bone Tissue Dissociation System, Bone OptiTDS™, add 1.0 ml of the Mouse Bone OptiTDS™ Reconstitution Buffer, Mix well.
7. Add 1.0 ml of the fresh enzyme working solution to one vial of Mouse Bone OptiTDS™ Digestion Buffer (9 ml). Warm the diluted enzyme working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 5-7 mg tissue samples per 10 ml diluted enzyme working solutions.
8. Place the pieces of bone in a small sterile bottle with a magnetic stirrer, and add 4 ml of digestion solution. (This amount should cover the bone specimens.)
9. Stir the solution containing bone fragments at room temperature for 45 min.
10. Remove the suspension of released cells and discard it, since these cells are most likely to contain fibroblasts.
11. Add a second aliquot of 4 ml of digestion solution to the bone fragments, and stir the mixture at room temperature for 30 min.
12. If necessary, the disassociation step is repeated several more times. With large amounts of bone, the digestion period can be increased to 1-3 h. .

2.3.5 Storage:

Reconstituted tissue dissociation systems should occur before use and can only be stored for 2-4 days at 4 ^{o}C. For long-term use, it should be aliquoted and stored at -20 ^{o}C. Avoid repeated freeze-thaw cycles.

2.4 Culture of Primary Mouse Osteoblasts

2.4.1 Medium Preparation.

Thaw out Mouse Bone PrimaCell™ Basal Culture Medium, Mouse Bone Osteoblasts PrimaCell™ Medium Supplements With Serum, and Mouse Bone Fibroblast Growth Inhibitors, Bone FibrOut™ on ice.

Mouse Complete Bone PrimaCell™ Culture Medium: To every 100 ml Mouse Bone PrimaCell™ Basal Culture Medium, add 10 ml Mouse Bone Osteoblasts PrimaCell™ Medium Supplements With Serum, mix thoroughly and warm the complete medium at a 37 ^{o}C water bath for 10 min prior use.

Mouse Complete Bone PrimaCell™ Culture Medium containing FibrOut™: To every 100 ml Mouse Bone PrimaCell™ Basal Culture Medium, add 10 ml Mouse Bone Osteoblasts PrimaCell™ Medium Supplements With Serum, one vial of Mouse Bone Fibroblast Growth Inhibitors, Bone FibrOut™, mix thoroughly and warm the complete medium at a 37 ^{o}C water bath for 10 min prior use. ***Important:*** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination.*

2.4.2 Primary Cell Isolation and Culture

13. Collect the digestion solution from bone fragments, and centrifuge it for 2 min at 580 g at room temperature.
14. After removing the supernatant, suspend the cells in 4 ml of Mouse Complete Bone PrimaCell™ Culture Medium containing FibrOut™, and count the cells.
15. Centrifuge the suspension at 580 g for 10 min, and resuspend the cells in 4 ml of Mouse Complete Bone PrimaCell™ Culture Medium containing FibrOut™. This suspension will become the inoculums.
16. Preincubate 75-cm^2 flasks for 20 min with 8 ml of Mouse Complete Bone PrimaCell™ Culture Medium containing FibrOut™ to equilibrate the tissue for digestion procedures.
17. Remove the preincubation solution and add 2 ml of Mouse Complete Bone PrimaCell™ Culture Medium containing FibrOut™.
18. Add 4 ml of medium containing the cell suspension. The inoculums should contain 6,000-10,000 cells per cm^2 of surface area.
19. Finally, add another 6 ml of Mouse Complete Bone PrimaCell™ Culture Medium containing FibrOut™, to give a total volume of 12 ml.
20. In the interim, add an additional 4 ml of digestion solution to the remaining pieces of bone, and repeat the digestion for 30 min. The released cells are harvested, and, if necessary, the digestion step is repeated several more times. With large amounts of bone, the digestion period can be increased to 1-3 h. Cell counts are performed after each digestion period, and the released cells are used to inoculate a different flask.

2.4.3 Subculture

21. Remove the pieces of explant.
22. Remove the medium and rinse the cell layer with Mouse Bone Tissue Washing Medium, 0.2 ml/ cm^2.
23. Add trypsin to the flask, 0.1 ml/ cm^2, and incubate at 37˚C until the cells have detached and separated from one another. Monitor cell detachment and separation on the microscope. In general, a10-min incubation is sufficient.
24. Transfer the released cells to a centrifuge tube with an equal volume of Mouse Complete Bone PrimaCell™ Culture Medium.
25. Centrifuge the cells at 600 g for 5 min.
26. Discard the supernatant, and resuspend the cells in Complete Bone PrimaCell™ Culture Medium by gentle, repeated pipetting.
27. Set one aliquot aside for the determination.
28. Inoculate the remaining cells into culture flasks or wells that have previously been equilibrated with medium. The cells should reattach within 24 h.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample; the best results are reported when cells from preconfluent primary cultures are used.

29. Detach cells as for the subculture, and centrifuge at 100 g for 10 min.
30. Resuspend cells in Mouse Complete Bone PrimaCell™ Culture Medium and count.
31. Dispense aliquots of 2×10^6 cells/ml in Mouse Complete Bone PrimaCell™ Culture Medium with 10% glycerol into cryopreservation tubes.

32. Equilibrate at 4℃ for 1-2 h.
33. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
34. To recover cells:
    a) Thaw cryotubes quickly in a 37℃ water bath.
    b) Dilute cells tenfold with medium.
    c) Centrifuge cells and resuspend them at an appropriate concentration in the desired Mouse Complete Bone PrimaCell™ Culture Medium, and seed culture vessel.

IV Fibroblast Contamination

There are several techniques have been published in the literature to deal with fibroblast contamination during colorectal primary cell culture. These include: (1) Physically remove a well-isolated fibroblast colony by scraping it with a sterile blunt instrument (e.g., a cell scraper). Care has to be taken to wash the culture up to six times to remove any fibroblasts that have detached in order to prevent them from reseeding and reattaching to the flask. (2) Differential trypsinization can be attempted with the carcinomas. (3) Dispase preferentially (but not exclusively) removes the epithelium during passaging and leaves behind most of the fibroblastic cells attached to the culture vessel. During subculture, cells that have been removed with dispase can be preincubated in plastic petri dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the osteoblasts. (4) Reduce the concentration of serum to about 2.5-5% if there are heavy concentrations of fibroblastic cells. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and that using any or all of the preceding techniques will eventually push the cells through so many divisions that any fibroblasts will senesce.

Mouse Bone PrimaCell™ system includes a fibroblast elimination system, the Mouse Bone Fibroblast Growth Inhibitors, Bone marrow FibrOut™. It contains a mixture of cis-OH-proline, collagenase, and gentamycin. This system can effectively eliminate Bone fibroblast contamination while has not affect on the behavior of osteoblasts.

V Characterization of osteoblasts

In order to characterize the mouse osteoblasts, various analysis need to be performed. Due to presence of large amount of rough endoplasmic reticulum, cytoplasm of osteoblasts appears to be basophilic via normal HE stain. A large Golgi apparatus is also present in the centre, and the nucleus is spherical and large. These include measurement of cell growth, alkaline phosphatase, and response to PTH by measuring cyclic adenosine monophosphate (cAMP) production. Several genes, such as alkaline phosphatase, osteocalcin, and Cbfa1/Osf2, are known to be regulated during osteoblastic differentiation and are commonly used as “osteoblast markers” for *in vitro* or in *vivo* studies. In addition, osteoblasts can be stained for procollagen type 1 (PICP), alpha 1(I) procollagen, Bone Gla Protein (BGP), Bone Sialoprotein (BSP), etc. In some case, one can determine the appearance of osteoblasts by immunohistochemical staining of AP and collagen I (Figure 8 and Figure 9).

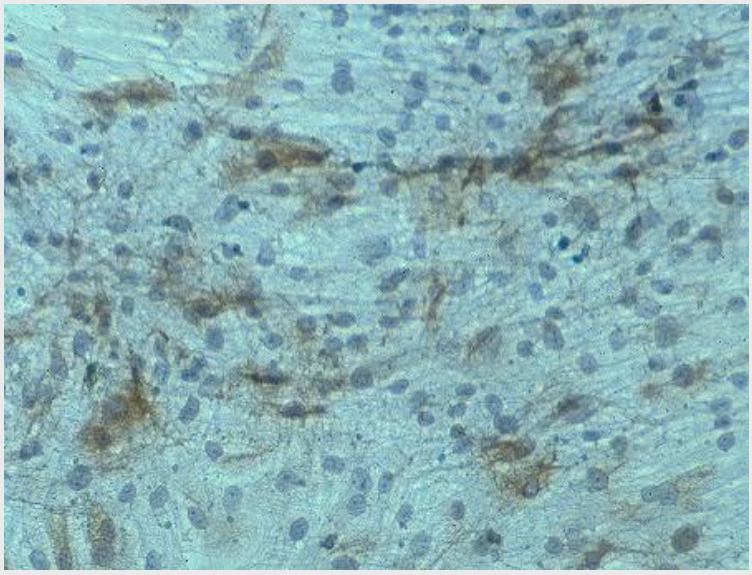


Figure 8: Histochemistry of AP in osteoblast cultures from the maxilla. Shown here are the blue / purple staining of AP positive cells (x300)(Günter Lauer, 2001, ref. 2).

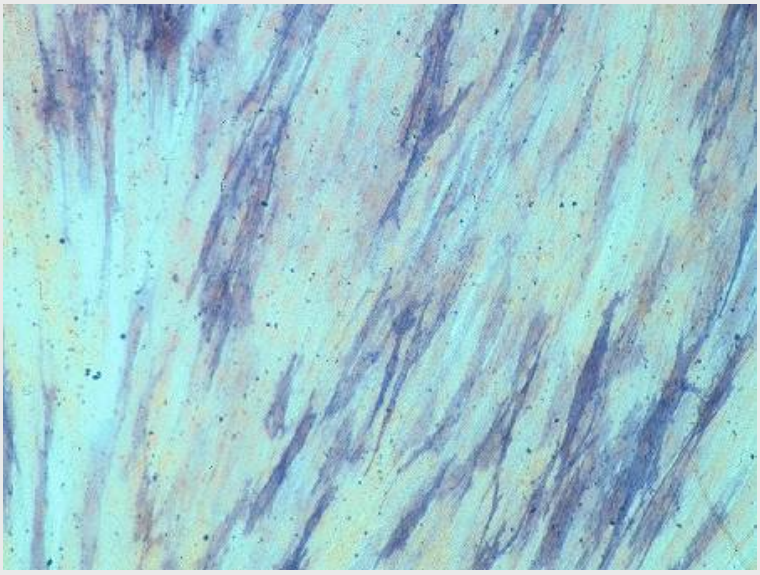


Figure 9: **ımmunohistochemistry of collagen I in osteoblast cultures from the maxilla** (× 150)(Günter Lauer, 2001, ref. 2).

VI References:

1. Garcia, T., Roman-Roman, S., Jackson, A., Theilhaber, J., Connolly, T., Spinella-Jaegle, S., Kawai, S., Courtois, B., Bushnell, S., Auberval, M*., et al.* (2002). *Behavior of osteoblast, adipocyte, and myoblast markers in genome-wide expression analysis of mouse calvaria primary osteoblasts in vitro.* Bone *31*, 205-211.
2. Günter Lauer, R.S. (2001). *Isolation of human osteoblasts and in vitro amplification for tissue engineering and subsequent bone repair.* Int Poster J Dent Oral Med.
3. Ongphiphadhanakul, B., Jenis, L.G., Braverman, L.E., Alex, S., Stein, G.S., Lian, J.B., and Baran, D.T. (1993). *Etidronate inhibits the thyroid hormone-induced bone loss in rats assessed by bone mineral density and messenger ribonucleic acid markers of osteoblast and osteoclast function.* Endocrinology *133*, 2502-2507.
4. Zhou, H., Choong, P., McCarthy, R., Chou, S.T., Martin, T.J., and Ng, K.W. (1994). *In situ hybridization to show sequential expression of osteoblast gene markers during bone formation in vivo.* J Bone Miner Res *9*, 1489-1499.

Human Bone PrimaCell™: Osteoblasts

Human Bone Primary Cell Culture

Cat No.	Description	Qt.	Price
2-96151	**Human Bone PrimaCell™ System**	**kit**	**$550**
7-66152	Human Bone Fibroblast Growth Inhibitors, Bone FibrOut™ (for 500 ml medium)	1 ml	$194
9-46015	Human Bone Osteoblasts PrimaCell™ Basal Culture Medium	500 ml	$73
9-36152	Human Bone Osteoblasts PrimaCell™ Medium Supplements With Serum (for 500 ml medium)	set	$160
4-26152	Human Bone Tissue Dissociation System, Bone OptiTDS™	ea	$146
9-86015	Human Bone Tissue Preparation Buffer Set	ea	$90

Human Bone Primary Cell Characterization

6-34011	**Human Osteoblast Primarker™ Kit**	**kit**	**$220**
6-34012	Human Osteoblast Primarker™ antibody set	set	$180
6-34013	Human Osteoblast Primarker™ buffer system	set	$90

Mouse Brain PrimaCell™ I: Cerebellar Granule Cells
(Cat No. 2-82005)

I. General Description

Neurons are extremely fastidious in their choice of substrate. Nuron cells usually grow very poorly on untreated glass or plastic surfaces, but demonstrate neurite outgrowth on collagen and poly-D-lysine. Neurite outgrowth is stimulated by the polypeptide Nerve Growth Factor (NGF) and several other unique growth factors secreted by glial cells. Most tissue culture conditions used for neurons do not support neuronal proliferation, even with neurons isolated from embryonic stages where evidence of mitosis was observed *in vivo*. Recently, however, breakthroughs in techniques have identified methods of propagating neurons in ways which allow proliferation *in vitro* and relocalization of those neurons *in vivo*.

Cerebellar granule cells in culture provide a well characterized neuronal cell population that is well suited for morphological and biochemical studies of cellular and molecular correlates of mechanisms of survival/apoptosis and neurodegeneration/neuroprotection. Cerebellar granule cell is one of the most reliable models for the study of neural development, function and pathology. The Mouse Brain PrimaCell kit (Cat No. 2-82005) allows the isolation of cerebellar granule cells from14-15 day old mice. Non-neuronal cells are prevented from interfering with the target cells by the addition of Brain Fibroblast Growth Inhibitors, FibrOut™ to the culture medium.

1.1 Components of the Mouse Brain PrimaCell™ I System

- **Mouse Brain Tissue Dissociation System, Brain OptiTDS™** (2 x 1 ml) --- *A mixture of collagenase, collagenase I, Trypsin and Mouse Brain OptiTDS™ Reconstitution Buffer.*
- **Mouse Brain OptiTDS™ Digestion Buffer,** (2 x 9 ml).
- **Mouse Brain Fibroblast Growth Inhibitors, Brain FibrOut™** --- Cervi× FibrOut™ (5 x 200 µl) --- *A mi×ture of cis-OH-proline, collagenase and gentamycin.*
- **Mouse Brain Cerebellar Granule Cells PrimaCell™ Basal Culture Medium,** (5 x 100 ml) --- A *Modified DMEM medium*.
- **Mouse Brain Cerebellar Granule Cells PrimaCell™ Medium Supplements,** (5 x 1 ml): *Glucose, L-glutamine, KCl, Insulin, P-aminobenzoic acid, and Gentamycin.*
- **Mouse Brain PrimaCell™ Serum,** (5 x 10 ml) --- *A modified heat inactivated fetal calf serum.*
- **Mouse Brain Tissue Washing Medium,** (1 x 100 ml): *HBSS with 200U/ml penicillin, 200 µg/ml streptomycin, 50 µg/ml gentamycin and 5% BSA.*
- **Brain PrimaCell™ Culture Dish Coating Solution,** (1 x 100 ml) --- *Poly-L-lysine solution.*

1.2 Required materials but NOT included

- PBS
- Water bath
- Tissue culture dishes

- 35-mm tissue culture Petri dishes
- Scalpels, scissors, and forceps
- Pasteur pipettes and 10-ml pipettes
- Test tubes, 12 and 50 ml

1.2 Siliconization of Pasteur pipettes

- Prepare a diluted Apuasil solution provided in this kit.
- Dip the pipettes into the solution and flush out the insides of the pipettes.
- Air-dry the pipettes for 24 h, or dry for several minutes at 100° C.
- Sterilize the pipettes by dry heat.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, fresh Brain PrimaCell™ Culture Dish Coating Solution-coated plate or culture dishes are recommended (see below for treatment of culture dishes).

Treatment of Culture Dishes:

.

1. Add 1 ml of Brain PrimaCell™ Culture Dish Coating Solution to each of the 35-mm tissue culture dishes.
2. Incubate the tissue culture dishes with the Brain PrimaCell™ Culture Dish Coating Solution for a minimum of 2 hrs, and aspirate.
3. Air dry tissue culture plates in a hood.

2.2 Procedure Outline

The cerebella from four to eight neonatal rats or mice are cut into small cubes and incubated with the Brain Tissue Dissociation System, Brain OptiTDS™. The provided Brain OptiTDS™ is diluted 1:10 in the provided Mouse Brain OptiTDS™ Digestion Buffer and the diluted Brain OptiTDS™ is incubated with brain tissue at a ratio of 10 ml diluted Brain OptiTDS™ solution per 4-5 g tissue sample for 30 min at 37° C. The cell suspension is seeded in freshly prepared Brain PrimaCell™ Culture Dish Coating Solution-coated culture dishes or flasks prepared above.

2.3 Mouse Cerebella Tissue Preparation

Mouse Brain: Brain from 7-8 day rats yield a large number of cells ($5\text{-}10 \times 10^6$), with a 30-40% plating efficiency. Rats are sacrificed by CO_2 narcosis or a method that is approved by the home institution. Carefully remove the skin and dissect out the area containing the cerebella.

4. Dissect out the cerebella aseptically and place in Mouse Brain Tissue Washing Medium at room temperature.
5. Mince the tissue using scalpels into small cubes measuring approximately 0.5 mm^3.
6. Transfer the minced tissue into 15 ml conical tubes and wash the tissue three times with Mouse Brain Tissue Washing Medium. Allow the tissue to settle to the bottom of the tubes

between each washing.

2.4 Cerebella Separation and Tissue Dissociation

2.4.1 Mouse Brain OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. Mouse Brain Tissue Dissociation System is suited for optimal dissociation of normal mouse cerebella tissues to yield maximum number of single Cerebellar Granule Cell (CGC).

2.4.2 Brain OptiTDS™ Compositions

- Collagenase: from *Clostridium Histolyticum*
- Collagenase I: from *Clostridium Histolyticum*
- Trypsin

2.4.3 System Components

- Mouse Brain Tissue Dissociation System, Brain OptiTDS™, (2 × 1 ml).
- Mouse Brain OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

7. Reconstitute the Mouse Brain Tissue Dissociation System stock solution by adding 1 ml Mouse Brain OptiTDS™ to 9 ml of Mouse Brain OptiTDS™ Digestion Buffer, mix well. We recommend using 10 ml diluted Brain OptiTDS™ solution per 3-5 g of brain tissue and follow by incubating in a water bath for 30 min at 37° C.
8. Disaggregate the tissue by trituration through a siliconized Pasteur pipette - until a single-cell suspension is obtained.

2.6 Cerebellar Granule Cell Isolation and Culture

2.6.1 Medium Preparation

Thaw out Mouse Brain Cerebellar Granule Cells PrimaCell™ Medium Supplements, Mouse Brain PrimaCell™ Serum, and Mouse Brain Fibroblast Growth Inhibitors, Brain FibrOut™ on ice.

Mouse Complete Brain PrimaCell™ I Culture Medium: To every 100 ml Mouse Brain PrimaCell™ Basal Culture Medium, add 10 ml Mouse Brain Cerebellar Granule Cells PrimaCell™ Medium Supplements, 10 ml Mouse Brain PrimaCell™ Serum, mix thoroughly and warm the complete medium at a 37 ^{o}C water bath for 10 min prior use.

Mouse Complete Brain PrimaCell™ I Culture Medium containing FibrOut™: To every 100 ml Mouse Brain PrimaCell™ Basal Culture Medium, add 10 ml Mouse Brain Cerebellar Granule Cells PrimaCell™ Medium Supplements, 10 ml Mouse Brain PrimaCell™ Serum, and one vial of Mouse Brain Fibroblast Growth Inhibitors, Brain FibrOut™, mix thoroughly and warm the complete medium at a 37 ^{o}C water bath for 10 min prior use. ***Important:*** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell*

contamination.

9. Once all cells have been separated, allow the cell suspension to remain in the 50 ml conical for 3-5 min, allowing the small clumps of tissue to settle to the bottom of the tube. Carefully remove these clumps with a Pasteur pipette.
10. Centrifuge the single-cell suspension at 200 g for 5 min, and carefully aspirate off the supernatant.
11. Re-suspend the pellet in Complete Brain Medium (as described in step 5), and seed the cells at a concentration of 2.5-3.0 × 10^6 cell/dish on previously treated culture plates.
12. After 2-4 days (best results are usually obtained after 2 days), continue to culture the cells with Complete Media containing the Mouse Brain Fibroblast Growth Inhibitors, Brain FibrOut™ for 24 h. FibrOut™ is used at 1:500 dilution in Complete Media.
13. After 24-48 hrs or culturing, switch to Complete Brain Medium without FibrOut™.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample; the best results are reported when cells from preconfluent primary cultures are used.

14. Detach cells as for the subculture, and centrifuge at 100 g for 10 min.
15. Resuspend cells in Mouse Complete Brain PrimaCell™ I Culture Medium and count.
16. Dispense aliquots of 2 × 10^6 cells/ml in Mouse Complete Brain PrimaCell™ I Culture Medium with 10% glycerol into cryopreservation tubes.
17. Equilibrate at 4℃ for 1-2 h.
18. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
19. To recover cells:

a) Thaw cryotubes quickly in a 37℃ water bath.
b) Dilute cells tenfold with medium.
c) Centrifuge cells and resuspend them at an appropriate concentration in the desired Mouse Complete Brain PrimaCell™ I Culture Medium, and seed culture vessel.

IV Characterization

Neurons can be identified immunologically using neuron-specific enolase antibodies or by using tetanus toxin as a neuronal marker. It is reported that alpha 6 subunit of gamma-aminobutyric type A receptors is used to detect the purity of cerebellar granule cells. Astrocyte contamination can be assessed by staining cultures with glial fibrillary acidic protein as a marker.

V References

1. Beattie, C.E., and Siegel, R.E. (1993). *Developmental cues modulate GABAA receptor subunit mRNA expression in cultured cerebellar granule neurons*. J Neurosci 13, 1784-1792.
2. Contestabile, A. (2002). *Cerebellar granule cells as a model to study mechanisms of neuronal apoptosis or survival in vivo and in vitro*. Cerebellum (London, England) 1, 41-55.
3. Hansen, S.L., Ebert, B., Fjalland, B., and Kristiansen, U. (2001). *Effects of GABA (A) receptor partial agonists in primary cultures of cerebellar granule neurons and cerebral cortical neurons reflect different receptor subunit compositions.* British journal of pharmacology 133, 539-549.
4. Jones, A., Bahn, S., Grant, A.L., Kohler, M., and Wisden, W. (1996). *Characterization of a*

cerebellar granule cell-specific gene encoding the gamma-aminobutyric acid type A receptor alpha 6 subunit. J Neurochem 67, 907-916.

5. Lindholm, D., Castren, E., Tsoulfas, P., Kolbeck, R., Berzaghi Mda, P., Leingartner, A., Heisenberg, C.P., Tessarollo, L., Parada, L.F., and Thoenen, H. (1993a). *Neurotrophin-3 induced by tri-iodothyronine in cerebellar granule cells promotes Purkinje cell differentiation.* J Cell Biol 122, 443-450.
6. Lindholm, D., Dechant, G., Heisenberg, C.P., and Thoenen, H. (1993b). *Brain-derived neurotrophic factor is a survival factor for cultured rat cerebellar granule neurons and protects them against glutamate-induced neurotoxicity.* Eur J Neurosci 5, 1455-1464.
7. New, D.R., Maggirwar, S.B., Epstein, L.G., Dewhurst, S., and Gelbard, H.A. (1998). *HIV-1 Tat induces neuronal death via tumor necrosis factor-alpha and activation of non-N-methyl-D-aspartate receptors by a NFkappaB-independent mechanism.* J Biol Chem 273, 17852-17858.
8. Popp, R.L., Lickteig, R.L., and Lovinger, D.M. (1999). *Factors that enhance ethanol inhibition of N-methyl-D-aspartate receptors in cerebellar granule cells.* The Journal of pharmacology and e×perimental therapeutics 289, 1564-1574.
9. Sui, Z., Sniderhan, L.F., Fan, S., Kazmierczak, K., Reisinger, E., Kovacs, A.D., Potash, M.J., Dewhurst, S., Gelbard, H.A., and Maggirwar, S.B. (2006). *Mouse immunodeficiency virus-encoded Tat activates glycogen synthase kinase-3beta to antagonize nuclear factor-kappaB survival pathway in neurons.* Eur J Neurosci 23, 2623-2634.

Mouse Brain PrimaCell™ I: Cerebellar Granule Cells

Mouse Brain Primary Cell Culture I

Cat No.	Description	Qt.	Price
2-82005	**Mouse Brain PrimaCell™ I System**	**kit**	**$499**
7-62041	Mouse Brain Fibroblast Growth Inhibitors, Brain FibrOut™ (for 500 ml medium)	1 ml	$146
9-42004	Mouse Brain Cerebellar Granule Cells PrimaCell™ Basal Culture Medium	500 ml	$61
9-32041	Mouse Brain Cerebellar Granule Cells PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
4-22041	Mouse Brain Tissue Dissociation System, Brain OptiTDS™	ea	$128
9-92004	Mouse Brain Tissue I Preparation Buffer Set	ea	$75

Mouse Primary Cerebellar Granule Cells Characterization

6-15011	**Mouse Cerebellar Granule Cell Primarker™ Kit**	**kit**	**$220**
6-15012	Mouse Cerebellar Granule Cell Primarker™ antibody set	set	$180
6-15013	Mouse Cerebellar Granule Cell Primarker™ buffer system	set	$90

Mouse Brain PrimaCell™ II: Olfactory Bulb Ensheathing Cells
(Cat No. 2-82006)

I. General Description

Olfactory bulb ensheathing cells have a unique biology and thus are widely used for experiments to study the activity of oncogenes in epithelial neoplasias, and the molecular mechanisms implicated in warts and other brain disorders. In addition, several *in vitro* brain models have been developed that accurately mimic the epidermis making it possible to study the brain in a physiologically relevant context. While these cells are extremely useful in the laboratory they are notoriously difficult to isolate and culture. The Mouse Brain PrimaCell™ II kit (Cat No. 2-82006) is designed for the successful isolation and culture of Olfactory bulb ensheathing cells from mouse brain allowing reproducible and dependable results.

1.1 Components of the Mouse Brain PrimaCell™ I System

- **Mouse Brain Tissue Dissociation System, Brain OptiTDS™** (2 × 1 ml) --- *A mixture of collagenase, collagenase II, Dexoyribonuclease I and Mouse Brain OptiTDS™ II Reconstitution Buffer.*
- **Mouse Brain OptiTDS™ Digestion Buffer,** (2 × 9 ml)
- **Mouse Brain Tissue Washing Medium,** (1 × 100 ml) --- (1 × 100 ml) --- *A modified Leibowitz L-15 with 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin.*
- **Mouse Brain Fibroblast Growth Inhibitors, Brain FibrOut™** --- Brain FibrOut™ (5 × 200 μl) --- *A mixture of cis-OH-proline, collagenase and gentamycin.*
- **Mouse Brain PrimaCell™ II Basal Culture Medium** (5 × 100 ml) --- A *Modified mixture of DMEM and glucose*.
- **Mouse Brain PrimaCell™ II Medium Supplements** (5 × 1 ml) --- *A mixture of gentamycin, BSA Pathocyte, Glutamine, Bovine pancreas insulin, Transferrin, Progesterone, Putrescine, L-thyroxine, Selenium, and 3,3'5-triiodo-L-thyronine.*
- **Mouse Brain PrimaCell™ Serum** (5 × 10 ml) --- *A modified Fetal Calf serum.*
- **Mouse Brain PrimaCell™ II Culture Dish Coating Solution** (3 × 10 ml): A modified buffer with Poly-L-lysine.

1.2 Required materials but NOT included:

- DMEM (Invitrogen Cat No. 10313-021)
- Fetal Bovine Serum (FBS, Sigma-Aldrich)
- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or mouse fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, fresh coated plate or culture dishes are recommended (see below for treatment of culture dishes). Culture plastics and glass coverslips with diluted **Mouse Brain PrimaCell™ II Culture Dish Coating Solution** (incubate for at least 1 h to a maximum of 24 h), wash the plastics and coverslips once with PBSA, and air dry them prior to use. A greater yield of cells is obtained from neonatal mouse rather than older animal, although it is possible to make preparations from animals of any age.

2.2 Principle

The protocol describes a rapid method for purifying a population of glial cells from the olfactory bulb by using cell adhesion character.

2.3 Mouse Brain Tissue Preparation

1. Sacrify the mice by decapitation.
2. Pin the head dorsal side up onto a dissecting board and spray with 70% ethanol. Dip all dissecting instruments in 70% ethanol prior to use, and shake the instruments dry.
3. Remove the skin from the head by using sharp curved scissors, and make a circular cut to remove the top of the skull, revealing the brain and the two olfactory bulbs at the tip of the nose.
4. Using curved forceps, gently release the olfactory bulbs from the brain, and place them in a Petri dish containing a 5-10 ml 70% ethanol for 1 min, followed by inoculating with 5-10 ml fresh Mouse Brain Tissue Washing Medium for 5 min.
5. Using a sterile scalpel blade, chop the olfactory bulbs into small pieces.

2.4 Epidermal Separation and Tissue Dissociation

2.4.1 Mouse Brain OptiTDS™ II

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Mouse Brain Tissue Dissociation System II is suited for optimal dissociation of normal newborn mouse brain tissues to yield maximum number of single Olfactory bulb ensheathing cells cells.

2.4.2 Enzyme Compositions

- Trypsin: from *Bovine Pancreas*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase II: from *Clostridium Histolyticum*
- Dexoyribonuclease I

2.4.3 System Components

- Mouse Brain Tissue Dissociation System II, Brain OptiTDS™ II, (2 × 1 ml).
- Mouse Brain OptiTDS™ II Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

6. Prepare fresh enzyme working solution: Add 1ml of Mouse Brain Tissue Dissociation System, Brain OptiTDS™ to one vial of Mouse Brain OptiTDS™ Digestion Buffer (9 ml). Warm the diluted Mouse Brain Tissue Dissociation System working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 5 ml Mouse Brain Tissue Dissociation System working solution.
7. Place the pieces into a small vial containing 5 ml of Mouse Brain Tissue Dissociation System working solution.
8. Incubate the pieces of olfactory bulbs at 37℃ for 30 - 45 min.

2.6 Olfactory bulb ensheathing Cells Isolation

9. Centrifuge the resultant suspension at 1,000 rpm for 5 min and remove the supernatant.
10. Resuspend the pelleted bulb tissue in 5 ml of Mouse Brain Tissue Washing Medium.
11. Glial cells are fragile; therefore, to produce a single-cell suspension, the olfactory bulb tissue must be dissociated gently, taking care not to produce air bubbles. Dissociate the tissue through 19G hypodermic needle followed by a 23G hypodermic needle.
12. Add 5 ml of Mouse Brain Tissue Washing Medium, and strain the cell mixture through a sterile cell strainer (70-100 μm) into a centrifuge tube to remove debris.

2.6 Primary Olfactory bulb ensheathing Cells Culture

2.6.1 Medium Preparation.

Thaw out Mouse Brain PrimaCell™ II Basal Culture Medium, Mouse Brain PrimaCell™ II Medium Supplements, and Mouse Brain Serum on ice.

Mouse Complete Brain PrimaCell™ II Culture Medium: To every 100 ml **Mouse Brain PrimaCell™ II Basal Culture Medium**, add 1 ml **Mouse Brain PrimaCell™ II Medium Supplements** and 10 ml **Mouse Brain PrimaCell™ Serum**, mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use.

Mouse Complete Brain PrimaCell™ II Culture Medium containing FibrOut™: To every 100 ml **Mouse Brain PrimaCell™ II Basal Culture Medium**, add 1 ml **Mouse Brain PrimaCell™ II Medium Supplements** and 10 ml **Mouse Brain PrimaCell™ Serum**, and 200 μl **Mouse Brain Fibroblast Growth Inhibitors, Brain FibrOut™** mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use. (***Important:*** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 1-2 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*)

2.6.2 Primary Cell Culture

13. Spin the suspension again at 1,000 rpm for 10 min.
14. Remove the supernatant and resuspend the cells in Mouse complete Brain PrimaCell™ II Basal Culture Medium/FibrOut yield a concentration of 8.5×10^5 cells/ml
15. The cell suspension was seeded into uncoated tissue culture plate or flask, and incubated at 37°C and 5% CO2 for 18 hours. Most of the fibroblasts attached during this first incubation period since fibroblasts will settle within one hour of seeding.

16. The supernatants from the step 15 were poured into uncoated tissue culture plate or flask, and incubated at 37°C and 5% CO2 to allow for the attachment of cells. Primary olfactory bulb ensheathing cells do not attach to uncoated slides for 96–120 hours.
17. After 36 hours of incubation, most of the ensheathing cells remained in the supernatant. This supernatant was used to seed poly-L-lysine-coated tissue culture plate or flask. After seeding, the ensheathing cells attached within 48 hours and neurons do not survive this culture environment.
18. Cells were maintained in an incubator (37°C, 5% CO_2) for 8 days, and the Mouse complete Brain PrimaCell™ II Culture Medium/FibrOut was changed every 2 days.

2.6 Subculture

Propagating Olfactory bulb ensheathing cells in culture can be somewhat challenging, however the following methods have worked consistently in many laboratories.

19. Remove complete Mouse complete Brain PrimaCell™ II Culture Medium and wash cells in 0.1% trypsin two times. Place just enough trypsin (0.1 % trypsin without EDTA) to moisten cell layer (200-500 µl depending on size of the dish).
20. Incubate for 1-5 min at 37°C. Gently pipette cells and resuspend in Mouse complete Brain PrimaCell™ II Culture Medium (FibrOut™ is not necessarily needed at this step) for counting and replatting on collagen coated tissue culture plates.
21. Gently pipette chondrocytes and resuspend in Mouse complete Brain PrimaCell™ II Culture Medium for counting and replatting on tissue culture plates.

III Cryopreservation

20. Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Olfactory bulb ensheathing cells cultures derived from preconfluent layers.
    (a) Trypsinize cells as above, and centrifuge at 100 g for 10 min.
    (b) Resuspend cells in Mouse complete Brain PrimaCell™ II Culture Medium and count cells.
    (c) Prepare aliquots of 2×10^6 cells/ml in Mouse complete Brain PrimaCell™ II Culture Medium and 10% glycerol into cryovials.
    (d) Equilibrate at 4℃ for 1-2 h.
    (e) Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
    (f) To recover cells:
        (i) Thaw cryovials quickly in a 37℃ water bath.
        (ii) Dilute cells tenfold with appropriate Mouse complete Brain PrimaCell™ II Culture Medium.
        (iii) Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in Mouse complete Brain PrimaCell™ II Culture Medium.

IV Characterization

Primary cultures of olfactory bulb ensheathing cells obtained from olfactory bulb synthesize carnosine (β-alanyl histidine). Olfactory bulb ensheathing cells can be characterized based on their specific for their glia phenotype to exclude contamination by neuronal cells which were anti-neuron specific enolase (NSE) positive. The source of tissue (embryonic, new-born, adult olfactory

bulb) as well as the culture conditions could influence the cell phenotype. The purity is determined by S100, and glial fibrillary acidic protein (GFAP), or p75 NGF receptor antibodies for the olfactory bulb ensheathing cells. Contaminating endothelial cells can be identified by antibodies against NSE, and the other cells are fibroblasts with flat or endothelial-like shape, which can be stained by antibody against Thy1.1.

V References

1. Bakardjiev, A., *Biosynthesis of carnosine in primary cultures of rat olfactory bulb.* Neurosci Lett, 1997. **227**(2): p. 115-8.
2. Wang, C.T., et al., *[Culture, purification and biological characters of olfactory ensheathing cells from adult transgenic mice].* Shi Yan Sheng Wu Xue Bao, 2005. **38**(4): p. 340-6.
3. Lipson, A.C., et al., *Neurotrophic properties of olfactory ensheathing glia.* Exp Neurol, 2003. **180**(2): p. 167-71.
4. Moreno-Flores, M.T., et al., *Immortalized olfactory ensheathing glia promote axonal regeneration of rat retinal ganglion neurons.* J Neurochem, 2003. **85**(4): p. 861-71.

Mouse Brain PrimaCell™ II: Olfactory Bulb Ensheathing Cells

Mouse Brain Primary Cell Culture II

Cat No.	Description	Qt.	Price
2-82006	**Mouse Brain PrimaCell™ II system**	**kit**	**$499**
4-22051	Mouse Brain Tissue Dissociation System, Brain OptiTDS™ (for 500 ml medium)	1 ml	$128
9-42005	Mouse Brain PrimaCell™ II Basal Culture Medium	500 ml	$73
9-32051	Mouse Brain PrimaCell™ II Medium Supplements with Serum (for 500 ml medium)	set	$140
7-62051	Mouse Brain Fibroblast Growth Inhibitors, Brain FibrOut™	ea	$146
9-92005	Mouse Brain Tissue II Preparation Buffer Set	ea	$75

Mouse Primary Olfactory Bulb Ensheathing Cells Characterization

6-16011	**Mouse Olfactory Bulb Ensheathing Cell Primarker™ Kit**	**kit**	**$220**
6-16012	Mouse Olfactory Bulb Ensheathing Cell Primarker™ antibody set	set	$180
6-16013	Mouse Olfactory Bulb Ensheathing Cell Primarker™ buffer system	set	$90

Mouse Breast PrimaCell™: Mammary Epithelium
(Cat No. 2-82004)

I. General Description:

This protocol is developed for attachment and growth of normal Mouse Breast epithelial cells from mouse mammary gland or breast tissues with Mouse Breast PrimaCell™ system (Cat No. 2-82004). This system provides an optimal condition of tissue dissociation system, Breast OptiTDS™ that yields 5-7 times of single cells more than most of the tissue dissociation protocols published in the literature. In addition, this system ensures a high viability of the target cells with improved gradient contained in the culture medium. With CHI's proprietary fibroblast inhibitory system, Breast FibrOut™, cells are growing with contamination of minimized amount of the non-epithelial cells.

The preparation of tissue specimens for cell culture is usually started within 1-2 h of removal from the animal. If this is impossible, fine cutting of the tissue into small pieces (1-2 mm) with scalpels and storage overnight at 4℃ in washing medium (see below) can also prove successful.

Mouse Breast PrimaCell™ system applies to all types of normal adult Mouse tissue samples. Tissue samples contain pathological organism (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Mouse Breast PrimaCell™ System

- **Mouse Breast Tissue Dissociation System, Breast OptiTDS™,** (2 x 1 ml) --- *A mixture of collagenase I, collagenase III, collagenase IV, collagenase, and trypsin*.
- **Mouse Breast OptiTDS™ Digestion Buffer**, (2 x 9 ml).
- **Mouse Breast Tissue Washing Medium**, (1 x 100 ml) --- *Basal Breast PrimaCell™ Culture Medium with 5% FCS, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Mouse Breast Fibroblast Growth Inhibitors, Breast FibrOut™** (5 x 200 μl) --- *A mixture of cis-OH-proline, toxin ricin, gentamycin and formulated serum substitutes*.
- **Mouse Breast PrimaCell™ Basal Culture Medium**, (5 x100 ml) --- *Modified formulation based on RPMI 1640 and DMEM medium*.
- **Mouse Breast PrimaCell™ Medium Supplements**, (5 x 1.0 ml) --- A mixture of *EGF, Insulin, Hydrocortisone, Cholera toxin, penicillin, and streptomycin*.
- **Mouse Breast PrimaCell™ Serum**, (1 x 50 ml) --- H*eat-inactivated and special-treated Fetal Calf Serum.*

1.2 Required Materials but not provided

- Pasteur pipettes
- Collagen I-coated Culture dishes
- Scalpels, scissors, and forceps
- Pasteur pipettes and 10-ml pipettes
- Test tubes, 12 and 50 ml

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, collagen I-coated plate (Corning, NY) MUST be pre-treated with 5 ml Coating Solutions for 5 min. Aspirate the coating solutions; let the dishes be air-dry in the ventilated cell culture hood for 5-10 min.

2.2 Surgical specimens

Biopsies of about 4-6 mm^3 are taken with biopsy forceps to portions of mammary glands. All surgical specimens should be immediately placed in Mouse Breast Tissue Washing Medium, transported on ice to the laboratory within 1 h and worked up immediately. With autoclaved scalpels, scissors, and forceps, carefully remove muscle and fat from specimens followed by washing procedures. Place specimens in a 10 ml falcon tube contain 5-10 ml fresh Breast Tissue Washing Medium followed by inoculating for 10 min at the room temperature. For large tissue specimens, 50 ml falcon tube and more wash medium is needed to ensure thoroughly washing. Aspirate the washing medium and repeat the washing procedures with fresh washing medium two more time. Washing tissue specimens sequentially in 70% ethanol for 1 min at the room temperature, in PBS for 5 min, and in fresh Breast Tissue Washing Medium for 10 min. Collecting tissue specimen by centrifugation prior to tissue dissociation procedures (see below).

2.3 Tissue Preparation and Dissociation

2.3.1 Mouse Breast OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Breast Tissue Dissociation System, OptiTDS™, is suited for optimal dissociation of normal adult Mouse biopsies samples to yield maximum number of single primary cells of Breast tissues.

2.3.2 Enzyme Compositions

- Collagenase I: from *Clostridium Histolyticum*
- Collagenase III: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*
- Collagenase: from *Clostridium Histolyticum*
- Trypsin: from *Bovine Pancreas*

2.3.3 System Components

- Breast Tissue Dissociation System, OptiTDS™, (2 x 1 ml).
- Breast OptiTDS™ Digestion Buffer, (2 x 9 ml).

2.3.4 Procedures For Tissue Preparation and Dissociation

1. Prepare fresh enzyme working solutions: add 1.0 ml of Breast Tissue Dissociation System, OptiTDS™, to one vial of Breast OptiTDS™ Digestion Buffer (9.0 ml). Warm the diluted enzyme working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 4-5 g tissue samples per 10 ml diluted enzyme working solutions.
2. Mince pre-washed tissue into pieces approximately 0.2-0.5 mm^2 in diameter with two scalpel and forceps.
3. Incubate minced tissues with the enzyme working solutions by incubating minced tissue samples (up to 5 g) in 10 ml fresh enzyme working solutions with slow magnetic stirring for 30 min at 37 ℃.
4. At the end of tissue dissociation period, gentle triturating tissue with a 10 ml pipette, constitutes filling the empty the barrel at a rate of 2-3 ml per second. Repeat this procedure for 5-6 times.
5. Collecting cells by filtration the mixture through a cell strainer to remove fatty layer and larger undigested tissues followed by centrifugation at 1 x 100 g. Carefully collect enzyme working solutions and resuspend the cell pellet with 1.0 ml complete culture medium.
6. Count the cells and seed cells in 3-4 T-25 collagen I-coated flasks (**Important:** pre-treat the flask with the provided Coating Solutions for 5 min and air-dry in hood prior to use) upon the density of viable cells (2.5-5 x 10^5 Cells/flask).
7. The undigested tissues can be repeated for digestion process by inoculating the tissue with the saved enzyme working solutions from step 5 for another 30 min, to obtain additional cells.

2.3.5 Storage:

Reconstituted tissue dissociation systems should occur before use and can only be stored for 2-4 days at 4 ^oC. For long-term use, it should be aliquoted and stored at -20 ^oC. Avoid repeated freeze-thaw cycles.

2.4 Culture of primary Mouse Mammary Epithelial cells

2.4.1 Medium Preparation.

Preparation of complete culture medium: thaw out one vial of **Mouse Mammary Epithelium PrimaCell™ Medium Supplements** and one vial of **Mouse Breast PrimaCell™ Serum**, one vial of **Mouse Breast Fibroblast Growth Inhibitors, Breast FibrOut™** on ice. To every 100 ml **Mouse Mammary Epithelium PrimaCell™ Basal Culture Medium**, add one vial of **Mouse Mammary Epithelium PrimaCell™ Medium Supplements**; **one vial Mouse Breast PrimaCell™ Serum**; and **one vial of Mouse Breast Fibroblast Growth Inhibitors**, **Breast FibrOut™**, mix thoroughly and warm the complete medium at a 37^oC water bath for 10 min prior use. (***Important:*** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*).

2.6.1 Treatment of Culture Dishes.

To enhance cell attachment to the culture dishes, collagen I-coated plate (Corning, NY) MUST be freshly pre-treated with the provided Coating Solutions by adding appropriate volume of Coating Solutions (enough to cover the whole cell-growth area) and incubate for 5 min. Aspirate the medium; let the dishes be air-dry in the ventilated cell culture hood for 5-10 min prior to use.

.

2.4.3 Standard primary culture conditions.

Inoculate epithelial tubules and clumps of cells derived from tissue specimens into T-25 flasks coated with collagen type I with pre-treatment of basal culture medium as described above at 37℃ in a 5%-CO_2 incubator with 4 ml of complete culture medium prepared in 2.4.1. Change the culture medium twice weekly. The tubules and cells start to attach to the substratum, and epithelial cells migrate out within 1-2 days. Most of the tubules and small clumps of epithelium attach within 4 days.

2.4.4 Alternative Primary Culture conditions.

The attachment of epithelium during primary culture and subculture is more reproducible and efficient when cells are inoculated onto collagen-coated flasks, and significantly better growth is obtained with 3T3 feeders than without. When the epithelial cells expand several passages, they become less dependent on 3T3 feeders, and no further addition of feeders is necessary. All medium and solutions described in the standard culture condition are applicable in this culture method.

2.4.5 Subculture and Propagation

Most mammary epithelium primary cultures cannot at present be passaged by routine trypsin / EDTA procedures. Disaggregation to single cells of the cultured Breast cells with 0.1% trypsin in 0.25 mM (0.1%) EDTA will result in extremely poor or even zero growth, so Dispase is used instead. One of the advantage using dispase is that dispase can only detach epithelial cell but not the fibroblast, thus increase the purity of epithelium.

8. Add 0.5% Dispase (Sigma, w/v) to the cell monolayer, just enough to cover the cells (~2.5 ml/25-cm^2 flask), and leave the solution to stand for 40-60 min for primary cultures and 20-40 min for cell lines.

9. Once the epithelial layers begin to detach (they do so as sheets rather than single cells), pipette to help detachment and disaggregation into smaller clumps.

10. Wash and replate the cells under standard culture conditions. It may take several days for clumps to attach, so replace the medium carefully when feeding.

III Fibroblast Contamination

There are several techniques have been published in the literature to deal with fibroblast contamination during colorectal primary cell culture. These include: (1) Physically remove a well-isolated fibroblasts by scraping it with a sterile blunt instrument (e.g., a cell scraper). Care has to be taken to wash the culture up to six times to remove any fibroblasts that have detached in order to prevent them from reseeding and reattaching to the flask. (2) Differential trypsinization can be attempted with the carcinomas. (3) Dispase preferentially (but not exclusively) removes the epithelium during passaging and leaves behind most of the fibroblastic cells attached to the culture vessel. During subculture, cells that have been removed with dispase can be preincubated in plastic Petri dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the epithelium. Clumps of epithelial cells still floating can be transferred to new flasks under standard culture conditions. This technique takes advantage of the

fact that fibroblasts in general attach much more quickly to plastic than do clumps of epithelial cells, so that a partial purification step is possible. (4) Reduce the concentration of serum to about 2.5-5% if there are heavy concentrations of fibroblastic cells. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and that using any or all of the preceding techniques will eventually push the cells through so many divisions that any fibroblasts will senesce.

Mouse Breast PrimaCell™ includes a fibroblast elimination system, the Mouse Breast Fibroblast Growth Inhibitors, Breast FibrOut™. It contains a mixture of cis-OH-proline, toxin ricin, gentamycin and formulated serum substitutes. This proprietary mixture kills contaminating fibroblasts, but shows no signs of toxicity toward the epithelium, whether derived from an adenoma, a carcinoma or normal Breast tissues.

IV References:

1. Kanazawa T, Enami J, Kohmoto K. *Effects of 1alpha, 25-dihydroxycholecalciferol and cortisol on the growth and differentiation of primary cultures of mouse mammary epithelial cells in collagen gel.* Cell Biol Int. 1999; 23(7):481-7.
2. Matitashvili E, Bauman DE. *Culture of primary bovine mammary epithelial cells.* In Vitro Cell Dev Biol Anim. 1999 Sep; 35(8):431-4.
3. Blatchford DR, Quarrie LH, Tonner E, McCarthy C, Flint DJ, Wilde CJ. *Influence of microenvironment on mammary epithelial cell survival in primary culture.* J Cell Physiol. 1999 Nov; 181(2):304-11.
4. Wang S, Haslam SZ. *Serum-free primary culture of normal mouse mammary epithelial and stromal cells.* In Vitro Cell Dev Biol Anim. 1994 Dec; 30A (12):859-66.
5. Taga M, Sakakura T, Oka T. *Identification and partial characterization of mesenchyme-derived growth factor that stimulates proliferation and inhibits functional differentiation of mouse mammary epithelium in culture.* Endocrinol Jpn. 1989 Aug; 36(4):559-68.
6. Imagawa W, Bandyopadhyay GK, Wallace D, Nandi S. *Phospholipids containing polyunsaturated fatty acyl groups are mitogenic for normal mouse mammary epithelial cells in serum-free primary cell culture.* Proc Natl Acad Sci U S A. 1989 Jun; 86(11):4122-6.

Mouse Breast PrimaCell™: Mammary Epithelium

Mouse Breast Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82004	**Mouse Breast PrimaCell™ system**	**kit**	**$499**
4-22031	Mouse Breast Tissue Dissociation System, Breast OptiTDS™ (for 500 ml medium)	1 ml	$128
9-42003	Mouse Mammary Epithelium PrimaCell™ Basal Culture Medium	500 ml	$61
9-32031	Mouse Mammary Epithelium PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
7-62031	Mouse Breast Fibroblast Growth Inhibitors, Breast FibrOut™	ea	$146
9-92003	Mouse Breast Tissue Preparation Buffer Set	ea	$75

Mouse Primary Mammary Epithelium Characterization

6-17011	**Mouse Mammary Epithelium Primarker™ Kit**	**kit**	**$220**
6-17012	Mouse Mammary Epithelium Primarker™ antibody set	set	$180
6-17013	Mouse Mammary Epithelium Primarker™ buffer system	set	$90

Mouse Cartilage PrimaCell™: Articular Cartilage
(Cat No. 2-82007)

I. General Description

Articular cartilage is a unique tissue where hydrostatic pressure is a significant component of the mechanical loading environment. Articular cartilage is sparsely populated by chondrocytes immobilized in the extracellular matrix. The chondrocytes, or cells of articular cartilage, represent 1% of the matrix volume, are responsible for secreting matrix molecules such as type II collagen and glycosaminoglycan (GAG) to maintain the correct size and mechanical properties of the tissue. Chondrocytes are highly specialized cells of mesenchymal origin that are responsible for synthesis, maintenance, and degradation of the cartilage matrix.Chondrocytes live in an unusual and constantly changing physicochemical environment. They receive signals during the loading of the tissue and produce, through a balance between macromolecular synthesis and degradation, a mechanically resilient extracellular matrix influenced by changes to the intracellular composition, such as cell volume, pH and ionic content. Chondrocytes have a unique biology and thus are widely used for experiments to study the molecular mechanisms implicated in cartilage associated disorders. A great deal of research in the field of rheumatology has been focused on understanding the mechanisms that induce metabolic changes in articular chondrobytes during osteoarthritis and rheumatoid arthritis. While these cells are extremely useful in the laboratory they are notoriously difficult to isolate and culture. They rapidly divide, become fibroblastic, and lose their biochemical characteristics The Mouse Cartilage PrimaCell™ kit (Cat No. 2-82007) is designed for the successful isolation and culture of chondrocytes from mouse cartilage allowing reproducible and dependable results.

1.1 Components of the Mouse Cartilage PrimaCell™ System

- **Mouse Cartilage Tissue Dissociation System , Cartilage OptiTDS™** (2 × 1 ml) --- *A mixture of collagenase I, collagenase II, Hyaluronidase I, trypsin and Mouse Cartilage OptiTDS™ Reconstitution Buffer,* (2 × 1 ml).
- **Mouse Cartilage OptiTDS™ Digestion Buffer,** (2 × 9 ml)
- **Mouse Cartilage PrimaCell™ Washing Medium** (1 × 100 ml) --- *A modified Ham's F12 with netilmycin, Vancomycin, ceftazidim, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin.*
- **Mouse Cartilage Fibroblast Growth Inhibitors, Cartilage FibrOut™** ---Cartilage FibrOut™ (5 × 200 µl) --- *A mixture of D-valine, collagenase and gentamycin.*
- **Mouse Cartilage Carilage Basic Culture Medium** (5 × 100 ml) --- A *Modified formulation based on Ham's F12 medium*.
- **Mouse Cartilage PrimaCell™ Medium Supplements** (5 × 1 ml): *gentamycin.*
- **Mouse Cartilage PrimaCell™ Washing Medium** (1 × 100 ml) --- *A modified Ham's F12 with netilmycin, Vancomycin, and ceftazidim.*
- **Mouse Cartilage PrimaCell™ Serum** (5 × 10 ml) --- *A modified Fetal Calf serum.*
- **Mouse Cartilage PrimaCell™ Gelation Solution** (5 × 10 ml) --- *A modified mixture of HEPES and $CaCl_2$.*
- **Mouse PrimaCell™ Solubilization Solution** (4 × 10 ml) --- *A modified mixture of HEPES and EDTA.*
- **Mouse PrimaCell™ Alginate Solution** (5 × 1 ml) --- *A modified mixture of HEPES Sodium*

alginateand, and NaCl.

❖ **Mouse PrimaCell™ Alginate Washing Solution** (5 × 10 ml) --- *NaCl.*

1.2 Required materials but NOT included:

- DMEM (Invitrogen Cat No. 10313-021)
- Fetal Bovine Serum (FBS, Sigma-Aldrich)
- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or mouse fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, fresh gelatin-coated plate or culture dishes are recommended (see below for treatment of culture dishes).

2.2 Principle

Among the various methods explored for maintaining the phenotype of chondrocytes, culture in alginate beads appears the most promising, since it has been shown that this culture system leads to the formation of a matrix similar to that of native articular cartilage. The system maintains the expression of the differentiated phenotype and is also able to restore it in dedifferentiated chondrocytes. Another advantage over other three-dimensional methods is that cells can easily be recovered after the culture is completed, allowing protein and gene expression studies.

2.3 Mouse Cartilage Tissue Preparation

Mouse Cartilage: Prepare cultures from knee, shoulder, and hip joints. Fetal or young donors are preferable to adults, as they provide higher quantities of cells and take longer to senesce.

1 Wash tissue pieces thoroughly with **Mouse Cartilage PrimaCell™ Washing Medium** before dissection. Dissection should begin without delay. Remove skin, muscle, and tendons from joints. Carefully take cartilage fragments from articulations that are free of connective tissue.
2 Using crossed scalpels, mince cartilage slices into 1-mm^3 pieces.
3 Transfer cartilage fragments into a 30-ml flat-bottomed vial.
4 Incubate tissue specimens with 5-10 ml 70% ethanol for 1 min, followed by inoculating with 5-10 ml fresh **Mouse Cartilage PrimaCell™ Dissection Medium** for 5 min.

2.4 Articular carilage Separation and Tissue Dissociation

2.4.1 Mouse Cartilage OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Mouse Cartilage Tissue Dissociation System is suited for optimal dissociation of normal newborn mouse cartilage tissues to yield maximum number of single chondrocyte.

2.4.2 Enzyme Compositions

- Trypsin: from *Bovine Pancreas*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase II: from *Clostridium Histolyticum*
- Hyaluronidase I

2.4.3 System Components

- Mouse Cartilage Tissue Dissociation System, Cartilage OptiTDS™, (2 × 1 ml).
- Mouse Cartilage OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

5 Prepare fresh enzyme working solution: Add 1ml of **Mouse Cartilage Tissue Dissociation System, Cartilage OptiTDS™** to one vial of **Mouse Cartilage OptiTDS™ Digestion Buffer** (9 ml). Warm the diluted **Mouse Cartilage Tissue Dissociation System** working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 5 ml **Mouse Cartilage Tissue Dissociation System** working solution.

6 Discard the **Mouse Cartilage PrimaCell™ Dissection Medium,** and add 5 – 10 ml of **Mouse Cartilage Tissue Dissociation System** working solution (prewarmed to 37℃) and incubate the fragments with moderate magnetic agitation for 30-60 min at 37 ℃ in a sealed vial at room temperature.

2.5 Chondrocytes Isolation

Note: Please read section 2.6 for specific information on chondrocytes culture and platting before preceding this section.

7 Transfer the cell suspension into a 50-ml centrifuge tube and mix on a vortex mixer for a few seconds.

8 Remove residual material left after digestion by passing the digested material through a 70-μm nylon filter.

9 Centrifuge the filtrate at 400 g for 10 min.

10 Resuspend the cell pellet in 20 ml of **Mouse Cartilage PrimaCell™ Dissection Medium**, and count the cells with a hemocytometer.

2.5 Primary chondrocytes Culture

2.5.1 Medium Preparation.

Thaw out **Mouse Cartilage PrimaCell™ II Basal Culture Medium**, **Mouse Cartilage PrimaCell™ II Medium Supplements**, and **Mouse Cartilage Serum** on ice.

Mouse Complete Cartilage PrimaCell™ II Culture Medium: To every 100 ml **Mouse Cartilage PrimaCell™ II Basal Culture Medium**, add 1 ml **Mouse Cartilage PrimaCell™ II Medium Supplements** and 10 ml **Mouse Cartilage PrimaCell™ II Serum**, mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

Mouse Complete Cartilage PrimaCell™ II Culture Medium/FibrOut: To every 100 ml **Mouse Cartilage PrimaCell™ II Basal Culture Medium**, add 1 ml **Mouse Cartilage PrimaCell™ II Medium Supplements** and 10 ml **Mouse Cartilage PrimaCell™ II Serum**, and 200 µl **Mouse Cartilage Fibroblast Growth Inhibitors, Cartilage FibrOut™** mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

2.5.2 Primary Cell Culture

(Important: Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination)

11 Centrifuge the cells at 400 g for 10 min.
12 Resuspend the cell pellet in 1 ml of Cartilage PrimaCell™ Alginate Solution, and then dilute the suspension progressively in more Cartilage PrimaCell™ Alginate Solution, until a cellular density of 2×10^6 cells/ml is reached. This progressive dilution is necessary to obtain a homogeneous cell suspension in alginate.
13 Express the cell suspension in drops through a 21 G needle into the Cartilage PrimaCell™ Gelation Solution with moderate magnetic stirring, and allow the alginate to polymerize for 10 min to form beads.
14 Wash the beads 3 times in 5 vol Cartilage PrimaCell™ Alginate Washing Solution.
15 Distribute the beads, 5 ml (1×10^7 cells), into 75-cm^2 flasks containing 20 ml of **Mouse Complete Cartilage PrimaCell™ Culture Medium/FibrOut™**.
16 Incubate the culture at 37˚C in a humidified atmosphere of 5% CO_2, and 95% air.

2.7 Recovery

17 Discard the medium.
18 Add 2 vol of Cartilage PrimaCell™ Solubilization Solution to the beads.
19 Incubate the culture 15 min at 37˚C.
20 Centrifuge the cells at 400 g for 10 min.
21 Resuspend the cell pellet in **Mouse Complete Cartilage PrimaCell™ Culture Medium/FibrOut™** containing 0.06% collagenase.
22 Incubate the cells for 30 min at 37˚C in a humidified atmosphere of 5% CO_2, and 95% air.
23 Centrifuge the cells at 400 g for 10 min.
24 Resuspend the cell pellet in **Mouse Cartilage PrimaCell™ Basal Culture Medium**, and count the cells with a hemocytometer.
25 Centrifuge the cells at 400 g for 10 min.
26 Repeat steps 28, 29, without counting the cells.

2.6 Subculture

Propagating chondrocytes in culture can be somewhat challenging, especially when chondrocytes are cultured alone on plastic, however the following methods have worked consistently in many laboratories.

27 Remove complete Mouse Chondrocytes Culture Medium and wash chondrocytes in 0.1% trypsin two times. Place just enough trypsin (0.1 % trypsin without EDTA) to moisten articular carilage layer (200-500 µl depending on size of the dish).

28 Incubate for 1-5 min at 37°C. Gently pipette chondrocytes and resuspend in complete Mouse Chondrocytes Culture Medium (FibrOut™ is not necessarily needed at this step) for counting and replatting on collagen coated tissue culture plates.

29 Gently pipette chondrocytes and resuspend in **Mouse Complete Cartilage PrimaCell™ Culture Medium** (FibrOut™ is not necessarily needed at this step) for counting and replatting on tissue culture plates.

III Cryopreservation

30 Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Chondrocytes cultures derived from preconfluent layers.

(a) Trypsinize cells as above, and centrifuge at 100 g for 10 min.

(b) Resuspend cells in **Mouse Complete Cartilage PrimaCell™ Culture Medium** and count cells.

(c) Prepare aliquots of 2×10^6 cells/ml in **Mouse Complete Cartilage PrimaCell™ Culture Medium** and 10% glycerol into cryovials.

(d) Equilibrate at 4℃ for 1-2 h.

(e) Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.

(f) To recover cells:

(g) Thaw cryovials quickly in a 37℃ water bath.

(h) Dilute cells tenfold with appropriate **Mouse Complete Cartilage PrimaCell™ Culture Medium**.

(i) Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in **Mouse Complete Cartilage PrimaCell™ Culture Medium**.

IV Characterization

The cells of articular cartilage live in an unusual and constantly changing physicochemical environment. Chondrocytes, the only cells found in cartilage, produce and maintain the cartilaginous matrix. Classic markers of a chondrocytic phenotype are—Sox9, collagen II, and aggrecan (Expression of chondrocyte markers by cells of normal and degenerate intervertebral discs). Sox9 is the major regulator of the chondrocytic phenotype. It is a potent promoter of collagen II gene expression, a molecule which is produced almost exclusively in the chondrocyte. The proteoglycan aggrecan is also a characteristic gene product of chondrocytes. Besides, there are various markers for differentiation of chondrocytes: 11-fibrau, a reliable and sensitive marker of chondrocyte phenotype, and a useful marker to characterize chondrocyte differentiation stage; Annexin VI, a marker of late chondrocyte differentiation; Cartilage oligomeric matrix protein (COMP), a sensitive marker for the differentiation state of articular primary chondrocytes; Cathepsin B, a marker of the dedifferentiated chondrocyte phenotype; cartilage matrix protein (CMP, or Matrilin-1), a mature chondrocyte marker; Collagen X and Collagen IX; etc.

V References:

1. Ikenoue, T., et al., Mechanoregulation of human articular chondrocyte aggrecan and type II collagen expression by intermittent hydrostatic pressure in vitro. J Orthop Res, 2003. 21(1): p. 110-6.
2. Ho, Y.C., et al., Highly efficient baculovirus-mediated gene transfer into rat chondrocytes. Biotechnol Bioeng, 2004. 88(5): p. 643-51.
3. Hauselmann, H.J., et al., Phenotypic stability of bovine articular chondrocytes after long-term culture in alginate beads. J Cell Sci, 1994. 107 (Pt 1): p. 17-27.
4. Hall, A.C., E.R. Horwitz, and R.J. Wilkins, The cellular physiology of articular cartilage. Exp Physiol, 1996. 81(3): p. 535-45.
5. Loeser, R.F., et al., Human chondrocyte expression of growth-arrest-specific gene 6 and the tyrosine kinase receptor axl: potential role in autocrine signaling in cartilage. Arthritis Rheum, 1997. 40(8): p. 1455-65.
6. Loeser, R.F., Jr. and R. Wallin, Vitamin K-dependent carboxylation in articular chondrocytes. Connect Tissue Res, 1991. 26(3): p. 135-44.
7. Magne, D., et al., The new IL-1 family member IL-1F8 stimulates production of inflammatory mediators by synovial fibroblasts and articular chondrocytes. Arthritis Res Ther, 2006. 8(3): p. R80.
8. Lotz, M., I. Clark-Lewis, and V. Ganu, HIV-1 transactivator protein Tat induces proliferation and TGF beta expression in human articular chondrocytes. J Cell Biol, 1994. 124(3): p. 365-71.
9. Guicheux, J., et al., Primary human articular chondrocytes, dedifferentiated chondrocytes, and synoviocytes exhibit differential responsiveness to interleukin-4: correlation with the expression pattern of the common receptor gamma chain. J Cell Physiol, 2002. 192(1): p. 93-101.

Mouse Cartilage PrimaCell™: Articular Cartilage

Mouse Cartilage Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82007	**Mouse Cartilage PrimaCell™ system**	**kit**	**$499**
4-22061	Mouse Cartilage Tissue Dissociation System, Cartilage OptiTDS™ (for 500 ml medium)	1 ml	$128
9-42006	Mouse Cartilage PrimaCell™ Basal Culture Medium	500 ml	$61
9-32061	Mouse Cartilage PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
7-62061	Mouse Cartilage Fibroblast Growth Inhibitors, Cartilage FibrOut™	ea	$146
9-98006	Mouse Cartilage Tissue Preparation Buffer Set	ea	$75

Mouse Primary Articular Cartilage Cell Characterization

6-18011	**Mouse Articular Cartilage Primarker™ Kit**	**kit**	**$220**
6-18012	Mouse Articular Cartilage Primarker™ antibody set	set	$180
6-18013	Mouse Articular Cartilage Primarker™ buffer system	set	$90

Mouse Cervix PrimaCell™: Cervical Epithelium
(Cat No. 2-82008)

I. General Description

The physiologic and pathologic conditions that affect the uterine cervix are of considerable contemporary interest. In particular, the recognition of the role of genital human papillomaviruses (HPVs) as the etiologic agent in cervical carcinoma has focused attention on the biology of cervical epithelium. Primary cervical epithelia are crucial target for pharmacotherapy. While these cells are extremely useful in the laboratory, they are notoriously difficult to isolate and culture. The Mouse Cervix PrimaCell™ kit (Cat No. 2- 82008) is designed for the successful isolation and culture of cervical epithelia from mouse cervix tissue allowing reproducible and dependable results.

1.1 Components of the Mouse Cervix PrimaCell™ System

- **Mouse Cervix Tissue Dissociation System, Cervix OptiTDS™** (2 × 1 ml) --- *A mixture of collagenase, collagenase I, collagenase III, collagenase IV, Hyaluronidase I, Trypsin, Dexoyribonuclease and Mouse Cervix OptiTDS™ Reconstitution Buffer.*
- **Mouse *Cervix OptiTDS™ Digestion Buffer,*** (2 × 9 ml)
- **Mouse Cervix Fibroblast Growth Inhibitors, Cervix FibrOut™---**Cervix FibrOut™ (5 x 200 µl) --- *A mixture of D-valine, collagenase and gentamycin.*
- **Mouse Cervical Epithelium PrimaCell™ Basal Culture Medium,** (5 × 100 ml) --- *A Modified Weymouth medium*.
- **Mouse Cervical Epithelium PrimaCell™ Medium Supplements,** (5 × 1 ml): *Mouse EGF, Hydrocortisone, Cholera toxin penicillin (100 U/ml), streptomycin and Weymouth medium.*
- **Mouse Cervix PrimaCell™ Serum,** (50 ml): A modified f*etal bovine serum.*
- **Mouse Cervix Tissue Washing Medium,** (1 × 100 ml): *A modified DMED medium with 10µg/ml amphotericin,* 10*µg/ml gentamycin, and 10% FCS.*

1.2 Required materials but NOT included:

- DMEM (Invitrogen Cat No. 10313-021)
- Fetal Bovine Serum (FBS, Sigma-Aldrich)
- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or mouse fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

1.3 Preparation of Swiss 3T3 Fibroblasts (optional):

(a) A large master stock of cells should be prepared and frozen in individual ampules of 1×10^6

cells. Cells should not be used for more than 20 passages.

(i) Grow 3T3s in DMEM/10% calf serum in 175-cm^2 tissue culture flasks. Inoculate cells at 1.5×10^4 cells/cm^2. Change the medium after 2d. Subculture every 4-5 d.

(ii) To avoid low-level contamination, maintain one master flask of cells on antibiotic-free medium; these cells are then used at each passage to inoculate the flasks required for that week's feeder cells.

(b) Feeder layers are inactivated by irradiation with 60 Gy (6,000 rad), either from an X-ray or 60Co source. Irradiated cells (XR-3T3) may be kept at 4℃ for 3-4 d.

(c) In the absence of a source of irradiation, inactivate feeder cells with mitomycin C.

(i) Expose 3T3 cells growing in monolayer to 400 μg/ml of mitomycin C for 1 h at 37℃.

(ii) Trypsinize the treated cells, resuspend and wash the cell pellet twice with fresh medium with serum, resuspend the cells at a suitable concentration in complete medium, and use.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, fresh gelatin-coated plate or culture dishes are recommended (see below for treatment of culture dishes).

2.2 Principle

Separation of the cervical epithelium from the cervix tissue is accomplished by enzymatic digestion using the Cervix Tissue Dissociation System supplied in this kit. The Cervix Tissue Dissociation System contains a mixture of collagenase, collagenase I, collagenase III, collagenase IV, Hyaluronidase I, Trypsin, and Dexoyribonuclease at the optimal concentrations to gently detach the fragile cervix layer from the cervix tissue. The isolated cervix tissue is then further disrupted to release individual cervical epithelium by enzymatic and mechanical agitation. The mixture is then filtered through Cell Strainers and seeded on flasks or specially coated tissue culture plates. The cervical epithelia are propagated in growth arrested feeder cells and the corresponding media. Sub-populations of cervical epithelia can then be isolated based on their selective attachment to specific basement matrix substrates.

2.3 Mouse Cervix Tissue Preparation

Mouse Cervix: Most cervical epithelium from cervix is obtained from hysterectomy specimens.

1. The female mouse is killed by decapitation and the uterine cervix dissected out under a dissecting microscope. Transfer biopsy immediately to Mouse Cervix Tissue Washing Medium. Rinse cervical biopsy two to three times with 5 ml of cold **Mouse Cervix Tissue Washing Medium**.
2. Place the biopsy, epithelial surface down, on a sterile culture dish.
3. Using surgical blade, cut and scrape away as much of the muscle and stroma as possible, leaving a thin, opaque epithelial strip.
4. Mince the epithelial strip finely with curved iris scissors.
5. Incubate tissue specimens with 5-10 ml 70% ethanol for 1 min, followed by inoculating with 5-10 ml fresh **Mouse Cervix Tissue Washing Medium** for 5 min.

2.4 Cervix Separation and Tissue Dissociation

2.4.1 Mouse Cervix OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the donor used, enzymes, culture mediums and growth supplements. The Mouse Cervix Tissue Dissociation System is suited for optimal dissociation of normal mouse cervix tissues to yield maximum number of single cervical epithelium.

2.4.2 Enzyme Compositions

- Collagenase: from *Clostridium Histolyticum*
- Collagenase I: from *Clostridium Histolyticum*
- collagenase III: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*
- Hyaluronidase I
- Trypsin
- Dexoyribonuclease

2.4.3 System Components

- Mouse Cervix Tissue Dissociation System, Cervix OptiTDS™, (2 × 1 ml).
- Mouse Cervix OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

6. Prepare fresh enzyme working solution: Add 1ml of the reconstituted tissue dissociation solution to one vial of Mouse Cervix OptiTDS™ Digestion Buffer (9 ml). Warm the diluted Mouse Cervix OptiTDS™ working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 5 ml Mouse Cervix OptiTDS™ working solutions.
7. Discard the **Mouse Cervix Tissue Washing Medium,** and float cervix samples on 5 – 10 ml of Mouse Cervix Tissue Dissociation System working solution (prewarmed to 37℃) to the epithelial mince, and transfer the tissue to a sterile glass universal containing a small plastic-coated magnetic stirrer bar.
8. Place the universal on a magnetic stirrer and stir slowly for 30-60 min at 37 ℃. This works particularly well with full-thickness cervix. Monitor the separation of the cervix carefully when using the rapid dissociation method.
9. Allow the suspension to stand at room temperature for 2-3 min
10. Remove the supernatant containing single cells to a 50-ml centrifuge tube, and spin down the cell mixture at 1000 rmp (800g) for 5 min. Collect the supernatant dissociation system working solution which can be reused, and add 10 ml of **Mouse Complete Cervix PrimaCell™ Culture Medium** to resuspend cells.
11. Add a further 5-10 ml of warm Mouse Cervix Tissue Dissociation System working solution to the fragments in the universal, and repeat the steps 8-10.

2.5 Cervixal Epithelia Isolation

Note: Please read section 2.6 for specific information on Cervical Epithelium culture and platting before preceding this section.

12. Combine the supension containing single cells, and strain the cell mixture through a sterile cell strainer (70-100μm) into a 50-ml centrifuge tube to remove debris. Cell strainers fit perfectly in 50 ml conical and are very convenient for this procedure. If cell strainers are not available, nylon gauze can be used after rinsed twice in PBS and placed at the opening of a 50 ml conical.
13. Centrifuge the strained mixture at 1000 rmp (800 g) for 5 min at 4°C.
14. Remove the supernatant; add 10 ml of complete medium to the pellet, resuspend the cells vigorously to give a single-cell suspension, and count the cells with a hemocytometer. Assess cell viability with trypan blue exclusion.

2.6 Primary Cervical Epithelia Culture

2.6.1 Medium Preparation.

Thaw out **Mouse Cervix PrimaCell™ Basal Culture Medium**, **Mouse Cervical Epithelium PrimaCell™ Medium Supplements**, and **Mouse Cervix PrimaCell™ Serum** on ice.

Mouse Complete Cervix PrimaCell™ Culture Medium: To every 100 ml **Mouse Cervix PrimaCell™ Basal Culture Medium**, add 10 ml **Mouse Cervix PrimaCell™ Medium Supplements** and 1 ml **Mouse Cervix PrimaCell™ Serum**, mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

Mouse Complete Cervix PrimaCell™ Culture Medium/FibrOut: To every 100 ml **Mouse Cervix PrimaCell™ Basal Culture Medium**, add 10 ml **Mouse Cervix PrimaCell™ Medium Supplements,** 1 ml **Mouse Cervix PrimaCell™ Serum**, and 1 ml **Mouse Cervix Fibroblast Growth Inhibitors, Cervix FibrOut™**,mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

2.5.3 Primary Cell Culture

(Important: Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination)

15. Primary Cervical Epithelia Primary Cervical Epithelia can be cultured alone or on a layer of feeder cells depending on the nature of the experiments.

- Cervical Epithelium Culture with feeder layer cells:
  - Prepare feeder layers by culturing 3T3 cells 3 days.When the cells have reached confluence irradiate the 3T3 at 30 Gy
  - Dilue the cervical cell suspension with **Mouse Complete Cervix PrimaCell™ Culture Medium/FibrOut**, and plate cells out at 2×10^4 cells/cm^2 together with 1×10^5 cells/cm^2 of lethally inactivated 3T3 cells (i.e., for 1×10^5 cervical cells, 5×10^5 XR-3T3/50-mm dish).
  - Incubate the cultures at 37℃ in 5% CO_2.
- Cervical Epithelium Culture without feeder layer:
  - Spin the suspension at 1,000 rpm (800 g) for 5 min. Remove the supernatant and resuspend the cells in 10 ml of **Mouse Cervix Tissue Washing Medium**.
  - Spin the suspension again and wash the cells once more with **Mouse Cervix Tissue**

Washing Medium. Resuspend the cells in 10 ml of **Mouse Complete Cervix PrimaCell™ Culture Medium/FibrOut**, and seed into culture dishes or flasks at a density of 2 $\times 10^5$ cells/cm2 (10^6 cells/50-mm Petri dish, 4 $\times 10^6$ /90-mm Petri dish).

- Incubate the cultures at 37℃ in 5% CO2

16. Seventy-two hours after the initial platin, replace the medium with a **Mouse Complete Cervix PrimaCell™ Culture Medium/FibrOut**. Check the cultures microscopically to ensure that the feeder layer is adequate. Add further feeder cells if necessary. Change the **Mouse Complete Cervix PrimaCell™ Culture Medium/FibrOut** twice weekly; keratinocyte colonies become visible on the microscope by days 9-12 and should be visible to the naked eye by days 14-16. Change **Mouse Complete Cervix PrimaCell™ Culture Medium/FibrOut** to **Mouse Complete Skeletal Muscle PrimaCell™ Culture Medium** after 3-5 cycles or an acceptable level of fibroblast cell contamination is observed. Cultures should be subcultured at this time.

Δ ***Safety Note.*** The rest of the biopsy and all tubes, pipettes, plates, etc., used in the procedure should be treated with hypochlorite before disposal.

2.7 Subculture

17. Propagating Cervical Epithelia in culture can be somewhat challenging, especially when Cervical Epithelia are cultured alone on plastic, however the following methods have worked consistently in many laboratories.

(a) Cervical Epithelia grown on a feeder layer:
- (i) Spin the suspension at 1,000 rpm (80 g) for 5 min. Remove the supernatant and resuspend the cells in 10 ml of **Mouse Cervix Tissue Washing Medium**.
- (ii) Spin the suspension again and wash the cells once more with **Mouse Cervix Tissue Washing Medium**. Resuspend the cells in **Mouse Complete Skeletal Muscle PrimaCell™ Culture Medium** and plate them onto culture dishes at 10^5 cells/cm^2 (5 $\times 10^5$ cells/50-mm Petri dish, 2 cm^2 $\times$ 10^6 cells/90-mm Petri dish).
- (iii) Cellsmay also be frozen at this stage for recovery at a later date

(b) Cultures in complete Mouse Cervical epithelium Culture Medium:
- (i) Remove the medium from the cell layer, and remove the feeders by rinsing rapidly with 0.01% EDTA. Wash twice with **Mouse Cervix Tissue Washing Medium.**
- (ii) To each culture dish, add enough prewarmed trypsin/EDTA to cover the cell sheet. Leave the cultures at 37℃ until the keratinocytes have detached; check for detachment with a microscope. Do not leave the cells in trypsin for more than 20 min.
- (iii) Remove the cell suspension from the plate and transfer it to a sterile centrifuge tube.
- (iv) Rinse the growth surface with **Mouse Complete Cervix PrimaCell™ Culture Medium** and add to the suspension. Mix and dispense the suspension with a 10-ml pipette.
- (v) Spin the cells at 1,000 rpm for 5 min, remove the supernatant, add 10 ml of **Mouse Complete Cervix PrimaCell™ Culture Medium**, and resuspend the cells vigorously with a 10-ml pipette to achieve a single-cell suspension.
- (vi) Count the cells with a hemocytometer.
- (vii) Cells may be replated on inactivated 3T3 cells and grown as just described or frozen for later recovery.

III Cryopreservation

18. Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Cervical Epithelia cultures derived from preconfluent layers.

(a) Trypsinize cells as above, and centrifuge at 100 g for 10 min.
(b) Resuspend cells in **Mouse Complete Cervix PrimaCell™ Culture Medium** and count cells.
(c) Prepare aliquots of 2 x 10^6 cells/ml in **Mouse Complete Cervix PrimaCell™ Culture Medium** and 10% glycerol into cryovials.
(d) Equilibrate at 4℃ for 1-2 h.
(e) Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
(f) To recover cells:
    (i) Thaw cryovials quickly in a 37℃ water bath.
    (ii) Dilute cells tenfold with **Mouse Complete Cervix PrimaCell™ Culture Medium**.
    (iii) Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in **Mouse Complete Cervix PrimaCell™ Culture Medium**.

IV Characterization

Studies on primary cultures of cervical epithelia have identified differences in morphology and gene expression between cells derived from endocervical and ectocervical regions. However, cervical epithelia have been relatively underinvestigated with regard to their in vitro growth characteristics, immune functions, and susceptibility to toxic agents and mechanisms of infection by pathogens other than HPV. The presence of specific keratins within cells is dependent on both the origin of the epithelial cells as well as the stage of differentiation. Alterations in keratin expression could serve as markers for the differentiation of cervical epithelial cells in vitro.

V References

1. Freshney, R.I.F.a.M.G., *CULTURE OF EPITHELIAL CELLS*, ed. 2. Vol. 5. 2002: Wiley-Liss, Inc.
2. Fichorova, R.N., J.G. Rheinwald, and D.J. Anderson, *Generation of papillomavirus-immortalized cell lines from normal human ectocervical, endocervical, and vaginal epithelium that maintain expression of tissue-specific differentiation proteins.* Biol Reprod, 1997. **57**(4): p. 847-55.
3. Wright, T.C., Jr., *Characterization of keratins from rat cervical epithelial cells in vivo and in vitro.* Cancer Res, 1987. **47**(24 Pt 1): p. 6678-85.

Mouse Cervix PrimaCell™: Cervical Epithelium

Mouse Cervix Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82008	**Mouse Cervix PrimaCell™ system**	**kit**	**$499**
4-22071	Mouse Cervix Tissue Dissociation System, Cervix OptiTDS™ (for 500 ml medium)	1 ml	$128
9-42007	Mouse Cervical Epithelium PrimaCell™ Basal Culture Medium	500 ml	$61
9-32071	Mouse Cervical Epithelium PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
7-62071	Mouse Cervix Fibroblast Growth Inhibitors, Cervix FibrOut™	ea	$146
9-92007	Mouse Cervix Tissue Preparation Buffer Set	ea	$75

Mouse Primary Cervical Epithelium Characterization

6-19011	**Mouse Cervical Epithelium Primarker™ Kit**	**kit**	**$220**
6-19012	Mouse Cervical Epithelium Primarker™ antibody set	set	$180
6-19013	Mouse Cervical Epithelium Primarker™ buffer system	set	$90

Mouse Colon PrimaCell™: Colorectal Epithelium
(Cat No. 2-82009)

I. General Description:

The Mouse Colon PrimaCell™ kit (Cat No. 2-82009) is developed to isolate and encourage the growth of normal mouse colonic epithelial cells derived from 1-3 mm of mouse colon tissues. This kit includes the Colon OptiTDS™ Tissue Dissociation System that provides the optimal enzymatic conditions for isolating viable cells from whole tissue and has been proven to yield 5-7 times more cells than most of the protocols published in the literature. The Mouse Colon PrimaCell™ kit also contains the optimal media and supplements that ensure the isolated colonic epithelial cells remain viable in tissue culture. The specific growth of colonic epithelial cells is further ensured by including Colon FibrOut™, a proprietary blend of enzymes and chemical reagents that inhibits the growth of aberrant fibroblasts that usually overtake primary cell cultures and crowd out the growth of colonic epithelial cells.

The Mouse Colon PrimaCell™ kit can be used to isolate primary colonic epithelial cells from normal adult mouse tissue samples. The results obtained from biopsy samples that may contain pathological organisms (virus, parasites, etc.) or malignancies may not yield optimal results using this system. Tissue specimens used for colon epithelial cells should ideally be used within 1-2 hrs of biopsy and, therefore, tissue removal from the patient. If this is not feasible, the biopsy sample can be cut into small pieces (1-2 mm) and stored at 4° C in Colon Washing Media (provided in this kit) overnight as described in the protocol below.

1.1 Components of Mouse Colon PrimaCell™ System

- **Colonic Tissue Dissociation System, Colon OptiTDS™,** (2 x 1 ml) --- *A* proprietary *mixture of collagenase I, collagenase III, collagenase IV, collagenase, and trypsin*.
- **Colon OptiTDS™ Reconstitution Buffer**, (2 x 1 ml).
- **Colon OptiTDS™ Digestion Buffer**, (2 x 9 ml).
- **Colonic Tissue Washing Medium**, (5 x 10 ml) --- *Basal Colon PrimaCell™ Culture Medium with 5% FBS, 200 u/ml of penicillin, 200 µg/ml of streptomycin, and 50 µg/ml of gentamycin*.
- **Mouse Colon Fibroblast Growth Inhibitors, Colon FibrOut™** (5 x 200 µl) --- *A* proprietary *mixture of* anti-*Thy-1 monoclonal antibody, D-valine, collagenase, and gentamycin*.
- **Mouse Colon PrimaCell™ Basal Culture Medium**, (5 x 100 ml) --- *Modified formulation based on NCTC 168 and Weymouth medium*.
- **Mouse Colon PrimaCell™ Medium Supplements**, (5 x 1.0 ml) --- A mixture of *ethanolamine, phosphoethanolamine, hydrocortisone, ascorbic acid, transferring, insulin, epidermal growth factor, pentagastrin, and deoxycholic acid*.
- **Mouse Colon PrimaCell™ Serum**, (1 x 50 ml) --- H*eat-inactivated and special-treated Fetal-bovine serum.*
- **Coating Solution**, (5 x 10 ml) --- *Basal growth medium containing 10 µg/ml BSA.*

1.2 Required Materials NOT included in the kit

- 70% sterile ethanol
- Pasteur pipettes, 10 ml pipettes
- Collagen I pre-coated tissue culture dishes
- Scalpels, scissors, and forceps
- Falcon Conicals, 15 and 50 ml

II. Procedures

2.1 Preparation of Tissue Culture Plates

All materials used in this experiment must be sterile to prevent contamination. To enhance cell attachment to tissue culture dishes, collagen I pre-coated plates (Corning, NY) MUST be further pre-treated with the Coating Solution included in this kit for 5 min. Aspirate the Coating Solution and allow the dishes to air-dry in a ventilated cell culture hood for 5-10 min before use.

2.2 Surgical specimens

Once isolated, tissue specimens should be placed immediately in 5-10 ml Colonic Tissue Washing Medium, transported on ice to the laboratory and worked up within 1 hr of isolation. Colon tissue specimens (1-3 mm) are further dissected to isolate the mucosal layer away from the muscle layer. With autoclaved scalpels, scissors, and forceps, carefully remove muscle and fat from tissue specimens. Place tissue in a 50 ml conical tube (Falcon) containing 5 ml fresh Colonic Tissue Washing Medium and incubate while shaking at room temperature for 10 min. For large tissue specimens, use a 50 ml conical tube (Falcon) and a larger volume of Colonic Tissue Washing Medium to ensure thorough washing. Aspirate the washing medium and repeat the washing procedures using fresh washing medium two more times. After the initial washes, incubate tissue in 70% ethanol for 1 min at room temperature, followed by incubating in 5-10 ml fresh Colonic Tissue Washing Medium for 5 min. Collect tissue pieces by gentle centrifugation and proceed to dissociation.

2.3 Tissue Preparation and Dissociation

2.3.1 Mouse Colon OptiTDS™

The isolation of primary cells is confounded by several important factors that can greatly affect yield and cell viability. The Colon Tissue Dissociation System, Colon OptiTDS™, is developed to produce the optimal conditions that allow for the dissociation of colonic epithelial cells from normal adult mouse tissue samples. This system uses a defined proprietary ratio of specific enzymes to yield the maximum number of single primary cells that remain viable in tissue culture.

2.3.2 Enzyme Compositions

- Collagenase I: from *Clostridium Histolyticum*
- Collagenase III: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*
- Hyaluronidase I: from *Bovine Testes*

2.3.3 System Components

- Colonic Tissue Dissociation System, OptiTDS™: 2 vials
- Colon OptiTDS™ Reconstitution Buffer: (2 x 1 ml)
- Colon OptiTDS™ Digestion Buffer: (2 x 9 ml)

2.3.4 Procedures For Tissue Preparation and Dissociation

1. Add 1.0 ml of the Colon OptiTDS™ Reconstitution Buffer to a vial of Colonic Tissue Dissociation System OptiTDS™ and mix well (Do not Vortex) with hand. It is important that the Tissue Dissociation System OptiTDS is freshly prepared and is provided in aliquots for your convenience.
2. Transfer the 1.0 ml of freshly prepared enzyme solution from step 1, to a vial containing 9.0 ml of Colon OptiTDS™ Digestion Buffer. Warm this enzyme solution at 37 ℃ for 10 min, just prior to use. For optimal results, we recommend a ratio of 100-150 mg tissue per 10 ml of enzyme solution.
3. Mince the pre-washed tissue into pieces measuring approximately 0.2-0.5 mm^2 in diameter with scalpel and forceps or scissors.
4. Incubate the minced tissue with the diluted enzyme solution with gentle stirring for 30 min at 37 ℃. Up to 5 mg of minced tissue can be incubated with 10 ml of the diluted enzyme solution.
5. Gently pipette sample with a 10 ml pipette, filling the barrel of the pipette at a rate of 2-3 ml per second. Repeat this for 5-6 times.
6. Filter the cell mixture through a cell strainer (100μM). Centrifuge the strained mixture at 100 g. Carefully remove the supernatant and resuspend the cell pellet with1.0 ml of Complete Colon Culture Medium (See 2.4.1 for preparation).
7. Count and seed the cells in 3 or 4 T-25 collagen I-coated flasks containing 4 ml of Complete Colon Culture Medium (**Important:** Collagen coated plates must be pre-treated with the provided Coating Solution, see section 2.1). This procedure will give you approximately 2.5-5 x 10^5 Cells/per T-25 flask.

2.3.5 Storage of Tissue Dissociation System:

Colonic Tissue Dissociation System, Colon OptiTDS™ should only be reconstituted when needed for cell preparation and can be stored for 2-4 days at 4 °C. For long-term use, it should be aliquoted and stored at -20 °C. Avoid repeated freeze-thaw cycles.

2.4 Culture of primary mouse colon cells

2.4.1 Medium Preparation

Thaw the Mouse Colon PrimaCell™ Medium Supplements and Mouse Colon PrimaCell™ Serum on ice. To prepare Complete Colon Culture Media dilute one vial of Mouse Colon PrimaCell™ Medium Supplements into 100 ml Mouse Colon PrimaCell™ Basal Culture Medium. Add 10 ml Mouse Colon PrimaCell™ Serum and one vial of Colon FibrOut™ (Mouse Colon Fibroblast Growth Inhibitors). Mix thoroughly and warm the Complete Colon Media in a 37° C water bath for 10 min prior use. (**Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*).

2.6.3 Treatment of Culture Dishes

To facilitate primary colon cell attachment to the tissue culture plates, the plates should be pre-coated with collagen I (available from Corning, NY) and MUST be pre-treated with the provided Coating Solution (Basal Growth Medium containing 10 µg/ml BSA). Cover the entire surface area of tissue culture plates with the Coating Solution and incubate for 5 min. Aspirate the Coating Solution and allow the dishes to air-dry in a ventilated cell culture hood for 5-10 min prior to use.

2.6.4 Standard primary culture conditions

Seed epithelial tubules and cell clumps isolated from colon tissues (from 2.3.4) into prepared T-25 flasks containing 4 ml of complete media and incubate a 37° C, 5%-CO_2 incubator. The Complete Colon Culture Medium should be changed twice a week. The tubules and cells will attach to the matrix on the plates and the colon epithelial cells will begin to migrate out within 1-2 days. Most of the tubules and cell clumps of epithelium will attach within 7 days, however, larger organoids can take up to 6 weeks to attach. It is important to note that the cells remain viable during this time.

2.6.5 Alternative Primary Culture conditions

Primary colon epithelium requires a substratum to efficiently attach during tissue culture propagation. While this can be achieved using pre-coated Collagen I plates, the growth of primary colon epithelium is significantly better when a layer of 3T3 feeder cells are used. As the initial colon epithelial colonies expand to several hundred cells per colony, the cells become less dependent on the 3T3 feeder cells, and can be cultured without the feeder cells. All media and solutions supplied in this kit can be used whether cells are grown on collagen coated plates or 3T3 feeder cells.

2.4.5 Subculture and Propagation

Most colon primary cells cannot be passaged by routine procedures using trypsin/EDTA. Colon epithelial cells can be de-attached using a mild dissociation enzyme, dispase as this treatment leads to more successful passaging. A further advantage of using dispase to passage cells is that dispase can only detach epithelial cell but not fibroblast. Thus this property decreases fibroblast contamination of the colon epithelial cells with each successive passage.

8. Add 0.5% Dispase (Sigma, w/v) to the cell monolayer. Use just enough dispase to cover the cells (~2.5 ml/25-cm^2 flask), and incubate for 40-60 min for primary colon cultures and 20-40 min for primary colon cell lines.
9. Once the epithelial layers begin to detach (they do so as sheets rather than single cells), pipette to encourage the detachment and dis-aggregation into smaller clumps.
10. Wash and replate the cells under standard culture conditions. It may take several days for clumps to attach, so take special care when changing the media and feeding the cells.

III Fibroblast Contamination

Several techniques have been published that help prevent fibroblast contamination of primary colon cell cultures. These include: (1) Physically removing isolated fibroblast colonies by scraping off the fibroblast colonies with a sterile cell scraper. This method requires extensive washing to ensure that all the lifted fibroblast that remain are washed away and not allowed to

reattach and repopulate the primary colon cultures. (2) Differential trypsinization can be attempted with the carcinomas but may not work well with normal primary colon cells. (3) Using dispase to lift off colon epithelial cells preferentially but not exclusively during routine passaging. In addition, cells can be seeded on mock plates for 4-6 hrs so that the fibroblasts attach, and floating colon cells can be transferred to collagen coated plates under standard culture conditions. This technique takes advantage of the fact that fibroblasts in general attach much more quickly to plastic than do clumps of epithelial cells. (4) Reducing the concentration of serum to about 2.5-5% to eliminate heavy concentrations of fibroblastic cells. The above methods can be used in combination knowing that normal primary fibroblasts have a finite growth span *in vitro* and if these measures are used persistently the fibroblasts will eventually be forced into senesce.
Important Note: The Mouse Colon PrimaCell™ kit includes a fibroblast elimination system Colon FibrOut™, an effective mixture of Mouse Colon Fibroblast Growth Inhibitors. It contains a mixture of anti-Thy-1 monoclonal antibody, toxin ricin and formulated serum substitutes. The principle behind the using this system is that Thy-1 antigen is present only on colorectal fibroblasts, but not colorectal epithelial cells. Using this approach, therefore, fibroblasts are eliminated without added toxicity to epithelial cells.

IV References:

1. Lan JG, Cruickshank SM, Singh JC, Farrar M, Lodge JP, Felsburg PJ, Carding SR. *Different cytokine response of primary colonic epithelial cells to commensal bacteria.* World J Gastroenterol. 2005 Jun 14;11(22):3375-84.
2. Griffiths EK, Sanchez O, Mill P, Krawczyk C, Hojilla CV, Rubin E, Nau MM, Khokha R, Lipkowitz S, Hui CC, Penninger JM. *Cbl-3-deficient mice exhibit normal epithelial development.* Mol Cell Biol. 2003 Nov;23(21):7708-18.
3. Whitehead RH, Brown A, Bhathel PS: *A method for the isolation and culture of human colonic crypts in collagen gels.* In Vitro 1986, 23:436-442.
4. Telega GW, Baumgart DC, Carding SR. *Uptake and presentation of antigen to T cells by primary colonic epithelial cells in normal and diseased states.* Gastroenterology. 2000 Dec;119(6):1548-59.
5. Branting C, Toftgard R, Hallstrom IP, Rafter J. *Role of protein kinase C in growth stimulation of primary mouse colonic epithelial cells.* In Vitro Cell Dev Biol Anim. 1995 Nov;31(10):790-5.

Mouse Colon PrimaCell™: Colorectal Epithelium

Mouse Colon Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82009	**Mouse Colon PrimaCell™ system**	**kit**	**$499**
4-22081	Mouse Colon Tissue Dissociation System, Colon OptiTDS™ (for 500 ml medium)	1 ml	$128
9-42008	Mouse Colorectal Epithelium PrimaCell™ Basal Culture Medium	500 ml	$61
9-32081	Mouse Colorectal Epithelium PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
7-62081	Mouse Colon Fibroblast Growth Inhibitors, Colon FibrOut™	ea	$146
9-92008	Mouse Colon Tissue Preparation Buffer Set	ea	$75

Mouse Primary Colorectal Epithelium Cell Characterization

6-11011	**Mouse Colorectal Epithelium Primarker™ Kit**	**kit**	**$220**
6-11012	Mouse Colorectal Epithelium Primarker™ antibody set	set	$180
6-11013	Mouse Colorectal Epithelium Primarker™ buffer system	set	$90

Mouse Endothelium PrimaCell™: Vascular Endothelial Cells
(Cat No. 2-82010)

I. General Description:

The Mouse Endothelium PrimaCell kit (Cat No. 2-82010) allows the isolation and tissue culture growth of normal mouse vascular endothelial cells from the mouse vascular endothelial tissues. This system provides the optimal conditions for endothelial cell culture and yields 5-7 times more cells than most available protocols published in the literature. In addition, this system ensures high cell viability and a pure endothelial cell population that is obtained using CHI's proprietary fibroblast inhibitory system, FibrOut™.

Endothelial cells exist in a single cell layer lining the inner surface of all blood vessels. Historically, the vessels most commonly used to obtain cultured endothelial cells are derived from bovine and mouse aorta, bovine adrenal capillaries, rat and mouse brain capillaries, human umbilical veins, and human dermal and adipose capillaries. Although all endothelia share some common properties, significant differences exist between the endothelial cells of large and small blood vessels.

The Mouse Vascular Endothelial PrimaCell™ kit can be used to isolate most types of endothelial cells from mice ranging from E16 through 3 weeks. E20-22 or 2-3 weeks are only recommended for convenience and obtaining a maximum yield. The preparation of aorta, capillaries or blood vessels for cell culture is usually started within 1-2 h of removal from the animal. If this is impossible, cut the vessels into fine pieces of about 10-15 mm and store overnight at 4° C in washing medium (see below). Endothelial samples containing pathological organism (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Mouse Vascular Endothelial PrimaCell™ System

- **Vascular Endothelial Tissue Dissociation System, Vascular Endothelial OptiTDS™, (**2 aliquots) --- *A proprietary mixture of collagenase, collagenase I, collagenase IV, and dispase*.
- **Vascular Endothelial Tissue OptiTDS™ Reconstitution Buffer, (**2 x 1 ml)
- **Vascular Endothelial Tissue OptiTDS™ Digestion Buffer, (**2 x 9 ml)
- **Vascular Endothelial Tissue Washing Medium**, (1 x 100 ml) --- *Basal Vascular Endothelial PrimaCell™ Culture Medium with 5% FBS, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Mouse Vascular Endothelial Fibroblast Growth Inhibitors, Vascular Endothelial FibrOut™, (**5 x 200 μl) --- *A proprietary mixture of cis-OH-proline, collagenase, D-valine, and special serum substitutes*.
- **Mouse Vascular Endothelial PrimaCell™ Basal Culture Medium,** (5 x100 ml) --- *Modified formulation based on medium 199 and DMEM medium*.
- **Mouse Vascular Endothelial PrimaCell™ Medium Supplements with Serum,** (5 x 10 ml) --- *A mixture of EGF, VEGF and Heparin, and CHI's proprietary mouse serum extracts*.
- **Vascular Endothelial PrimaCell™ I Culture Dish Coating Solution,** (5 x 10 ml) --

- 1.5% gelatin solution, 0.5% bovine serum albumin in PBS.

1.2 Required Materials NOT provided

- 70% sterile ethanol
- Tissue culture dishes
- Pasteur pipettes and 10-ml pipettes
- Falcon Conicals: 15 and 50 ml
- Two clamps or hemostats, 25 mm
- Sharp scissors, 50 mm

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, fresh gelatin-coated plate or culture dishes are recommended (see below for treatment of culture dishes).

2.2 Treatment of Culture Dishes

1. Tissue culture ware used to seed endothelial cells has to be pretreated using Vascular Endothelial PrimaCell™ I Culture Dish Coating Solution.
2. Incubate the plates or dishes with 2-3 ml (enough to cover the medium reaching areas) Vascular Endothelial PrimaCell™ I Culture Dish Coating Solution overnight at room temperature in the tissue culture hood.
3. Remove the Vascular Endothelial PrimaCell™ I Culture Dish Coating Solution (without washing the surface), immediately add complete Mouse Vascular Endothelial PrimaCell™ Medium (see 2.4.1 for Medium Preparation) and incubate the medium until the cells are ready for platting.

- (Note: *Pre-coated gelatin plates or dishes must be used within 72 after preparation.*)

2.3 Vascular Endothelial Tissue Preparation

4. Mice at ages of E20-22 mice through 2-3 weeks are recommended for convenient of procedures and yielding maximum amount of viable target cells. Mice are sacrificed by CO_2 narcosis.
5. Aseptically isolated blood vessels, preferably in 10-15 mm sections, approximately 5 mm in diameter. If asepsis cannot be guaranteed, clamp both ends of the blood vessel.
6. Incubate blood vessels for up to 10 min in Vascular Endothelial Tissue Washing Medium to prevent infection. (This procedure will not affect endothelial cells viability.)
7. Incubate tissues in 10 ml 70% sterile ethanol for 30 sec.
8. Rinse tissue twice in Vascular Endothelial Tissue Washing Medium for 5 min each and kept on ice.

2.3 Tissue Dissociation

2.3.1 Mouse Vascular Endothelial OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Mouse Vascular Endothelial Tissue Dissociation System, OptiTDS™, is suited for optimal dissociation of normal adult Vascular Endothelial tissues to yield maximum number of single endothelial cells.

2.3.2 Enzyme Compositions

- Dispase: from *Bacillus polymyxa*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase II: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*

2.3.3 System Components

- Vascular Endothelial Tissue Dissociation System, OptiTDS™, 2 vials.
- Vascular Endothelial OptiTDS™ Reconstitution Buffer, (2 x 1 ml).
- Vascular Endothelial OptiTDS™ Digestion Buffer, (2 x 9 ml).

2.3.4 Procedures For Tissue Preparation and Dissociation

9. Add 1.0 ml of Vascular Endothelial Tissue Dissociation System, OptiTDS™ to one vial of Vascular Endothelial OptiTDS™ Digestion Buffer (9.0 ml). Warm the diluted Vascular Endothelial Tissue OptiTDS™ digestion solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 10 ml diluted Vascular Endothelial OptiTDS™ working solutions.
10. Ligate one end of a 10-cm section of blood vessel 2-10 mm in diameter to a 5-ml plastic syringe.
11. Run the Vascular Endothelial Tissue OptiTDS™ digestion solution through the blood vessel until it appears at the bottom end, clamp that end with a hemostat, and incubate the vessel containing the fresh enzyme working solution at room temperature for 30 min with rocking.
12. Cut the vessel above the clamp with sharp scissors, and collect the Vascular Endothelial Tissue OptiTDS™ digestion solution in a 10-cm Petri dish.
13. Rinse the lumen of the vessel with 10 ml of Vascular Endothelial Tissue Washing Medium, and add this to the Vascular Endothelial Tissue OptiTDS™ digestion solution collected from step 4.
14. Repeat procedure 3-5 until finish all blood vessels and collecting all enzymes digestion solution containing cells.
15. Collect cells by centrifugation at 350 g, washing cells with 10 ml PBS or complete culture medium twice. At the end of washing process, collect cells and dilute cell in 0.5-1.0 ml Complete Mouse Vascular Endothelial PrimaCell™ I Culture Medium (see 2.4.1 for Medium Preparation).
16. Count viable cells.
17. Seed cells at 37℃ in Complete Mouse Vascular Endothelial PrimaCell™ I Culture Medium at desired densities (see 2.4.1 for Medium Preparation).

Alternative Method: The vessels can be cut along the length to reveal the inner layer of endothelial cells. The vessels can be attached to a Styrofoam board with the interior of the vessel facing up. Then the entire board revealing the exposed vessel lumen can be inverted

into the Vascular Endothelial Tissue OptiTDS™ digestion solution to dissociate the endothelial cells.

2.3.5 Storage:

Diluting tissue dissociation systems should occur before use and can only be stored for 2-4 days at 4 ^{o}C. For long-term use, it should be aliquoted and stored at -20 ^{o}C. Avoid repeated freeze-thaw cycles.

2.4 Culture of Vascular Endothelial Cells

2.4.1 Medium Preparation.

Thaw out the Vascular Endothelial PrimaCell™ Medium Supplements with Serum on ice. To every 100 ml Mouse Vascular Endothelial PrimaCell™ Basal Culture Medium, add 10 ml of Vascular Endothelial PrimaCell™ Medium Supplements with Serum; and one vial of Mouse Vascular Endothelial Fibroblast Growth Inhibitors, Vascular Endothelial FibrOut™, mix thoroughly and warm the Complete Vascular Endothelial Media in a 37^{o} C water bath for 10 min prior use. (**Important:** Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination).

2.6.6 Primary Culture Protocols.

18. Resuspend the final pellet collected from last step described in 2.3.4 in Complete Vascular Endothelial Media, and seed the cells into fresh prepared gelatin-coated dishes or flasks, with approximately 5 x 10^5 cells derived from one 10-15 cm section of blood vessel, 5 mm in diameter, per 72-cm flask or 10-cm-diateter dish.
19. Subculture by conventional trypsinization method.

2.4.3 Subculture and Propagation

20. Gently rinse the culture dish twice with 1X PBS.
21. Add 3 ml of 0.25% trypsin/0.1% (2.5 mM) EDTA, and incubate at 37℃. Examine the dish under phase microscopy every 5 min to detect cell detachment.
22. When most cells have detached, add 10 ml Complete Vascular Endothelial Media to inactive the trypsin activity.
23. Pipette the contents of the dish to ensure complete endothelial cell detachment.
24. Aspirate and centrifuge the cells for 5 min at 350 g.
25. Aspirate the supernatant, resuspend the cells in a complete growth medium, and re-plate at 2-4 × 10^5 cells per 100-mm dish.
26. Feed the culture twice a week with Complete Vascular Endothelial Media.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample; the best results are reported when cells from preconfluent primary cultures are used.

27. Detach cells as for the subculture, and centrifuge at 350 g for 10 min.

28. Resuspend cells in complete culture medium with serum, and count.
29. Dispense aliquots of 2 x 10^6 cells/ml in Complete Vascular Endothelial Media with additional 15% FCS and 10% glycerol into cryopreservation tubes.
30. Equilibrate at 4℃ for 1-2 h.
31. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
32. To recover cells:
    (i) Thaw cryotubes quickly in a 37℃ water bath.
    (ii) Dilute cells tenfold with medium.
    (iii) Centrifuge cells and resuspend them at an appropriate concentration in the desired culture medium, and seed culture vessel.

Mouse Vascular Endothelial cells can be grown in all media for 4-7 weeks and can be subcultured only 4-5 times.

IV Fibroblast Contamination

Mouse Vascular Endothelial PrimaCell™ system includes a fibroblast elimination system, the Mouse Vascular Endothelial Fibroblast Growth Inhibitors, Vascular Endothelial FibrOut™. It contains a mixture of cis-OH-proline, collagenase, D-valine, and formulated serum substitutes. This system can effectively eliminate Vascular Endothelial fibroblast contamination while has not affect on the behavior of endothelial cells. (**Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*).

V Confirmation of Vascular Endothelial Cells

Vascular endothelial cells are typically identified by the production of factor VIII, angiotensin-converting, the uptake of acetylated low-density lipoprotein, the presence of Weibel-Palade bodies, and the expression of endothelial-specific cell surface antigens.

VI References:

1. Zetter BR. The endothelial cells of large and small blood vessels. Diabetes. 1981;30(Suppl 2):24-8.
2. Kern PA, Knedler A, Eckel RH. *Isolation and culture of microvascular Vascular Endothelial from human adipose tissue. J Clin Invest. 1983 Jun;71(6):1822-9.*
3. Davison PM, Bensch K, Karasek MA. *Isolation and long-term serial cultivation of endothelial cells from the microvessels of the adult human dermis. In Vitro. 1983 Dec;19(12):937-45.*
4. Jaffe EA, Nachman RL, Becker CG, Minick CR. *Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest. 1973 Nov;52(11):2745-56*
5. Folkman J, Haudenschild CC, Zetter BR. *Long-term culture of capillary endothelial cells. Proc Natl Acad Sci U S A. 1979 Oct;76(10):5217-21.*
6. Bowman PD, Betz AL, Ar D, Wolinsky JS, Penney JB, Shivers RR, Goldstein GW. *Primary culture of capillary Vascular Endothelial from rat brain. In Vitro. 1981 Apr;17(4):353-62.*
7. Booyse FM, Sedlak BJ, Rafelson ME Jr. *Culture of arterial endothelial cells:*

characterization and growth of bovine aortic cells. Thromb Diath Haemorrh. 1975 Dec 15;34(3):825-39.

8. Kobayashi M, Inoue K, Warabi E, Minami T, Kodama T. *A simple method of isolating mouse aortic endothelial cells.* J Atheroscler Thromb. 2005;12(3):138-42.

Mouse Endothelium PrimaCell™: Vascular Endothelial Cells

Mouse Endothelium Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82010	**Mouse Endothelium PrimaCell™ system**	**kit**	**$499**
4-22111	Mouse Vascular Endothelial Tissue Dissociation System, Vascular Endothelial OptiTDS™ (for 500 ml medium)	1 ml	$128
9-42011	Mouse Vascular Endothelial PrimaCell™ Basal Culture Medium	500 ml	$61
9-32111	Mouse Vascular Endothelial PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
7-62111	Mouse Vascular Endothelial Fibroblast Growth Inhibitors, Vascular Endothelial FibrOut™	ea	$146
9-92011	Mouse Endothelium Tissue Preparation Buffer Set	ea	$75

Mouse Primary Vascular Endothelium Characterization

6-11111	**Mouse Vascular Endothelium Primarker™ Kit**	**kit**	**$220**
6-11112	Mouse Vascular Endothelium Primarker™ antibody set	set	$180
6-11113	Mouse Vascular Endothelium Primarker™ buffer system	set	$90

Mouse Eye PrimaCell™: Corneal Epithelial Cells
(Cat No. 2-82011)

I. General Description:

This protocol is developed for attachment and growth of normal mouse corneal epithelial cells from adult Mouse Eye with Mouse Eye PrimaCell™ system (Cat No. 2-82011). This system provides an optimal condition of tissue dissociation system, Mouse Eye OptiTDS™ that yields 4-7 times of single cells more than most of the tissue dissociation protocols published in the literature. In addition, this system ensures a high viability of the target cells with improved gradient contained in the culture medium. With CHI's proprietary fibroblast inhibitory system, FibrOut™, cells are growing with contamination of minimized amount of the non-epithelial cells.

Mouse Eye PrimaCell™ system applies to all type tissue samples from mouse at all age though adult tissue samples are recommended for yielding maximum amount of viable target cells. However, tissue samples contain pathological organism (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Mouse Eye PrimaCell™ System

- **Mouse Eye Tissue Dissociation System, Eye OptiTDS™,** (2 x 1 ml) --- *A mixture of Collagenase II, Collagenase III, Collagenase IV, Elastase, Hyaluronidase I, Trypsin, Papain.*
- **Mouse Eye OptiTDS™ Digestion Buffer**, (2 x 9 ml).
- **Mouse Eye Tissue Washing Medium**, (1 x 100 ml) --- *Corneal Epithelial Cells PrimaCell™ Basal Culture Medium with 5% FBS, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Mouse Eye Fibroblast Growth Inhibitors, Eye FibrOut™** (5 x 200 μl) --- *A mixture of cis-OH-proline, collagenase, D-valine, and formulated serum substitutes*.
- **Mouse Corneal Epithelial Cells PrimaCell™ Basal Culture Medium**, (5 x100 ml) --- *Modified formulation based on Weymouth medium*.
- **Mouse Corneal Epithelial Cells PrimaCell™ Medium Supplements**, (5 x 1.0 ml) --- A mixture of *Mouse EGF, Insulin, Hydrocortisone, bovine pituitary extract, penicillin, and streptomycin*.
- **Mouse Eye PrimaCell™ Serum**, (5 x 10 ml) --- *Highly purified* s*pecial-treated Fetal-bovine serum.*

1.2 Required Materials but not provided

- PBS containing 5% sterilized BSA
- 70% sterile ethanol
- Trypsin-EDTA: Trypsin, 0.05%, EDTA, 0.5 mM
- Rat-tail collagen, type I-coated 6-well plate, (Becton Dickinson)
- Fibronectin-collagen-coated (FNC) culture dishes, 60 mm and 100 mm (Becton Dickinson)
- Scalpels No. 1621 (Becton Dickinson)

- Pipettes (10 and 25 ml)
- Gloves sterilized with autoclave (mouse tissue can be contaminated with biologically hazardous agents)
- Controlled atmosphere chamber
- Phase-contrast inverted microscope

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, culture plate (Corning, NY) must be coated with the provided coating solution.

2.2 Surgical specimens

- Carefully remove eye tissue from donor in a way that is minimized damages to the whole corneal area.
- Incubate the eye tissue sequentially in 70% sterile ethanol for 1 min; in Eye Tissue Washing Medium for 10 min. (This procedure will not affect cell viability.)
- Carefully separate the corneas from connective tissues.
- Place corneas epithelial side up on a sterile surface (e.g., a regular cell culture dish), and cut them into 12 triangular shaped wedges, using a single cut of the scalpel and avoiding any sawing motion.

2.3 Tissue Preparation and Dissociation

2.3.1 Mouse Eye OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Mouse Eye Tissue Dissociation System, OptiTDS™, is suited for optimal dissociation of normal adult cornea samples to yield maximum number of single primary cells of eye tissues.

2.3.2 Enzyme Compositions

- Collagenase II: from *Clostridium Histolyticum*
- Collagenase III: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*
- Elastase: from *Porcine Pancreas*
- Hyaluronidase I: from *Bovine Testes*
- Trypsin: from *Bovine Pancreas*
- Papain: from *Bovine Testes*

2.3.3 System Components

- Mouse Eye Tissue Dissociation System, OptiTDS™, (2 x 1 ml)
- Mouse Eye OptiTDS™ Digestion Buffer, (2 x 9 ml).

2.3.4 Procedures For Tissue Preparation and Dissociation

1 Prepare Mouse Eye OptiTDS™ Digestion solution: Add 1.0 ml of Mouse Eye Tissue Dissociation System, OptiTDS™ to one vial of Mouse Eye OptiTDS™ Digestion Buffer (9.0 ml).
2 Prepare complete tissue dissociation solution: Mix equal volume of Mouse Eye OptiTDS™ Digestion solution and Mouse Corneal Epithelial Cells PrimaCell™ Basal Culture Medium (without serum, the kit supplied with extra 50 ml basal culture medium).
3 Warm the complete tissue dissociation solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 10 ml complete tissue dissociation solution.
4 Turn each corneal segment epithelial side down, and place four segments in each well of a six-well tray (precoated with rat-tail collagen, type I, Biocoat, Becton Dickinson).
5 Press each segment down gently with forceps to ensure good contact between the tissue and the tissue culture surface. Allow the tissue to dry for 20 min.
6 Place one drop of complete tissue dissociation solution from step 3 carefully upon each segment. The solution should be applied to fully cover but not to spread over the cornea segments.
7 Incubate the culture for 30 min at 37℃ in 5% CO_2.

2.3.5 Storage:

Reconstituting tissue dissociation solution should occur before use and can only be stored for 2-4 days at 4 ^{o}C. For long-term use, it should be aliquoted and stored at -20 ^{o}C. Avoid repeated freeze-thaw cycles.

2.4 Culture of Primary Mouse Corneal Epithelial Cells

2.4.1 Medium Preparation.

Thaw out the Mouse Corneal Epithelial Cells PrimaCell™ Medium Supplements and one vial of Mouse Eye Fibroblast Growth Inhibitors, Mouse Eye FibrOut™ on ice. To every 100 ml Mouse Corneal Epithelial Cells PrimaCell™ Basal Culture Medium, add one vial of Mouse Corneal Epithelial Cells PrimaCell™ Medium Supplements; and one vial of Mouse Eye Fibroblast Growth Inhibitors, Mouse Eye FibrOut™, mix thoroughly and warm the complete culture medium at a 37^{o}C water bath for 10 min prior use. (**Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*).

2.6.7 Primary Culture Conditions.

8 At the end of 30 min incubation, carefully remove the complete tissue dissociation solution remained on each segment by pipetting.
9 Add 1.5 ml of complete culture medium to each well and continue culturing for overnight at 37℃ in 5% CO_2.
10 Next day, exam cells under microscope. When cells are observed to emigrate

only from the limbal region of the cornea. Asparate medium and washing cell once with 1X PBS.

11 Carefully remove the tissue segment with forceps and add 3 ml of complete culture medium. After removal of the donor tissue, adherent cells remained in the plate continuing to proliferate, and within 2 weeks from the time of establishment of the culture, confluent monolayers form, displaying the typical cobblestone morphology associated with epithelia. The yield is approximately 7.5-8 $\times 10^6$ cells/cornea.

2.4.3 Subculture and Propagation

12 Following the initial outgrowth period, feed the cultures twice per week.

13 At 70-80% confluence, rinse the cells in Dulbecco's phosphate-buffered saline (PBS), and release with trypsin/EDTA (0.05% trypsin, 0.53 mM EDTA) for 4 min at 37℃.

14 Stop the reaction with 10% FBS in PBS.

15 Wash the cells (centrifugation followed by resuspension in complete culture medium), count them, and plate at 1×10^4 cells/cm^2 onto tissue culture surfaces coated with FNC.

16 Incubate the culture at 37℃ in 95% air and 5% CO_2.

17 Exchange the culture medium with fresh complete culture medium 1 d after trypsinization and reseeding. Immediately after passage, cells appear more spindles shaped, are refractile, and are highly migratory. Within 6-8 d, control cultures become 70-80% confluent, continue to display cobblestone morphology, and, if allowed to become postconfluent, retain the ability to stratify in discrete areas.

18 Although corneal epithelial cultures can be subcultured up to five times (approximately 9-10 population doublings), most of the proliferation occurs between the first and third passages. Approximate yields are 2.5-3.0 $\times 10^6$ cells/cornea. Senescence always ensues by P5.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample; the best results are reported when cells from preconfluent primary cultures are used.

19. Detach cells as for the subculture, and centrifuge at 100 g for 10 min.
20. Resuspend cells in complete culture medium with serum, and count.
21. Dispense aliquots of 2 x 10^6 cells/ml in complete growth medium with additional 10% FBS and 10% glycerol into cryopreservation tubes.
22. Equilibrate at 4℃ for 1-2 h.
23. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
24. To recover cells:

(i) Thaw cryotubes quickly in a 37℃ water bath.
(ii) Dilute cells tenfold with medium.
(iii) Centrifuge cells and resuspend them at an appropriate concentration in the desired culture medium, and seed culture vessel.

Mouse corneal epithelial cells can be grown in the complete culture media for 5-6 weeks and can be subcultured only 5-7 times.

IV Fibroblast Contamination

There are several techniques have been published in the literature to deal with fibroblast contamination during cornea primary cell culture. These include: (1) Physically remove a well-isolated fibroblast colony by scraping it with a sterile blunt instrument (e.g., a cell scraper). Care has to be taken to wash the culture up to six times to remove any fibroblasts that have detached in order to prevent them from reseeding and reattaching to the flask. (2) Differential trypsinization can be attempted with the carcinomas. (3) Dispase preferentially (but not exclusively) removes the epithelium during passaging and leaves behind most of the fibroblastic cells attached to the culture vessel. During subculture, cells that have been removed with dispase can be preincubated in plastic petri dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the epithelium. This technique takes advantage of the fact that fibroblasts in general attach much more quickly to plastic than do clumps of epithelial cells, so that a partial purification step is possible. (4) Reduce the concentration of serum to about 2.5-5% if there are heavy concentrations of fibroblastic cells. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and that using any or all of the preceding techniques will eventually push the cells through so many divisions that any fibroblasts will senesce.

Mouse Eye PrimaCell™ system includes a fibroblast elimination system, the Mouse Eye Fibroblast Growth Inhibitors, Mouse Eye FibrOut™. It contains a mixture of of cis-OH-proline, collagenases, D-valine, and formulated serum substitutes. This system can effectively eliminate Eye fibroblast contamination while has not affect on the behavior of cornea epithelial cells.

V References:

1. Lyu J, Joo CK. *Expression of Wnt and MMP in epithelial cells during corneal wound healing.* Cornea. 2006 Dec;25(10 Suppl 1):S24-8.
2. Tseng SC, Kruse FE, Merritt J, Li DQ. *Comparison between serum-free and fibroblast-cocultured single-cell clonal culture systems: evidence showing that epithelial anti-apoptotic activity is present in 3T3 fibroblast-conditioned media.* Curr Eye Res. 1996 Sep;15(9):973-84.
3. Hazlett L, Masinick S, Mezger B, Barrett R, Kurpakus M, Garrett M. *Ultrastructural, immunohistological and biochemical characterization of cultured mouse corneal epithelial cells.* Ophthalmic Res. 1996;28(1):50-6.
4. Chen WY, Mui MM, Kao WW, Liu CY, Tseng SC. *Conjunctival epithelial cells do not transdifferentiate in organotypic cultures: expression of K12 keratin is restricted to corneal epithelium.* Curr Eye Res. 1994 Oct;13(10):765-78.
5. Castro-Munozledo F, Valencia-Garcia C, Kuri-Harcuch W. *Cultivation of rabbit corneal epithelial cells in serum-free medium.* Invest Ophthalmol Vis Sci. 1997 Oct;38(11):2234-44.
6. Koizumi N, Cooper LJ, Fullwood NJ, Nakamura T, Inoki K, Tsuzuki M, Kinoshita S. *An evaluation of cultivated corneal limbal epithelial cells, using cell-suspension culture.* Invest Ophthalmol Vis Sci. 2002 Jul;43(7):2114-21.

Mouse Eye PrimaCell™: Corneal Epithelial Cells

Mouse Eye Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82011	**Mouse Eye PrimaCell™ system**	**kit**	**$499**
4-22091	Mouse Eye Tissue Dissociation System, Eye OptiTDS™ (for 500 ml medium)	1 ml	$128
9-42009	Mouse Corneal Epithelial Cells PrimaCell™ Basal Culture Medium	500 ml	$61
9-32091	Mouse Corneal Epithelial Cells PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
7-62091	Mouse Eye Fibroblast Growth Inhibitors, Eye FibrOut™	ea	$128
9-92009	Mouse Eye Tissue Preparation Buffer Set	ea	$75

Mouse Primary Corneal Epithelial Cells Characterization

6-12111	**Mouse Corneal Epithelial Cell Primarker™ Kit**	**kit**	**$220**
6-12112	Mouse Corneal Epithelial Cell Primarker™ antibody set	set	$180
6-12113	Mouse Corneal Epithelial Cell Primarker™ buffer system	set	$90

Mouse Fat PrimaCell™: Adipose Cells
(Cat No. 2-82012)

I. General Description

Fat cells, found in adipose tissue, also called adipose cells (adipocytes), are terminally differentiated specialized cells whose primary physiological role hss classically been described as an energy reservoir for the body. The cytoplasm usually being compressed into a thin envelope, with the nucleus at one point in the periphery. The chief chemical constituents of this fat are the neutral glycerol esters of stearic, oleic, and palmitic acids. The size of adipose cells can vary considerably from fat depot to fat depot. Abnormalities in adipose tissue can contribute directly to the pathogenesis of common dieases such as diabetes, hypertension and obesity. Adipocytes are crucial target for pharmacotherapy. While these cells are extremely useful in the laboratory, they are notoriously difficult to isolate and culture. The protocol described in this kit can be scaled up or down as needed and has been used to provide insulin-responsive cells suitable for DNA transfer by electroporation. The Mouse Fat PrimaCell™ kit (Cat No. 2- 82012) is designed for the successful isolation and culture of adipocytes from mouse adipose tissue allowing reproducible and dependable results.

1.1 Components of the Mouse Fat PrimaCell™ System

- **Mouse Fat Tissue Dissociation System, Fat OptiTDS™** (2 × ml) --- *A mixture of collagenase, collagenase I, and trypsin and Mouse Fat OptiTDS™ Reconstitution Buffer.*
- **Mouse *Fat OptiTDS™ Digestion Buffer,*** (2 × 9 ml)
- **Mouse Fat Fibroblast Growth Inhibitors, Fat FibrOut™**---Fat FibrOut™ (5 x 200 µl) --- *A mixture of cis-OH-proline, collagenase and gentamycin.*
- **Mouse Adipose Cells PrimaCell™ Basal Culture Medium,** (5 x 100 ml) --- A *Modified DMEM*.
- **Mouse Adipose Cells PrimaCell™ Medium Supplements with Serum,** (5 x 1 ml): *Glucose, Glutamine, ®-N6-(1-methyl-2-phenylethyl) adenosine, antibiotics (penicillin, 100 U/ml), streptomycin. Gentamycin, BSA and HEPES.*
- **Mouse Adipose Washing Medium,** (1 x 100 ml): *Basal Kerbs-Ringer medium with 10mM NaHCO3, 30mM HEPES, 200nM adenosine and 5% BSA.*

1.2 Required materials but NOT included:

- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or mouse fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, fresh gelatin-coated plate or culture dishes are recommended (see below for treatment of culture dishes).

2.2 Principle

Separation of the adipocytes from the adipose tissue is accomplished by enzymatic digestion using the Fat Tissue Dissociation System supplied in this kit. The Fat Tissue Dissociation System contains a mixture of collagenases and trypsin at the optimal concentrations to gently detach the fragile adipose layer from the adipose tissue. The isolated adipose is then further disrupted to release individual adipocytes by enzymatic and mechanical agitation. The mixture is then filtered through Cell Strainers and seeded on specially coated tissue culture plates. The Adipocytes are propagated in serum-free, low-calcium media or growth arrested feeder cells and the corresponding media. Sub-populations of adipocytes can then be isolated based on their selective attachment to specific basement matrix substrates.

2.3 Mouse Adipose Tissue Preparation

Mouse Adipose: Samples of subcutaneous adipose tissues can be used. Each mouse adipose yield large number of cells (2-4 x 10^6/adipose), with a 35-40% plating efficiency.

1. Anesthetize mouse in a plastic box with a gas mixture of 70% CO_2, and 30% O_2.
2. Decapitate mouse using a guillotine and exsanguinate.
3. Soak the bodies briefly in 70% ethanol.
4. Remove the epididymal fat pads while maintaining the highest level of sterility possible.
   (a) Cut through the skin on the lowest abdomen with one pair of scissors to expose the peritoneum.
   (b) Using a second pair of scissors, open the peritoneum and pull up the testes with a pair of forceps.
   (c) Trim fat pads from epididymides, taking care to leave the blood vessels behind.
   (d) Transport tissue to5-10 ml 70% ethanol and incubate for 1 min, followed by inoculating with 5-10 ml fresh **Mouse Adipose Washing Medium** for 5 min.
5. Add 4 g of fat pads (approximately equivalent to 8 fat pads) to a 30-ml low-density polypropylene vial containing **Mouse Adipose Washing Medium**. Mince fat pads into pieces approximately 2 mm in diameter with scissors.

2.4 Adipose Separation and Tissue Dissociation

2.4.1 Mouse Fat OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Mouse Fat Tissue Dissociation System is suited for optimal dissociation of normal mouse adipose tissues to yield maximum number of single adipocytes.

2.4.2 Enzyme Compositions

- Trypsin: from *Bovine Pancreas*
- Collagenase: from *Clostridium Histolyticum*
- Collagenase I: from *Clostridium Histolyticum*

2.4.3 System Components

- Mouse Fat Tissue Dissociation System, Fat OptiTDS™, (2 × ml).
- Mouse Fat OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Dissociation

6. Prepare fresh enzyme working solution: Add 1ml of the reconstituted tissue dissociation solution to one vial of Mouse Cervix OptiTDS™ Digestion Buffer (9 ml). Warm the diluted Mouse Fat OptiTDS™ working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-4 g tissue samples per 5 ml Mouse Fat OptiTDS™ working solutions.

7. Discard the **Mouse Fat Washing Medium,** and add 5 ml of Mouse Fat Dissociation System working solution to the vial containing the minced fat pads, and incubate the pieces in a shaking water bath at 37˚C for approximately 1 h, until the cell mixture takes on a creamy consistency.
8. After Mouse Fat Dissociation System digestion, add 10 ml of **Mouse Adipose Washing Medium** at 37˚C to the vial.
9. Mix cells in the vial by swirling, and gently pass the cells through a 250-μm nylon mesh filter into a 50-ml conical tube.

2.5 Adipocytes Isolation

Note: Please read section 2.6 for specific information on Adipocytes culture and platting before preceding this section.

10. Wash the cells by adding 30 ml of **Mouse Adipose Washing Medium** buffer at 37˚C to the tube and centrifuging briefly at 200 g in a tabletop centrifuge. Remove infranatant with a pipette. Note that adipose cells will be floating on top of the aqueous buffer.
11. Repeat the washing of cells by adding 40 ml of **Mouse Adipose Washing Medium**, centrifuging, and removing infranatant two additional times.
12. Wash cells twice with 40 ml of **Mouse Adipose Cells PrimaCell™ Basal Culture Medium** at 37˚C.
13. After the final wash, resuspend the cells from the surface of the medium in **Mouse Complete Adipose PrimaCell™ Culture Medium/FibrOut™** at a cytocrit of approximately 40%.

2.6 Primary Adipocytes Culture

2.6.1 Medium Preparation.

Thaw out **Mouse Adipose Cells PrimaCell™ Basal Culture Medium**, **Mouse Adipose Cells PrimaCell™ Medium Su**pplements with Se**rum** on ice.

Mouse Complete Adipose Cells PrimaCell™ Culture Medium: To every 100 ml **Mouse Adipose Cells PrimaCell™ Basal Culture Medium**, add 1 ml **Mouse Adipose Cells**

PrimaCell™ Medium Supplements with Serum, mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

Mouse Complete Adipose Cells PrimaCell™ Culture Medium/FibrOut: To every 100 ml **Mouse Adipose Cells PrimaCell™ Basal Culture Medium**, add 1 ml **Mouse Adipose Cells PrimaCell™ Medium Supplements with Serum** and 1 ml **Mouse Fat Fibroblast Growth Inhibitors, Fat FibrOut™**, mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

2.6.2 Primary Cell Culture

(Important: Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination)

14. Transfer 2 ml of the 40% cytocrit **Mouse Complete Adipose PrimaCell™ Culture Medium/FibrOut** suspension, using 200-µl wide-bore pipette tips, into one 60-mm tissue culture dish (Becton Dickinson).
15. Place the cells in a humid incubator at 37˚C with 5% CO_2 for 1.5 h.
16. Add 5 ml of **Mouse Complete Adipose PrimaCell™ Culture Medium/FibrOut** to each dish. Change the **Mouse Complete Adipose PrimaCell™ Culture Medium/FibrOut** every 2-3 days;

Δ ***Safety Note.*** The rest of the biopsy and all tubes, pipettes, plates, etc., used in the procedure should be treated with hypochlorite before disposal.

2.7 Subculture

Propagating adipocytes in culture can be somewhat challenging, especially when adipocytes are cultured alone on plastic, however the following methods have worked consistently in many laboratories.

17. The primary cultures grew exponentially until confluency was attained.
18. Remove the medium from the cell layer, and wash twice with **Mouse Adipose Tissue Washing Medium.**
19. To each culture dish, add enough prewarmed 0.01% EDTA/0.1% trypsin. to cover the cell sheet. Leave the cultures at 37℃ until the adipocytes have detached; check for detachment with a microscope. Do not leave the cells in trypsin for more than 20 min.
20. Remove the cell suspension from the plate and transfer it to a sterile centrifuge tube.
21. Rinse the growth surface with **Mouse Complete Cervix PrimaCell™ Culture Medium** and add to the suspension. Mix and dispense the suspension with a 10-ml pipette.
22. Spin the cells at 1,000 rpm for 5 min, remove the supernatant, add 10 ml of **Mouse Complete Cervix PrimaCell™ Culture Medium**, and resuspend the cells vigorously with a 10-ml pipette to achieve a single-cell suspension.
23. Count the cells and plate them onto culture dishes at 10^5 cells/cm^2.
24. Cells may be replated and grown as just described or frozen for later recovery.

III Cryopreservation

25. Cryopreservation is often necessary to maintain large quantities of cells derived from the same

tissue sample. The best results have been reported from Adipocytes cultures derived from preconfluent layers.

(a) Trypsinize cells as above, and centrifuge at 100 g for 10 min.

(b) Resuspend cells in **Mouse Complete Adipose PrimaCell™ Culture Medium** and count cells.

(c) Prepare aliquots of 2 x 10^6 cells/ml in **Mouse Complete Adipose PrimaCell™ Culture Medium** and 10% glycerol into cryovials.

(d) Equilibrate at 4℃ for 1-2 h.

(e) Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.

(f) To recover cells:

(i) Thaw cryovials quickly in a 37℃ water bath.

(ii) Dilute cells tenfold with **Mouse Complete Adipose PrimaCell™ Culture Medium**.

(iii) Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in **Mouse Complete Adipose PrimaCell™ Culture Medium**.

IV Characterization

Morphologically, pre-adipocytes are distinguishable from other primary fibroblasts. There are no biochemical arkers unique to pre-adipocytes in routine use. Morphological changes in pre-adipocytes are apparent when cells start to differentiate. Cells “round up” and lose their fibroblastic morphology, and retain only sparse and tenuous cell-cell contact. As differentiation progresses, intra-cytoplasmic lipid droplets form. It is easy to detect under phase-contrast microscopy, and stain with Oil red-O or Nile red, a simple means of assessing intracytoplasmic lipid accumulation, the classical morphological marker of differentiation. As differentiation continues, more droplets form such that the cytoplasm increases in size and becomes packed with small lipid. Cells tend to differentiate in clumps, with those cells that are refractory to differentiation reverting to a more fibroblastic morphology. The viability of the cells can be checked by glucose uptake assay. Adipocyte markers such as aP2 and adipsin are used in biochemical assays. Lipoprotein lipase (LPL) and pOb24 are widely used as an early marker, and Glycerol 3-phosphate dehydrogenase (G3PDH), along with adipsin and leptin is expressed much later in differentiation. Glucose transporter GLUT4, mediates insulin-stimulated glucose uptake in adipocytes by rapidly moving from intracellular storage sites to the plasma membrane, is a common used adipocyte specific marker gene expression assays, such as Northern bloting, real time PCR, etc., are used to detect early or late markers of differentiation.

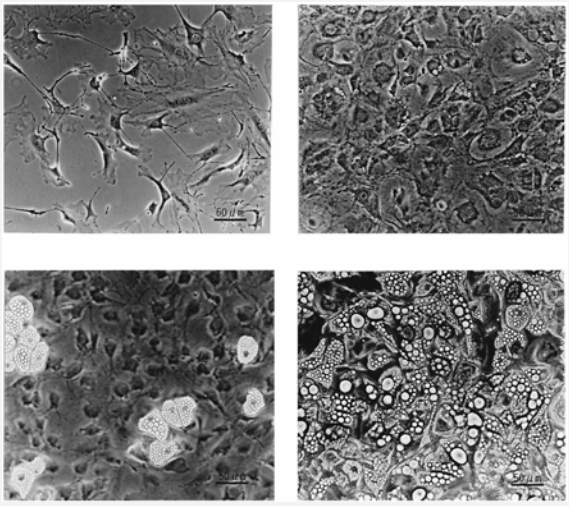


Fig. Adipocytes differentiation: upleft: Preadipocyte cells after subculture; upright: Confluent cells after 6-7 days; downleft: Some cells begin to show lipid droplets in their cytoplasm 14-20 days; downright: Adipocyte differentiation finished. [7]

V References

1. Freshney, R.I., *Culture of Animal Cells: A Manual of Basic Technique, 4th Edition* 4ed. 2001: Cold Spring Harbor Laboratory Press. 600.
2. Salans, L.B., S.W. Cushman, and R.E. Weismann, *Studies of human adipose tissue. Adipose cell size and number in nonobese and obese patients.* J Clin Invest, 1973. **52**(4): p. 929-41.
3. Dani, C., et al., *Expression and regulation of pOb24 and lipoprotein lipase genes during adipose conversion.* J Cell Biochem, 1990. **43**(2): p. 103-10.
4. Tong, Q., et al., *Function of GATA transcription factors in preadipocyte-adipocyte transition.* Science, 2000. **290**(5489): p. 134-8.
5. Abel, E.D., et al., *Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver.* Nature, 2001. **409**(6821): p. 729-33.
6. Park, S.Y., et al., *Calorie restriction improves whole-body glucose disposal and insulin resistance in association with the increased adipocyte-specific GLUT4 expression in Otsuka Long-Evans Tokushima fatty rats.* Arch Biochem Biophys, 2005. **436**(2): p. 276-84.
7. Doi, H., et al., *A new preadipocyte cell line, AP-18, established from adult mouse adipose tissue.* Tohoku J Exp Med, 2005. **207**(3): p. 209-16.
8. Bjorntorp, P., et al., *Differentiation and function of rat adipocyte precursor cells in primary culture.* J Lipid Res, 1980. **21**(6): p. 714-23.

Mouse Fat PrimaCell™: Adipose Cells

Mouse Fat Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82012	**Mouse Fat PrimaCell™ system**	**kit**	**$499**
4-22101	Mouse Fat Tissue Dissociation System, Fat OptiTDS™ (for 500 ml medium)	1 ml	$128
9-42010	Mouse Adipose Cells PrimaCell™ Basal Culture Medium	500 ml	$61
9-32101	Mouse Adipose Cells PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
7-62101	Mouse Fat Fibroblast Growth Inhibitors, Fat FibrOut™	ea	$146
9-92010	Mouse Fat Tissue Preparation Buffer Set	ea	$75

Mouse Fat Primary Cell Characterization

6-11311	**Mouse Adipose Cell Primarker™ Kit**	**kit**	**$220**
6-11312	Mouse Adipose Cell Primarker™ antibody set	set	$180
6-11313	Mouse Adipose Cell Primarker™ buffer system	set	$90

Mouse Glomerular PrimaCell™: Glomerular endothelial cells
(Cat No. 2-84404)

I. General Description

Glomerular endothelial cells (GEC) are specialized cells with important roles in physiological filtration and glomerular disease. While these cells are extremely useful in the laboratory and clinic research, they are notoriously difficult to isolate and culture. They rapidly divide, become fibroblastic, and lose their biochemical characteristics The Mouse GEC PrimaCell™ kit (Cat No. 2-84404) is designed for the successful isolation and culture of GECs from mouse glomerular tissue allowing reproducible and dependable results.

1.1 Components of the Mouse Glomerular PrimaCellTM System

- **Mouse Glomerular Tissue Dissociation System, Glomerular OptiTDS™** (2 × 1 ml) --- A mixture of collagenase, Trypsin, Dexoyribonuclease and Mouse Glomerular OptiTDS™ Reconstitution Buffer.
- **Mouse Glomerular OptiTDS™ Digestion Buffer**, (2 × 9 ml)
- **Mouse Glomerular Fibroblast Growth Inhibitors, Glomerular FibrOut™---** Glomerular FibrOutTM (5 x 200 µl) --- A mixture of D-valine, collagenase and gentamycin.
- **Mouse GEC PrimaCell™ Basal Culture Medium**, (5 × 100 ml) --- A Modified Ham F12.
- **Mouse GEC PrimaCell™ Medium Supplements**, (5 × 1 ml): Mouse EGF, Hydrocortisone, Cholera toxin penicillin (100 U/ml), streptomycin and Weymouth medium.
- **Mouse GEC PrimaCell™ Serum**, (50 ml): A modified fetal bovine serum.
- **Mouse Glomerular Tissue Washing Medium**, (1 × 100 ml): A modified DMED medium with 10 µg/ml amphotericin, 10 µg/ml gentamycin, and 10% FCS.

1.2 Required materials but NOT included:

- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or mouse fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To

enhance cell attachment to the culture dishes, fresh gelatin-coated plate or culture dishes are recommended (see below for treatment of culture dishes).

2.2 Principle

Separation of the GEC from the glomerular tissue is accomplished by enzymatic digestion using the Glomerular Tissue Dissociation System supplied in this kit. The Glomerular Tissue Dissociation System contains a mixture of collagenase, and Trypsin at the optimal concentrations to gently detach the fragile glomerular layer from the glomerular tissue. The isolated glomerular tissue is then further disrupted to release individual GEC by enzymatic and mechanical agitation. The mixture is then filtered through Cell Strainers and seeded on flasks or specially coated tissue culture plates. The GECs are propagated in growth arrested feeder cells and the corresponding media. Sub-populations of GECs can then be isolated based on their selective attachment to specific basement matrix substrates.

2.3 Mouse Glomerular Tissue Preparation

We isolated GEC from mice, which were maintained under specific pathogen-free conditions,

1. Glomerular tissues were obtained from freshly sacrificed mice perfused through the heart with cold **Mouse Glomerular Washing Medium** and glomeruli were prepared by a serial sieving method.
2. Glomerular specimens were incubated in 5-10 ml 70% ethanol for 1 min, followed by inoculating with 5-10 ml fresh Mouse Glomerular Washing Medium for 5 min.
3. Minced renal cortex tissue was serially passed through 150, 106 and 75 μm mesh stainless steel screens and glomeruli were collected using 53 μm mesh.
4. The glomeruli, which contain small debris of renal tubule, were suspended in **Mouse Glomerular Washing Medium**, washed twice by brief centrifugation (800 x g, 1 min)

2.4 GEC Separation and Tissue Dissociation

2.4.1 Mouse Glomerular OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the donor used, enzymes, culture mediums and growth supplements. The Mouse Glomerular Tissue Dissociation System is suited for optimal dissociation of normal mouse glomerular tissues to yield maximum number of single GEC.

2.4.2 Enzyme Compositions

- Collagenase: from Clostridium Histolyticum
- Trypsin

2.4.3 System Components

- Mouse Glomerular Tissue Dissociation System, Glomerular OptiTDS™, (2 × 1 ml).
- Mouse Glomerular OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

5. Prepare fresh enzyme working solution: Add 1ml of the reconstituted tissue dissociation solution to one vial of Mouse Glomerular OptiTDS™ Digestion Buffer (9 ml). Warm the diluted Mouse Glomerular OptiTDS™ working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 5 ml Mouse Glomerular OptiTDS™ working solutions.
6. Discard the Mouse Glomerular Washing Medium, and float glomeruli on 5 – 10 ml of 1 mg/ml collagenase Dissociation System working solution (prewarmed to 37℃) and transfer the tissue to a sterile glass universal containing a small plastic-coated magnetic stirrer bar. Place the universal on a magnetic stirrer and stir slowly for 30-60 min at 37 ℃. (or incubate in tube at 37 with occasional vortexing)
7. Allow the suspension to stand at room temperature for 2-3 min (or briefly centrifuge Undigested glomeruli were pelleted by).

2.5 GECs Isolation

Note: Please read section 2.6 for specific information on Cervical Epithelium culture and platting before preceding this section.

8. Combine the supernatant containing single cell suspension of GEC, and filter mixture of cells through a 200-mm nylon mesh.
9. Centrifuge at 200 g for 2 min, and the resulting pellet was resuspended in Mouse Glomerular PrimaCell™ Basal Culture Medium.
10. Wash GECs twice with Mouse Glomerular PrimaCell™ Basal Culture Medium and centrifuge at 200 g for 2 min.

2.7 Primary GECs Culture

2.7.1 Medium Preparation.

Thaw out Mouse Glomerular PrimaCell™ Basal Culture Medium, Mouse GEC PrimaCell™ Medium Supplements, and Mouse Glomerular PrimaCell™ Serum on ice.

Mouse Complete Glomerular PrimaCell™ Culture Medium: To every 100 ml Mouse Glomerular PrimaCell™ Basal Culture Medium, add 10 ml Mouse Glomerular PrimaCell™ Medium Supplements and 1 ml Mouse Glomerular PrimaCell™ Serum, mix thoroughly and warm the complete medium at a 37oC water bath for 10 min prior use.

Mouse Complete Glomerular PrimaCell™ Culture Medium/FibrOut: To every 100 ml Mouse Glomerular PrimaCell™ Basal Culture Medium, add 10 ml Mouse Glomerular PrimaCell™ Medium Supplements, 1 ml Mouse Glomerular PrimaCell™ Serum, and 1 ml Mouse Glomerular Fibroblast Growth Inhibitors, Glomerular FibrOutTM,mix thoroughly and warm the complete medium at a 37oC water bath for 10 min prior use.

2.7.2 Primary Cell Culture

(Important: *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*)

11. Pellets containing single cell suspension of GEC was suspended in Mouse Complete

Glomerular PrimaCell™ Culture Medium/FibrOut and plated on fibronectin-coated dishes.

12. Small colonies of GEC were observed within 1 week after plating. To remove contaminating mesangial and epithelial cells, brief trypsinization was performed until a culture with purity over 90% of endothelial cells was achieved. The cells were maintained in collagen-coated culture dish.

Δ Safety Note. The rest of the biopsy and all tubes, pipettes, plates, etc., used in the procedure should be treated with hypochlorite before disposal.

2.7 Subculture

Propagating GECs in culture can be somewhat challenging, especially when GECs are cultured alone on plastic, however the following methods have worked consistently in many laboratories.

13. Remove culture medium, and wash cells with Mouse Glomerular PrimaCell™ Basal Culture Medium.
14. Add a small volume of EDTA gently to the cells and remove it immediately.
15. Add sufficient trypsin solution (0.25%) to form a thin layer over the cells.
16. When cells detach, add 5 to 10 ml of Mouse Complete Thyroid PrimaCell™ Culture Medium, pass the culture very gently in and out of a pipette, and then centrifuge the cells for 10 min at 350 g.
17. Count an aliquot and seed the cells at the chosen concentration.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Skeletal muscle cells cultures derived from preconfluent layers.

18. Trypsinize cells as above, and centrifuge at 100 g for 10 min.
19. Resuspend cells in Mouse Complete Glomerular PrimaCell™ Culture Medium and count cells.
20. Prepare aliquots of 2 × 106 cells/ml in Mouse Complete Glomerular PrimaCell™ Culture Medium and 10% glycerol into cryovials.
21. Equilibrate at 4℃ for 1-2 h.
22. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
23. To recover cells:
    (i) Thaw cryovials quickly in a 37℃ water bath.
    (ii) Dilute cells tenfold with appropriate Mouse Complete Glomerular PrimaCell™ Culture Medium.
    (iii) Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in Mouse Complete Glomerular PrimaCell™ Culture Medium.

IV Characterization

The GECs can be characterized as endothelial cells by homogeneous monolayer of phase contrast image and positive staining with anti-CD31 in the junctional area and negative with anti-desmin. The negative staining with anti-desmin indicates that there was little contamination of mesangial cells in the primary culture. In addition, RT－PCR analysis is used to confirm CD31 expression in the GEC.

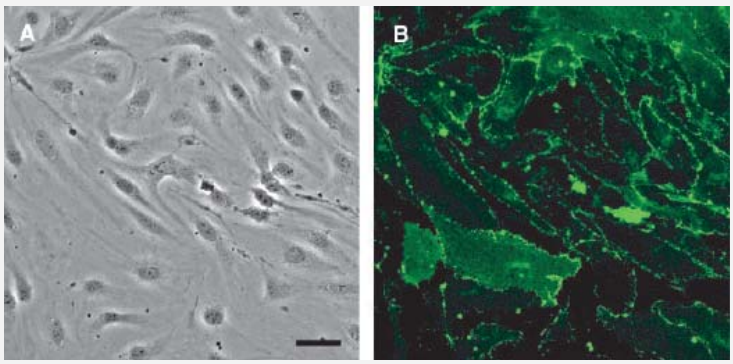


Figure 1. Phase contrast image of isolated GEC (A) and immunofluorescence image for CD31 (B)[1]

V Reference

1. Nagao, T., et al., *Up-regulation of adhesion molecule expression in glomerular endothelial cells by anti-myeloperoxidase antibody.* Nephrol Dial Transplant, 2007. **22**(1): p. 77-87.
2. Adler, S., et al., *Complement membrane attack complex stimulates production of reactive oxygen metabolites by cultured rat mesangial cells.* J Clin Invest, 1986. **77**(3): p. 762-7.
3. Hugo, C., et al., *The plasma membrane-actin linking protein, ezrin, is a glomerular epithelial cell marker in glomerulogenesis, in the adult kidney and in glomerular injury.* Kidney Int, 1998. 54(6): p. 1934-44.

Mouse Glomerular PrimaCell™: Glomerular Endothelial cells

Mouse Glomerular Primary Cell Culture

Cat No.	Description	Qt.	Price
2-84404	**Mouse Glomerular PrimaCell™ system**	**kit**	**$499**
4-26431	Mouse Glomerular Tissue Disassociation System, Glomerular OptiTDS™ (for 500 ml medium)	1 ml	$128
9-48403	Mouse Glomerular Endothelial Cells PrimaCell™ Basal Culture Medium	500 ml	$ 61
9-37431	Mouse Glomerular Endothelial Cells PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$ 140
7-67431	Mouse Glomerular Fibroblast Growth Inhibitors, Glomerular FibrOut™	ea	$146
9-98403	Mouse Glomerular Tissue Preparation Buffer Set	ea	$75

Mouse Primary Glomerular Endothelial Cells Characterization

6-12211	**Mouse Glomerular Endothelial Cell Primarker™ Kit**	**kit**	**$220**
6-12212	Mouse Glomerular Endothelial Cell Primarker™ antibody set	set	$180
6-12213	Mouse Glomerular Endothelial Cell Primarker™ buffer system	set	$90

Mouse Heart PrimaCell™ II: Cardiomyocyte
(Cat No. 2-84204)

I. General Description

The muscle cells of heart muscle tissue are called cardiomyocytes. Primary culture of cardiomyocytes has been widely used as a valuable tool for pharmacological and toxicological studies. However, the fact that heart is a solid organ and cardiomyocytes do not proliferate after birth makes the primary myocardial culture a tedious job. The two isolation steps are essential for a successful culture. One is the enzyme digestion step for dissociating cells from heart tissue. The other is the purification step for eliminating non-muscle cells. The latter is critical for ensuring a constant proportion of myocytes.

The Mouse Heart PrimaCell™ kit (Cat No. 2-84204) is designed for the successful isolation and culture of Cardiomyocytes from mouse heart tissue allowing reproducible and dependable results.

1.1 Components of the Mouse Heart PrimaCell™ System

- **Mouse Heart Tissue Dissociation System, Heart OptiTDS™** (2 × 1 ml) --- *A mixture of collagenase, and Mouse Heart OptiTDS™ Reconstitution Buffer.*
- **Mouse *Heart OptiTDS™ Digestion Buffer*,** (2 × 9 ml)
- **Mouse Heart Fibroblast Growth Inhibitors, Heart FibrOut™**---Heart FibrOut™ (5 x 200 µl) --- *A mixture of D-valine, collagenase and gentamycin.*
- **Mouse Cardiomyocyte PrimaCell™ Basal Culture Medium,** (5 × 100 ml) --- *A Modified Ham F12*.
- **Mouse Cardiomyocyte PrimaCell™ Medium Supplements,** (5 × 1 ml): *Mouse EGF, Hydrocortisone, Cholera toxin penicillin (100 U/ml), streptomycin and Weymouth medium.*
- **Mouse Cardiomyocyte PrimaCell™ Serum,** (50 ml): A modified f*etal bovine serum.*
- **Mouse Heart Tissue Washing Medium,** (1 × 100 ml): *A modified DMED medium with 10 µg/ml amphotericin,* 10 *µg/ml gentamycin, and 10% FCS.*

1.2 Required materials but NOT included:

- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or mouse fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination.

2.2 Principle

Separation of the Cardiomyocyte from the heart is accomplished by enzymatic digestion using the Heart Tissue Dissociation System supplied in this kit. The Heart Tissue Dissociation System contains a mixture of collagenase and other materials at the optimal concentrations to gently detach the fragile heart layer from the heart tissue. The isolated heart tissue is then further disrupted to release individual Cardiomyocyte by enzymatic and mechanical agitation. The mixture is then filtered through Cell Strainers and seeded on flasks.

2.3 Mouse Heart Tissue Preparation

1. Mouse pups (Sprague-Dawley or Wistar rats) at the age of postnatal day 1–3 were sacrificed by ethyl ether
2. The animals were decontaminated with 75% ethanol, and transferred to a Luminer flow hood.
3. Surgically remove the beating heart from animals immediately, and keep it in cold **Mouse Heart Tissue Washing Medium**
4. Ventricles were excised and transferred to fresh ice-cold **Mouse Heart Tissue Washing Medium** and were minced with fine scissors into 1–3 mm3 pieces after washing blood away from the heart lumen. Red blood cells were removed by instant centrifugation for two times.

2.4 Heart Separation and Tissue Dissociation

2.4.1 Mouse Heart OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the donor used, enzymes, culture mediums and growth supplements. The Mouse Heart Tissue Dissociation System is suited for optimal dissociation of normal mouse heart tissues to yield maximum number of single Cardiomyocyte.

2.4.2 Enzyme Compositions

- Collagenase: from *Clostridium Histolyticum*
- Trypsin

2.4.3 System Components

- Mouse Heart Tissue Dissociation System, Heart OptiTDS™, (2 × 1 ml).
- Mouse Heart OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

5. Prepare fresh enzyme working solutions: to each vial of **Mouse Heart Tissue Dissociation System, Bone OptiTDS™**, add 1.0 ml of the **Mouse Heart OptiTDS™ Reconstitution Buffer**, Mix well.
6. Add 1.0 ml of the fresh enzyme working solution to one vial of **Mouse Heart OptiTDS™ Digestion Buffer** (9 ml). Warm the diluted enzyme working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 5-7 mg tissue samples per 10 ml diluted enzyme working solutions.

7. Transfer the minced tissue to a small sterile bottle with a magnetic stirrer, and add 4 ml of digestion solution. The flask was then settled on ice for 20 min, and shaken every 3 min for better mixing.
8. Stir the solution containing bone fragments at 37℃ for 30 min.
9. Gently pipeting mixture to dispersed cells from the tissue. The cell suspension was settled on ice for 2-3 min
10. Transfer the supernatant to a centrifuge tube and and centrifuge it for 2 min at 580 g at room temperature.
11. Remove the supernatant digestion solution buffer which can be reused, and resuspend the cells in 5 ml of **Mouse Heart PrimaCell™ Culture Medium/FibrOut**.
12. In the interim, add an additional 5 ml of digestion solution to the remaining pieces of tissue, and repeat the digestion for 30 min. The released cells are harvested as described in step 12, and, if necessary, the digestion step is repeated several more times.

2.5 Cardiomyocytes Isolation

Note: Please read section 2.6 for specific information on Cervical Epithelium culture and platting before preceding this section.

13. Combine the supension containing single cells, and strain the cell mixture through a sterile cell strainer (70-100μm) into a 50-ml centrifuge tube to remove debris. Cell strainers fit perfectly in 50 ml conical and are very convenient for this procedure. If cell strainers are not available, nylon gauze can be used after rinsed twice in PBS and placed at the opening of a 50 ml conical.
14. Centrifuge the strained mixture at 1000 rmp (800 g) for 5 min at 4°C.
15. Remove the supernatant; add 10 ml of **Mouse Heart PrimaCell™ Culture Medium/FibrOut** to the pellet, resuspend the cells vigorously to give a single-cell suspension.

2.6 Primary Cardiomyocytes Culture

2.6.1 Medium Preparation.

Thaw out **Mouse Cardiomyocyte PrimaCell™ Basal Culture Medium**, **Mouse Cardiomyocyte PrimaCell™ Medium Supplements**, and **Mouse CardiomyocytePrimaCell™ Serum** on ice.

Mouse Complete Cardiomyocyte PrimaCell™ Culture Medium: To every 100 ml **Mouse CardiomyocytePrimaCell™ Basal Culture Medium**, add 10 ml **Mouse Cardiomyocyte PrimaCell™ Medium Supplements** and 1 ml **Mouse Cardiomyocyte PrimaCell™ Serum**, mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use.

Mouse Complete Cardiomyocyte PrimaCell™ Culture Medium/FibrOut: To every 100 ml **Mouse Cardiomyocyte PrimaCell™ Basal Culture Medium**, add 10 ml **Mouse Cardiomyocyte PrimaCell™ Medium Supplements,** 1 ml **Mouse Cardiomyocyte PrimaCell™ Serum**, and 1 ml **Mouse Heart Fibroblast Growth Inhibitors, Heart FibrOut™**,mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use.

2.6.2 Primary Cell Culture

(Important: Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination)

16. The cells were plated in a 40 ml tissue culture flask and incubated at 37 °C in a humidified atmosphere (5% CO2, 95% air).
17. Since non-myocardiocytes attach to the substrata more readily than myocardiocytes, cells were incubated for 1.5 h to allow the attachment of non-myocardiocytes.
18. The majority of myocardiocytes remained in culture medium. The suspended cells were collected and plated at a density of 2 • 105 ml_1 into a new tissue culture flask.
19. Generally, cells isolated from 2 to 3 hearts can be seeded in one 40 ml culture flask.The culture medium was replaced with fresh media every 2-3 days.

Δ ***Safety Note.*** The rest of the biopsy and all tubes, pipettes, plates, etc., used in the procedure should be treated with hypochlorite before disposal.

2.7 Subculture

Propagating Cardiomyocytes in culture can be somewhat challenging, especially when Cardiomyocytes are cultured alone on plastic, however the following methods have worked consistently in many laboratories.

20. Remove culture medium, and wash cells with **Mouse Cardiomyocyte PrimaCell™ Basal Culture Medium.**
21. Add a small volume of EDTA gently to the cells and remove it immediately.
22. Add sufficient trypsin solution (0.25%) to form a thin layer over the cells.
23. When cells detach, add 5 to 10 ml of **Mouse Complete Cardiomyocyte PrimaCell™ Culture Medium**, pass the culture very gently in and out of a pipette, and then centrifuge the cells for 10 min at 350 g.
24. Count an aliquot and seed the cells at the chosen concentration.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Skeletal muscle cells cultures derived from preconfluent layers.

25. Trypsinize cells as above, and centrifuge at 200 g for 10 min.
26. Resuspend cells in **Mouse Complete Cardiomyocyte PrimaCell™ Culture Medium** and count cells.
27. Prepare aliquots of 2×10^6 cells/ml in **Mouse Complete Cardiomyocyte PrimaCell™ Culture Medium** and 10% glycerol into cryovials.
28. Equilibrate at 4℃ for 1-2 h.
29. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
30. To recover cells:
    (i) Thaw cryovials quickly in a 37℃ water bath
    (ii) Dilute cells tenfold with appropriate **Mouse Complete Cardiomyocyte PrimaCell™ Culture Medium**.
    (iii) Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in **Mouse Complete Cardiomyocyte PrimaCell™ Culture Medium.**

IV Characterization

A-sarcomeric actin is considered as a specific protein in cardiomyocytes, anti-a-sarcomeric actin is applied as the primary antibody to identify cardiomyocytes. Besides, Cardiac troponin I (cTnI), Caveolin-3 is cardiomyocyte specific markers, eHAND, a cardiomyocyte-specific transcription factor, is used to identify cardiomyocyte.

V References

1. Fu J., G.J., Pi R. and Liu P., *An optimized protocol for culture of cardiomyocyte from neonatal rat.* Cytotechnology, 2005. **49**: p. 109-116.
2. Kodama, H., et al., *Cardiomyogenic differentiation in cardiac myxoma expressing lineage-specific transcription factors.* Am J Pathol, 2002. **161**(2): p. 381-9.
3. Jiajia Fu, J.G., Rongbiao Pi and Peiqing Liu, *An optimized protocol for culture of cardiomyocyte from neonatal rat.* Cytotechnology, 2005. **49**: p. 109-116.

Mouse Heart PrimaCell™ II: Cardiomyocyte

Mouse Heart Primary Cell II Culture

Cat No.	Description	Qt.	Price
2-84204	**Mouse Heart PrimaCell™ II system**	**kit**	**$499**
4-26231	Mouse Heart Tissue Disassociation System, Heart OptiTDS™ (for 500 ml medium)	1 ml	$128
9-48203	Mouse Cardiomyocyte PrimaCell™ Basal Culture Medium	500 ml	$ 61
9-37231	Mouse Cardiomyocyte PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$ 140
7-67231	Mouse Heart Fibroblast Growth Inhibitors, Heart FibrOut™	ea	$146
9-98203	Mouse Heart Tissue Preparation Buffer Set	ea	$75

Mouse Primary Cardiomyocytes Characterization

6-12311	**Mouse Cardiomyocyte Primarker™ Kit**	**kit**	**$220**
6-12312	Mouse Cardiomyocyte Primarker™ antibody set	set	$180
6-12313	Mouse Cardiomyocyte Primarker™ buffer system	set	$90

Mouse Intestine PrimaCell™: Intestinal Epithelial Cells
(Cat No. 2-2-92201)

I. General Description

Small Intestine Epithelial Cells (IECs) have been difficult to maintain in culture, remaining viable for only hours to several days. Although long term cultures of human and rat small IEC have been established, IEC derived from mice have depended on immortalization by simian virus 40 transfection to remain viable. Successful cultivation of small IEC is dependent on a number of factors such as method of digestion and dissociation; the fine treated and applied serum, and treatment of the culture dishes.

This protocol is developed for attachment and growth of normal Mouse Intestine epithelial cells from newborn or adult Mouse Intestine with Mouse Intestine PrimaCell™ system (Cat No. 2-92201). This system provides an optimal condition of tissue dissociation system, Intestine OptiTDS™ that yields 5-7 times of single cells more than most of the tissue dissociation protocols published in the literature. In addition, this system ensures a high viability of the target cells with improved gradient contained in the culture medium. With CHI's proprietary fibroblast inhibitory system, FibrOut™, cells are growing with contamination of minimized amount of the non-epithelial cells.

The Intestine PrimaCellTM I system is suited for long-term culturing for the monolayer small intestinal epithelium of mouse. This kit applies to all types of normal adult mouse biopsies samples. Biopsies samples contain pathological organism (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Mouse Kidney PrimaCell™ System

- **Mouse Intestine Tissue Dissociation System, Intestine OptiTDS™** ((2 x 1 ml)) --- *A proprietary mixture of Collagenase XIa, Dispase I, Soybean trypsin inhibitor, and BSA.*
- **Mouse Intestine OptiTDS™ Digestion Buffer,** (2 x 9 ml)
- **Mouse Intestine Fibroblast Growth Inhibitors**, **Intestine FibrOut™** (5 x 200 µl) --- *A mixture of D-valine, collagenase, toxin ricin, and formulated serum.*
- **Mouse Intestine Epithelium Basic Culture Medium** (5 x 100 ml) --- *Modified formulation based on DMEM and Ham's F-12*.
- **Mouse Intestine Epithelium Culture Medium Supplements with Serum** (5 x 1 ml) --- *A mixture of Insulin, Dexamethasone, Selenium, Transferrin, Triiodothyronine, EGF, HEPES, Glutamine and D-glucose.*
- **Intestinal Epithelium PrimaCell™ Serum** (5 x 10 ml): Charcoal-stripped and highly purified FBS.
- **Buffer Systems for Mouse Intestine Preparation** (2 x 100 ml)

1.2 Required Materials NOT provided

- Water bath.
- Pasteur pipettes

- Collagen-I coated culture dishes
- 35-mm tissue culture Petri dishes
- Scalpels, scissors, and forceps
- Pasteur pipettes and 10-ml pipettes
- Test tubes, 12 and 50 ml

II. Procedures

2.1 Preparation of Tissue Culture Plates

All materials used in this experiment must be sterile to prevent contamination. To enhance cell attachment to tissue culture dishes, collagen I pre-coated plates (Corning, NY) MUST be further pre-treated with the Coating Solution included in this kit for 5 min. Aspirate the Coating Solution and allow the dishes to air-dry in a ventilated cell culture hood for 5-10 min before use.

2.2 Surgical specimens

Mouse Small Intestines: Adult or newborn (prefer 14-16 days) mouse yields a large number of cells (5-10 x 10^7), with a 30-40% plating efficiency. Mice are sacrificed by CO_2 narcosis or a method that is approved by user's institution. Once the intestines is isolated, tissue specimens should be placed immediately in 5-10 ml Intestinal Tissue Washing Medium, transported on ice to the laboratory and worked up within 1 hr of isolation. Intestine tissue specimens are opened longitudinally. With autoclaved scalpels, scissors, and forceps, carefully remove muscle and fat from tissue specimens. Place intestine in a 50 ml conical tube (Falcon) containing 5 ml fresh Intestinal Tissue Washing Medium and incubate while shaking at room temperature for 10 min. For large tissue specimens, use a 50 ml conical tube (Falcon) and a larger volume of Intestinal Tissue Washing Medium to ensure thorough washing. Aspirate the washing medium and repeat the washing procedures using fresh washing medium two more times. After the initial washes, incubate tissue in 70% ethanol for 1 min at room temperature, followed by incubating in 5-10 ml fresh Intestinal Tissue Washing Medium for 5 min. Collect tissue by gentle centrifugation and proceed to dissociation.

2.3 Tissue Preparation and Dissociation

2.3.1 Mouse Intestine OptiTDS™

The isolation of primary cells is confounded by several important factors that can greatly affect yield and cell viability. The Intestine Tissue Dissociation System, Intestine OptiTDS™, is developed to produce the optimal conditions that allow for the dissociation of intestinal epithelial cells from normal adult mouse tissue samples. This system uses a defined proprietary ratio of specific enzymes to yield the maximum number of single primary cells that remain viable in tissue culture.

2.3.2 Enzyme Compositions

- Collagenase XIa: from *Clostridium Histolyticum*
- Dispase I: from *Bacillus polymyxa*
- Soybean trypsin inhibitor: from *Glycine Max*

2.3.3 System Components

- Intestinal Tissue Dissociation System, OptiTDS™: (2 x 1 ml)
- Intestine OptiTDS™ Digestion Buffer: (2 x 9 ml)

2.3.4 Procedures For Tissue Preparation and Dissociation

1. Prepare Mouse Intestine OptiTDS™ working solution: Add 1.0 ml of Mouse Intestine Tissue Dissociation System, Intestine OptiTDS™ to 9.0 ml of Mouse Intestine OptiTDS™ Digestion Buffer and mix well (Do not Vortex) with hand. It is important that the Mouse Intestine OptiTDS™ working solution is freshly prepared and is provided in aliquots for your convenience.
2. Warm the Mouse Intestine OptiTDS™ working solution at 37 ℃ for 10 min, just prior to use. For optimal results, we recommend a ratio of 4-5 g intestinal tissue per 10 ml of enzyme solution.
3. Mince the pre-opened and washed intestinal tissue into pieces measuring approximately 2-5 cm in length with scalpel and forceps or scissors.
4. Incubate the minced tissue with the Mouse Intestine OptiTDS™ working solution with gentle stirring for 30 min at 37 ℃. Up to 5 g of minced tissue can be incubated with 10 ml of the diluted enzyme solution.
5. Gently pipette sample with a 10 ml pipette, filling the barrel of the pipette at a rate of 2-3 ml per second. Repeat this for 5-6 times.
6. Filter the cell mixture through a cell strainer (100μM). Centrifuge the strained mixture at 100 g. Carefully remove the supernatant and resuspend the cell pellet with1.0 ml of Complete Intestine Culture Medium (See 2.4.1 for preparation).
7. Count and seed the cells in 3 or 4 T-25 collagen I-coated flasks containing 4 ml of Complete Intestine Culture Medium (**Important:** Collagen coated plates must be pre-treated with the provided Coating Solution, see section 2.1). This procedure will give you approximately 2.5-5 x 10^5 Cells/per 5 cm intestinal tissues in length.

2.3.5 Storage of Tissue Dissociation System:

Intestinal Tissue Dissociation System, Intestine OptiTDS™ should only be reconstituted when needed for cell preparation and can be stored for 2-4 days at 4 ^{o}C. For long-term use, it should be aliquoted and stored at -20 ^{o}C. Avoid repeated freeze-thaw cycles.

2.4 Culture of primary mouse intestine cells

2.4.1 Medium Preparation

Thaw the Mouse Intestinal Epithelium PrimaCell™ Medium Supplements and Mouse Intestinal Epithelium PrimaCell™ Serum on ice. To prepare Complete Intestine Culture Media, add one vial of Mouse Intestinal Epithelium PrimaCell™ Medium Supplements into 100 ml Mouse Intestinal Epithelium PrimaCell™ Basal Culture Medium. Add 10 ml Intestinal Epithelium PrimaCell™ Serum and one vial of Intestine Fibroblast Growth Inhibitors, Intestine FibrOut™. Mix thoroughly and warm the Complete Intestine Media in a 37^{o} C water bath for 10 min prior use. (**Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using*

FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination).

2.6.1 Treatment of Culture Dishes

To facilitate primary intestine cell attachment to the tissue culture plates, the plates should be pre-coated with collagen I (available from Corning, NY) and MUST be pre-treated with the provided Coating Solution (Basal Growth Medium containing 10 µg/ml BSA). Cover the entire surface area of tissue culture plates with the Coating Solution and incubate for 5 min. Aspirate the Coating Solution and allow the dishes to air-dry in a ventilated cell culture hood for 5-10 min prior to use.

2.6.1 Standard primary culture conditions

Seed epithelial tubules and cell clumps isolated from intestine tissues (from 2.3.4) into prepared T-25 flasks containing 4 ml of complete media and incubate a 37° C, 5%-CO_2 incubator. The Complete Intestine Culture Medium should be changed twice a week. The tubules and cells will attach to the matrix on the plates and the intestine epithelial cells will begin to migrate out within 1-2 days. Most of the tubules and cell clumps of epithelium will attach within 7 days, however, larger organoids can take up to 6 weeks to attach. It is important to note that the cells remain viable during this time.

2.6.1 Alternative Primary Culture conditions

Primary intestine epithelium requires a substratum to efficiently attach during tissue culture propagation. While this can be achieved using pre-coated Collagen I plates, the growth of primary intestine epithelium is significantly better when a layer of 3T3 feeder cells are used. As the initial intestine epithelial colonies expand to several hundred cells per colony, the cells become less dependent on the 3T3 feeder cells, and can be cultured without the feeder cells. All media and solutions supplied in this kit can be used whether cells are grown on collagen coated plates or 3T3 feeder cells.

2.4.5 Subculture and Propagation

Most intestine primary cells cannot be passaged by routine procedures using trypsin/EDTA. Intestine epithelial cells can be de-attached using a mild dissociation enzyme, dispase as this treatment leads to more successful passaging. A further advantage of using dispase to passage cells is that dispase can only detach epithelial cell but not fibroblast. Thus this property decreases fibroblast contamination of the intestine epithelial cells with each successive passage.

8. Add 0.5% Dispase (Sigma, w/v) to the cell monolayer. Use just enough dispase to cover the cells (~2.5 ml/25-cm^2 flask), and incubate for 40-60 min for primary intestine cultures and 20-40 min for primary intestine cell lines.
9. Once the epithelial layers begin to detach (they do so as sheets rather than single cells), pipette to encourage the detachment and dis-aggregation into smaller clumps.
10. Wash and replate the cells under standard culture conditions. It may take several days for clumps to attach, so take special care when changing the media and feeding the cells.

III Fibroblast Contamination

Several techniques have been published that help prevent fibroblast contamination of primary intestine cell cultures. These include: (1) Physically removing isolated fibroblast colonies by scraping off the fibroblast colonies with a sterile cell scraper. This method requires extensive washing to ensure that all the lifted fibroblast that remain are washed away and not allowed to reattach and repopulate the primary intestine cultures. (2) Differential trypsinization can be attempted with the carcinomas but may not work well with normal primary intestine cells. (3) Using dispase to lift off intestine epithelial cells preferentially but not exclusively during routine passaging. In addition, cells can be seeded on mock plates for 4-6 hrs so that the fibroblasts attach, and floating intestine cells can be transferred to collagen coated plates under standard culture conditions. This technique takes advantage of the fact that fibroblasts in general attach much more quickly to plastic than do clumps of epithelial cells.
(4) Reducing the concentration of serum to about 2.5-5% to eliminate heavy concentrations of fibroblastic cells. The above methods can be used in combination knowing that normal primary fibroblasts have a finite growth span *in vitro* and if these measures are used persistently the fibroblasts will eventually be forced into senesce. (**Important Note:** The Mouse Intestine PrimaCell™ kit includes a fibroblast elimination system Intestine FibrOut™, an effective mixture of Mouse Intestine Fibroblast Growth Inhibitors. It contains a mixture of D-valine, collagenase, toxin ricin, and formulated serum. The principle behind the using this system is that low concentration of D-valine is toxic to fibroblast but not epithelial-type cells. Using this approach, therefore, fibroblasts are eliminated without added toxicity to epithelial cells.)

V References:

1. Golaz JL, Vonlaufen N, Hemphill A, Burgener IA. Establishment and characterization of a primary canine duodenal epithelial cell culture.
2. Spottl T, Hausmann M, Gunckel M, Herfarth H, Herlyn M, Schoelmerich J, Rogler G. A new organotypic model to study cell interactions in the intestinal mucosa. Eur J Gastroenterol Hepatol. 2006 Aug;18(8):901-9.
3. Dzierzewicz Z, Orchel A, Swierczek-Zieba G, Latocha M, Cwalina B, Wilczok T. Normal colonocytes in primary culture--an experimental model for molecular pharmacology and biology of large intestine. Acta Pol Pharm. 2000 Nov;57 Suppl:23-5.
4. Follmann W, Weber S, Birkner S. Primary cell cultures of bovine colon epithelium: isolation and cell culture of colonocytes. Toxicol In Vitro. 2000 Oct;14(5):435-45.
5. Slorach EM, Campbell FC, Dorin JR. A mouse model of intestinal stem cell function and regeneration. J Cell Sci. 1999 Sep;112 Pt 18:3029-38.
6. Perreault N, Beaulieu JF. Primary cultures of fully differentiated and pure human intestinal epithelial cells. Exp Cell Res. 1998 Nov 25;245(1):34-42.

Mouse Intestine PrimaCell™: Intestinal Epithelial Cells

Mouse Intestine Primary Cell Culture

Cat No.	Description	Qt.	Price
2-52009	**Mouse Intestine PrimaCell™ system**	**kit**	**$499**
4-52081	Mouse Intestine Tissue Dissociation System, Intestine OptiTDS™ (for 500 ml medium)	1 ml	$128
9-52008	Mouse Intestinal Epithelium PrimaCell™ Basal Culture Medium	500 ml	$61
9-52081	Mouse Intestinal Epithelium PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
7-52081	Mouse Intestine Fibroblast Growth Inhibitors, Intestine FibrOut™	ea	$146
9-52008	Mouse Intestinal Tissue Preparation Buffer Set	ea	$75

Mouse Primary Colorectal Epithelium Cell Characterization

6-51011	**Mouse Intestinal Epithelium Primarker™ Kit**	**kit**	**$220**
6-51012	Mouse Intestinal Epithelium Primarker™ antibody set	set	$180
6-51013	Mouse Intestinal Epithelium Primarker™ buffer system	set	$90

Mouse Kidney PrimaCell™ I: Kidney Epithelium
(Cat No. 2-82013)

I. General Description

The Mouse Kidney PrimaCell™ kit (Cat No. 2-82013) allows the isolation and growth of mouse kidney epithelial cells from newborn or adult mice with typical yields of 5-8 times more cells than most protocols published in the literature. In addition, the specially formulated media in the Mouse Kidney PrimaCell™ kit ensures high cell viability and the proprietary Kidney Fibroblast Growth Inhibitors, Kidney FibrOut™ minimizes fibroblast contamination of epithelial cultures.

The preparation of tissues is usually started within 1-2 hrs of removal from mouse. If this is impossible, tissue can be cut into 1 mm cubes with sterile scalpels and stored overnight at 4°C in the provided washing medium. The Mouse Kidney PrimaCell™ system can be used to isolate cells from mouse kidneys aged 2-5 months. Kidney samples containing pathological organisms (virus, parasites, etc.) or tumor may not suitable for this system.

1.2 Components of Mouse Kidney PrimaCell™ System

- **Kidney Tissue Dissociation System**, **Kidney OptiTDS™,** (2 x 1 ml) --- A proprietary mixture of collagenase, collagenase I, collagenase II, collagenase IV, Soybean Trypsin Inhibitor, Dexoyribonuclease I, Trypsin, and Protease.
- **Kidney OptiTDS™ Digestion Buffer,** (2 x 9 ml)
- **Kidney Tissue Washing Medium, (**1 x 100 ml) --- *Basal Kidney PrimaCell™ Culture Medium with 5% FBS, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Kidney Fibroblast Growth Inhibitors, Kidney FibrOut™,** (5 x 200 μl) --- *A proprietary mixture of cis-OH-proline, collagenase, D-valine, and formulated serum substitutes*.
- **Mouse Kidney Epithelium PrimaCell™ Basal Culture Medium, (**5 x100 ml) --- *Modified formulation based on medium DMEM and F-12*.
- **Mouse Kidney PrimaCell™ Medium Serum, (**1 x 50 ml) --- *Highly purified and special-treated Fetal-bovine serum.*
- **Mouse Kidney Epithelium PrimaCell™ Medium Supplements with Serum**, (5 x 10 ml) --- A mixture of insulin, sodium selenite, transferring, 3,3'5-triiodothyronine, highly purified bovine serum albumin, and glutamine, dexamethasone, antibiotics (penicillin, streptomycin), and charcoal-stripped Fetal Bovine Serum.

1.2 Required Materials NOT provided

- Falcon Conicals, 50 ml
- Nylon gauze cell strainer (70-100 μm)
- Plastic tissue culture dishes, collagenase -I coated, 100 mm (Corning, NY)
- Scalpels, curved forceps
- 70% ethanol

- 0.05% EDTA (pH 7.4), sterile
- 0.25% trypsin/0.1% (2.5 mM) EDTA, sterile
- PBSA (PBS containing 10% BSA), sterile

II. Procedures

2.1 Procedure Overview and Materials Preparation

Tissue fragments are excised from the outer cortex of the kidney, minced, washed, and incubated with agitation in Kidney OptiTDS™ solution. The Kidney Dissociation System provides the most optimal conditions to isolate kidney epithelial cells. The kidney epithelium is further dispersed by additional incubations and mechanical disruption. The cells are filtered and platted onto specially treated tissue culture plates. All materials and equipment used in these experiments should be sterilized and rinsed with PBSA prior use.

2.2 Surgical specimens

1. Kidneys from two mice (males and/or females between 2-5 months) are sacrificed using an approved method. The mice are doused with 70% ethanol to minimize contamination. Kidneys are removed using scissors and forceps, immersed in 70% ethanol for 1 min, and immediately placed in a 100-mm tissue culture dish containing 10 ml of Kidney Tissue Washing Medium (each kidney is placed in a separate dish). After all the kidneys are removed, transfers into fresh 100-mm dishes containing 10 ml of Kidney Tissue Washing Medium. Whole kidneys are minced into 1 mm cubes using sterile razor blades. The minced tissues are transferred into sterile 15 ml conical tubes containing Kidney Tissue Washing Medium. After allowing the minced tissue to settle, aspirate off the Kidney Tissue Washing Medium, and wash once more with Kidney Tissue Washing Medium.
2. Incubate kidney pieces sequentially in 10 ml 70% ethanol for 1 min, in 10 ml PBSA for 2 min, and in 20 ml Kidney Tissue Washing Medium for 10 min. These steps reduce the risk of infection and do not interfere with cell viability.
3. Keep tissues on ice until ready to proceed with tissue dissociation.

2.3 Tissue Preparation and Dissociation

2.3.1 Mouse Kidney OptiTDS™

Several important factors can affect the yield and viability of primary cells, including the tissue type, species, and age of the animals, enzymes, culture media and growth supplements. The Mouse Kidney Tissue Dissociation System, OptiTDS™, is optimized for the efficient dissociation of kidney epithelial cells from normal adult and newborn mouse kidneys yielding the maximum number of single primary cells.

2.3.2 Enzyme Compositions

- Collagenase: from *Clostridium Histolyticum*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase II: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*

- Soybean Trysin Inhibitor: from *Glycine Max*
- Dexoyribonuclease I: from *Bovine Pancreas*
- Trypsin: from *Bovine Pancreas*
- Protease: from *Staph Aureus*

2.3.3 System Components

- **Kidney Tissue Dissociation System, OptiTDS™,** (2 x 1 ml).
- **Kidney OptiTDS™ Digestion Buffer,** (2 x 9 ml).

2.3.4 Procedures For Tissue Preparation and Dissociation

1. Prepare fresh enzyme working solutions: Add 1.0 ml of Kidney Tissue Dissociation System, OptiTDS™ to 9 ml of Kidney OptiTDS™ Digestion Buffer. Warm this diluted enzyme solution at 37° C for 10 min prior to use. For optimal results we recommend using 10 ml diluted enzyme solution per 2-3 g of tissue.
2. Mince pre-washed tissue into pieces measuring approximately 1 mm with scalpels or sterile razors.
3. Incubate minced tissue with the fresh Kidney Dissociation enzyme working solution (the diluted enzyme solutions prepared in step 1) by one of the following steps:

    a. Rapid dissociation: Incubate kidney tissue in Kidney Dissociation enzyme working solution with rocking for 2-3 hrs at 37° C. This works particularly well with thicker/older kidneys.
    b. Slow dissociation: Incubate kidney tissue in ice-cold Kidney Dissociation enzyme working solution with rocking at 4° C for 15-24 hrs.

4. Prepare Nylon cell strainers by placing inside a 50 ml conical tube. Alternatively, rinse sterile woven cloth 2X in sterile PBSA and place into sterile funnel on top of sterile beaker and use to strain the cell mixture in order to remove debris. Save the Kidney Dissociation enzyme working solution.
5. Tirturate the cell mixture with a sterile pipette several times to facilitate a single cell mixture. Collect the cells by passing the mixture through the Nylon cell strainers into a 50 ml conical.
6. The collected cells are centrifuged at 800 g for 5 min. The resulting pellet contains the primary kidney epithelial cells. This pellet can be resuspended in 5 ml of Complete Kidney Epithelium Medium (see medium preparation below 2.4.1). Save the supernatant for repeating tissue dissociation process in step 4.
7. To the remaining pieces of tissue, repeat the tissue dissociation process with the saved Kidney Dissociation working solutions from step 4 to yield additional cells. This process can repeat up to 3 times if it is necessary without changing the enzyme working solution. In general, kidneys from each 5-6 month old mouse should produce six to eight 60-mm dishes of primary culture cells.
8. Count viable cells and plate cells at a density of 1×10^5 cells per 100-mm collagenase -I coated culture dish.

Note: Kidneys can be pooled for this preparation; however, it is very important that the concentration of the dissociation solutions be kept constant at 10 ml /2-3 g kidney tissues.

2.3.5 Storage:

The tissue dissociation systems should be reconstituted just before use and can only be stored for 2-4 days at 4° C. For long-term use, the reconstituted tissue dissociation system solutions should be aliquoted and stored at -20° C. Avoid repeated freeze-thaw cycles.

2.4 Culture of Primary Mouse Kidney Epithelium

2.4.1 Medium Preparation

Thaw out Mouse Kidney Epithelium PrimaCell™ Basal Culture Medium, 1 ml Mouse Kidney Epithelium PrimaCell™ Medium Supplements, and 1 ml Mouse Kidney Fibroblast Growth Inhibitors, Kidney FibrOut™ on ice. To every 100 ml Mouse Kidney Epithelium PrimaCell™ Basal Culture Medium, add 1 ml of Mouse Kidney PrimaCell™ Medium Supplements, 1 ml of Mouse Kidney Fibroblast Growth Inhibitors, Kidney FibrOut™, mix thoroughly and warm the Complete Kidney Media at a 37° C water bath for 10 min prior use. (**Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*).

2.4.2 Primary Cell Culture

Primary cultures are incubated at 37 °C, 5% CO^2 incubator for 24 hrs to allow cells to adhere. After 24 hrs the cells should be washed twice with sterile PBS to remove non-adherent cells (i.e. non epithelial cells) and tissue fragments. Return cells to 37° C incubator in Complete Kidney Media described above for another 2–3 days, or until they reach 60–80% confluency. At this time, cells from the same organ are trypsinized, combined, and split into the number of 60-mm tissue-culture dishes required for a single experiment (usually,17–24 dishes). Cells usually require an additional 3-4 days of growth before the appropriate density is reached for experimentation. Please note that individual kidneys can be kept separate or pooled if the concentrations of reagents are scaled up as described per kidney.

2.4.3 Subculture and Propagation

9. Gently rinse the culture dish twice with 0.02% (0.7 mM) EDTA.
10. Add 3 ml of 0.25% trypsin/0.1% (2.5 mM) EDTA, and incubate at 37° C. Examine the dish under a microscopy every 5 min to detect cell detachment.
11. When most cells have detached, add 10 ml Complete Kidney Media to inactive the trypsin.
12. Pipette the contents of the dish to ensure all the cells have detached.
13. Collect the cells and centrifuge at 350 g for 5 min.
14. Aspirate the supernatant; resuspend the cell pellet in a Complete Kidney Media, and plate at a density of 1×10^5 cells per cm^2.
15. Feed the cultures twice a week with Complete Kidney Media.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample; the best results are obtained from preconfluent primary cultures.

16. Trypsinize cells using method used above, and centrifuge cells at 100 g for 10 min.

17. Resuspend the cell pellet in Complete Kidney Media with serum, and count cells.
18. Dispense aliquots of 2 x 10^6 cells/ml in Complete Kidney Medium supplemented with an additional 10% FBS and 10% glycerol into cryopreservation vials.
19. Equilibrate at 4° C for 1-2 hrs.
20. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of one degree per min.

To recover cells:

(i) Thaw cryovials quickly in a 37° C water bath.
(ii) Dilute cells tenfold with medium.
(iii) Centrifuge cells; resuspend them at an appropriate density in Complete Kidney Media and plate.

Mouse cells can be grown for several weeks and can be subcultured only 4-6 passages, in Complete Kidney Media.

IV Fibroblast Contamination

Several techniques have been published in the literature to address fibroblast contamination of primary kidney primary cell cultures. These include (1) Physically removing well-isolated fibroblast colonies by scraping and following with several careful washes to remove any fibroblasts that have detached (2) Differential trypsinization of carcinomas (3) Using dispase to preferentially (but not exclusively) to remove epithelial cells during passaging. During subculture, cells that have been removed with dispase can be pre-incubated in plastic culture dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the epithelium. This technique takes advantage of the fact that fibroblasts in general attach much more quickly to plastic than do clumps of epithelial cells, so that a partial purification step is possible. (4) Reduce the concentration of serum between 2.5-5% to discourage fibroblast growth. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and that using any or all of the preceding techniques will eventually push fibroblasts through enough divisions to allow the fibroblasts to senesce.

The Mouse Kidney PrimaCell™ I system includes a unique fibroblast elimination system, the Mouse Kidney Fibroblast Growth Inhibitors, Kidney FibrOut™. It contains a proprietary mixture of cis-OH-proline, collagenase, D-valine, and formulated serum substitutes that most effectively eliminate kidney fibroblast contaminations and does not interfere with the propagation and biology of kidney epithelial cells.

V References:

7. Kempson SA, Ying AL, McAteer JA, Murer H. 1989. Endocytosis and Na+/solute cotransport in renal epithelial cells. *J Biol Chem*. Nov 5;264(31):18451-6.
8. Andreoli SP, McAteer JA. 1990. Reactive oxygen molecule-mediated injury in endothelial and renal tubular epithelial cells *in vitro*. *Kidney Int*. Nov;38(5):785-94.
9. Yusufi AN, Szczepanska-Konkel M, Kempson SA, McAteer JA, Dousa TP. 1986. Inhibition of human renal epithelial Na+/Pi cotransport by phosphonoformic acid. *Biochem Biophys Res Commun*. Sep 14;139(2):679-86.
10. Bayley SA, Stones AJ, Smith CG. 1988. Immortalization of mouse kidney by transfection with polyomavirus large T gene. *Exp Cell Res*. 1988 Jul;177(1):232-6.
11. Fukase M, Birge SJ Jr, Rifas L, Avioli LV, Chase LR. 1982. Regulation of 25 hydroxyvitamin D3 1-hydroxylase in serum-free monolayer culture of mouse kidney. *Endocrinology*. Mar;110(3):1073-5.

12. Alenghat FJ, Nauli SM, Kolb R, Zhou J, Ingber DE. 2004. Global cytoskeletal control of mechanotransduction in kidney epithelial cells. *Exp Cell Res.* Nov 15;301(1):23-30.
13. Akis N, Madaio MP. 2004. Isolation, culture, and characterization of endothelial cells from mouse glomeruli. *Kidney Int.* Jun;65(6):2223-7.

Mouse Kidney PrimaCell™ Kidney Epithelium

Mouse Kidney Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82013	**Mouse Kidney PrimaCell™ system**	**kit**	**$499**
4-22121	Mouse Kidney Tissue Dissociation System, Kidney OptiTDS™ (for 500 ml medium)	1 ml	$128
9-42012	Mouse Kidney Epithelium PrimaCell™ Basal Culture Medium	500 ml	$67
9-32121	Mouse Kidney Epithelium PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
7-62121	Mouse Kidney Fibroblast Growth Inhibitors, Kidney FibrOut™	ea	$152
9-92012	Mouse Kidney Tissue I Preparation Buffer Set	ea	$75

Mouse Primary Kidney Epithelium Characterization

6-11411	**Mouse Kidney Epithelium Primarker™ Kit**	**kit**	**$220**
6-11412	Mouse Kidney Epithelium Primarker™ antibody set	set	$180
6-11413	Mouse Kidney Epithelium Primarker™ buffer system	set	$90

Mouse Kidney PrimaCell™ II: Proximal Tubular Cells
(Cat No. 2-84604)

I. General Description

Renal cell culture remains an essential tool to investigate kidney cell function, transport processes and a variety of cytotoxic or ischemic effects. Conventional primary cultures of mouse proximal tubular cells (PTC) are time-consuming, have low yields of starting material, and expose the cells to oxidative or mechanical aggression that influence cell differentiation. While these cells are extremely useful in the laboratory they are notoriously difficult to isolate and culture. They rapidly divide, become fibroblastic, and lose their biochemical characteristics. Our protocol is a combination of careful dissection of the kidney, enzymatic digestion and sieving.

The Mouse Kidney Cortice PrimaCell™ kit (Cat No. 2-84604) is designed for the successful isolation and culture of PTCs from mouse kidney cortice tissue allowing reproducible and dependable results.

1.1 Components of the Mouse Kidney cortice PrimaCell™ System

- **Mouse Kidney Cortice Tissue Dissociation System, Kidney cortice OptiTDS™** (2 × 1 ml) --- *A mixture of collagenase, and Mouse Kidney cortice OptiTDS™ Reconstitution Buffer.*
- **Mouse *Kidney cortice OptiTDS™ Digestion Buffer*,** (2 × 9 ml)
- **Mouse Kidney cortice Fibroblast Growth Inhibitors, Kidney cortice FibrOut™---** Kidney cortice FibrOut™ (5 x 200 µl) --- *A mixture of D-valine, collagenase and gentamycin.*
- **Mouse PTC PrimaCell™ Basal Culture Medium,** (5 × 100 ml) --- *A Modified Ham F12*.
- **Mouse PTC PrimaCell™ Medium Supplements,** (5 × 1 ml): *Mouse EGF, Hydrocortisone, Cholera toxin penicillin (100 U/ml), streptomycin and Weymouth medium.*
- **Mouse PTC PrimaCell™ Serum,** (50 ml): A modified f*etal bovine serum.*
- **Mouse Kidney Cortice Tissue Washing Medium,** (1 × 100 ml): *A modified DMED medium with 10µg/ml amphotericin,* 10*µg/ml gentamycin, and 10% FCS.*

1.2 Required materials but NOT included:

- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or mouse fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination.

2.2 Principle

Separation of the PTC from the Kidney Cortice is accomplished by enzymatic digestion using the Kidney cortice Tissue Dissociation System supplied in this kit. The Kidney cortice Tissue Dissociation System contains a mixture of collagenase and other materials at the optimal concentrations to gently detach the fragile kidney cortice layer from the kidney cortice tissue. The isolated kidney cortice tissue is then further disrupted to release individual PTC by enzymatic and mechanical agitation. The mixture is then filtered through Cell Strainers and seeded on flasks.

2.3 Mouse Kidney cortice Tissue Preparation

1. Mice were killed at 3-4 weeks of age.
2. Surgically remove the renal cortices from animals immediately, and keep it in cold **Mouse Kidney cortice Tissue Washing Medium**
3. Renal cortices were dissected visually in fresh ice-cold **Mouse Kidney Cortice Tissue Washing Medium** and sliced into pieces of approximately 1mm wide.

2.4 Kidney cortice Separation and Tissue Dissociation

2.4.1 Mouse Kidney cortice OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the donor used, enzymes, culture mediums and growth supplements. The Mouse Kidney cortice Tissue Dissociation System is suited for optimal dissociation of normal mouse kidney cortice tissues to yield maximum number of single PTC.

2.4.2 Enzyme Compositions

- Collagenase: from *Clostridium Histolyticum*
- Trypsin: from *Clostridium Histolyticum*

2.4.3 System Components

- Mouse Kidney Cortice Tissue Dissociation System, Kidney Cortice OptiTDS™, (2 × 1 ml).
- Mouse Kidney Cortice OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

4. Prepare fresh enzyme working solutions: to each vial of **Mouse Kidney Cortice Tissue Dissociation System, Bone OptiTDS™**, add 1.0 ml of the **Mouse Kidney Cortice OptiTDS™ Reconstitution Buffer**, Mix well.
5. Add 1.0 ml of the fresh enzyme working solution to one vial of **Mouse Kidney Cortice OptiTDS™ Digestion Buffer** (9 ml). Warm the diluted enzyme working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 mg tissue

samples per 5 ml diluted enzyme working solutions.
6. Transfer the minced tissue to a small sterile bottle with a magnetic stirrer, and add 5 ml of digestion solution.
7. Stir the solution containing bone fragments at 37℃ for 30 min.
8. The cell suspension was settled on ice for 2-3 min

2.5 PTCs Isolation

Note: Please read section 2.6 for specific information on Cervical Epithelium culture and platting before preceding this section.

9. The supernatant containing single cells, and strain the cell mixture through two sterile cell strainers (250μm and 80μm) to remove debris and substantial contamination of other nephron segments or glomeruli. The longer proximal tubule fragments remained in the 80μm sieve.
10. Wash cells in 80μm sieve with **Mouse Kidney cortice Tissue Washing Medium.**
11. Resuspend cells by flushing the sieve in the reverse direction with **Mouse Kidney cortice Tissue Washing Medium**.
12. Centrifuge the PTCs at 200 g for 5 min at 4°C and resuspend cells with **Mouse Kidney cortice Tissue Washing Medium**.

2.6 Primary PTCs Culture

2.6.1 Medium Preparation.

Thaw out **Mouse PTC PrimaCell™ Basal Culture Medium**, **Mouse PTC PrimaCell™ Medium Supplements**, and **Mouse PTCPrimaCell™ Serum** on ice.

Mouse Complete PTC PrimaCell™ Culture Medium: To every 100 ml **Mouse PTCPrimaCell™ Basal Culture Medium**, add 10 ml **Mouse PTC PrimaCell™ Medium Supplements** and 1 ml **Mouse PTC PrimaCell™ Serum**, mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use.

Mouse Complete PTC PrimaCell™ Culture Medium/FibrOut: To every 100 ml **Mouse PTC PrimaCell™ Basal Culture Medium**, add 10 ml **Mouse PTC PrimaCell™ Medium Supplements,** 1 ml **Mouse PTC PrimaCell™ Serum**, and 1 ml **Mouse Kidney Cortice Fibroblast Growth Inhibitors, Kidney Cortice FibrOut™**,mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use. (**Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*)

2.6.2 Primary Cell Culture

13. The PTCs present in **Mouse Kidney cortice Tissue Washing Medium** were centrifuged for 5min at 200 x g, and then resuspended into **Mouse PTC PrimaCell™ Culture Medium/FibrOut**.
14. The cells were plated in a collagen-coated permeable PTFE-filter supports and incubated at 37 °C in a humidified atmosphere (5% CO2, 95% air).
15. The culture medium was replaced with fresh media every 2-3 days. After 7 days, cell cultures were organized as a confluent monolayer.

Δ ***Safety Note.*** The rest of the biopsy and all tubes, pipettes, plates, etc., used in the procedure should be treated with hypochlorite before disposal.

2.7 Subculture

Propagating PTCs in culture can be somewhat challenging, especially when PTCs are cultured alone on plastic, however the following methods have worked consistently in many laboratories.

16. Remove culture medium, and wash cells with **Mouse Thyroid PrimaCell™ Basal Culture Medium.**
17. Add a small volume of EDTA gently to the cells and remove it immediately.
18. Add sufficient trypsin solution (0.25%) to form a thin layer over the cells.
19. When cells detach, add 5 to 10 ml of **Mouse Complete Thyroid PrimaCell™ Culture Medium**, pass the culture very gently in and out of a pipette, and then centrifuge the cells for 10 min at 350 g.
20. Count an aliquot and seed the cells at the chosen concentration.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Skeletal muscle cells cultures derived from preconfluent layers.

21. Trypsinize cells as above, and centrifuge at 200 g for 10 min.
22. Resuspend cells in **Mouse Complete PTC PrimaCell™ Culture Medium** and count cells.
23. Prepare aliquots of 2×10^6 cells/ml in **Mouse Complete PTC PrimaCell™ Culture Medium** and 10% glycerol into cryovials.
24. Equilibrate at 4℃ for 1-2 h.
25. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
26. To recover cells:
    a. Thaw cryovials quickly in a 37℃ water bath.
    b. Dilute cells tenfold with appropriate **Mouse Complete PTC PrimaCell™ Culture Medium**.
    c. Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in **Mouse Complete PTC PrimaCell™ Culture Medium.**

IV Characterization

At low magnification, single cells grew as a homogeneous monolayer of cells with cobblestone-like appearance. The cells were structurally polarized: the apical pole, facing the free surface, differed from the basal pole, toward the underlying support layer. The presence of specific markers, like transporter proteins or brush border enzymes, was established to characterize these primary cultures. The PTC shows a clear immunoreactivity against AQP1, while no positive signal for early distal tubule expressing AQP2 or NCC was obtained. The multiligand receptor, megalin, is located at the brush border of PT cells and participates in protein reabsorption from the primitive urine. The chloride channel, ClC-5, and the vacuolar proton pump, V-ATPase, are both localized in endosomes ensuring correct endosomal acidification and protein trafficking in PT cells. All of these components of the endocytic apparatus were detected in primary PT cells

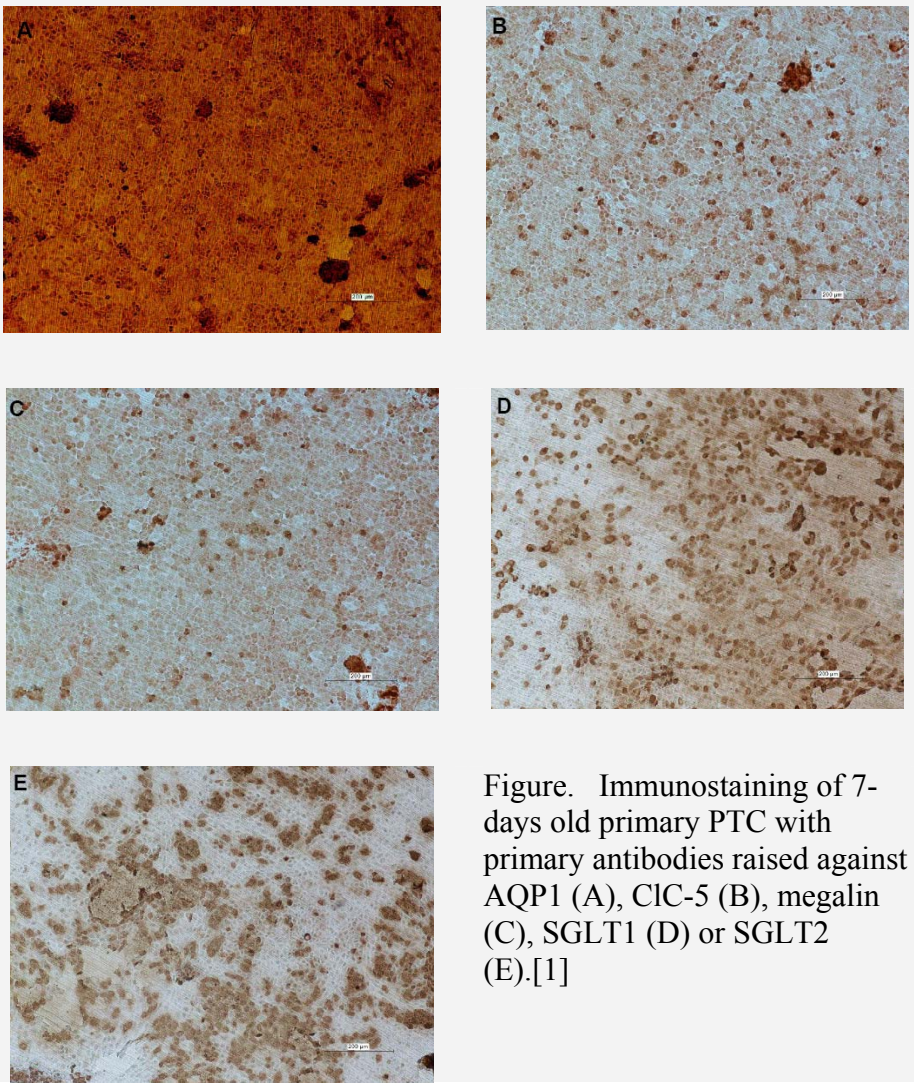


Figure. Immunostaining of 7-days old primary PTC with primary antibodies raised against AQP1 (A), ClC-5 (B), megalin (C), SGLT1 (D) or SGLT2 (E).[1]

V Reference

1. Terryn, S., et al., *A primary culture of mouse proximal tubular cells, established on collagen-coated membranes.* Am J Physiol Renal Physiol, 2007.
2. Pollock, C.A. and M.J. Field, *Compensatory renal hypertrophy: tubular cell growth and transport studied in primary culture.* Nephron, 1993. **64**(4): p. 615-20.
3. Fine, L.G. and L.M. Sakhrani, *Proximal tubular cells in primary culture.* Miner Electrolyte Metab, 1986. **12**(1): p. 51-7.
4. Qi, W., et al., *Isolation, propagation and characterization of primary tubule cell culture from human kidney.* Nephrology (Carlton), 2007. **12**(2): p. 155-9.

Mouse Kidney PrimaCell™ II: Proximal Tubular Cells

Mouse Kidney Primary Cell II Culture

Cat No.	Description	Qt.	Price
2-84604	**Mouse Kidney PrimaCell™ II system**	**kit**	**$499**
4-26631	Mouse Kidney Tissue Disassociation System II, Kidney OptiTDS™ (for 500 ml medium)	1 ml	$128
9-48603	Mouse Kidney Proximal Tubular Cells PrimaCell™ Basal Culture Medium	500 ml	$ 61
9-37631	Mouse Kidney Proximal Tubular Cells PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$ 140
7-67631	Mouse Kidney Fibroblast Growth Inhibitors, Kidney FibrOut™	ea	$146
9-98603	Mouse Kidney Tissue II Preparation Buffer Set	ea	$75

Mouse Primary Kidney Proximal Tubular Cells Characterization

6-12411	**Mouse Proximal Tubular Cell Primarker™ Kit**	**kit**	**$220**
6-12412	Mouse Proximal Tubular Cell Primarker™ antibody set	set	$180
6-12413	Mouse Proximal Tubular Cell Primarker™ buffer system	set	$90

Mouse Liver PrimaCell™: Hepatocytes
(Cat No. 2-82014)

I. General Description:

The basic two-step perfusion procedure can be used to isolate hepatocytes from the livers of mice, rabbits, guinea pigs, or woodchucks, by proportionally adapting the volume and flow rate of the solutions to the size of the liver. The technique used for human livers involves perfusion of the whole liver or a biopsy sample (15-30 ml/min, depending on the size of the tissue sample). Complete isolation of hepatocytes into a single-cell suspension can be obtained by an additional incubation at 37℃ with collagenase under gentle stirring for 10- 20 min (especially for human liver). Fish hepatocytes can be isolated by cannulation of the intestinal vein and incision of the heart to avoid excessive pressure. Perfusion is performed at room temperature at a flow rate of 12-15 ml per min.

This protocol has been developed for the attachment and growth of normal hepatocytes from adult mouse liver tissue using the Mouse Liver PrimaCell™ system (Cat No. 2-82014). This system provides the optimal tissue dissociation system, Liver OptiTDS™ that yields 4-7 times the single cells than most tissue dissociation protocols published in the literature. CHI's proprietary fibroblast inhibitory system, Liver FibrOut™, allows for minimal to no contamination of the hepatocyte cultures by non-epithelial cells types. In addition, the media and supplements provided have been supplied to ensure a robust culture of hepatocytes for experimentation.

The preparation of tissue specimens for cell culture should be started within 1-2 h of organ removal or sacrifice of the animal. If this is impossible, store the tissue overnight at 4℃ in washing medium (see below) as this has also given satisfactory results.

Mouse Liver PrimaCell™ system is well suited for use in normal adult mouse liver samples. Livers containing pathological organisms (virus, parasites, etc.) or tumor may not be suitable for use with this system.

1.1 Components of Mouse Liver PrimaCell™ System

- **Liver Tissue Dissociation System, Liver OptiTDS™,** (2 x 1 ml) *A proprietary mixture of collagenase, collagenase I, collagenase II, collagenase IV, Hyaluronidase I, and Dispase*
- **Liver OptiTDS™ Digestion Buffer**, (4 x 100 ml)
- **Liver Tissue Washing Medium**, (2 x 100 ml)
- *Basal Liver PrimaCell™ Culture Medium with NaCl, KCl, Na2HPO4.12H2O, HEPES, and 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*
- **Mouse Liver Fibroblast Growth Inhibitors, Liver FibrOut™** (5 x 200 µl) --- *A mixture of D-valine, collagenase, and formulated serum substitutes*
- **Mouse Liver PrimaCell™ Basal Culture Medium**, (5 x100 ml) *Modified formulation based on Ham's F12 and Weymouth medium*
- **Mouse Liver PrimaCell™ Medium Supplements with Serum**, (5 x 1.0 ml) A mixture of *Basal culture medium containing bovine albumin (grade V), bovine Insulin,*

1.2 Required Materials not provided in the kit

- Tygon tube (ID, 3.0 mm; OD, 5.0mm)
- Disposable scalp vein infusion needles, 20G
- Sewing thread for cannulation
- Graduated bottles and Petri dishes
- Surgical instruments (Sharp, straight, and curved scissors and clips)
- 2 × 1-ml disposable syringes
- Collagen I-coated plate (Corning, NY)
- Chronometer
- Peristaltic pump (10 to 200 rpm)
- Water bath

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, collagen I-coated plate (Corning, NY) MUST be pre-treated with the Mouse Liver PrimaCell™ Basal Culture Medium and incubated for 5 min. After 5 min aspirate the Mouse Liver PrimaCell™ Basal Culture Medium and allow the dishes to air-dry in a ventilated cell culture hood for 5-10 min.

2.2 Surgical specimens

Perfusion of liver tissue must be performed within 2 hours of the mouse being anesthetized to ensure a smooth and efficient perfusion. Immediately after the mouse is anesthetized, open the abdomen, ties a loose ligature around the portal vein (approximately 5 mm from the liver), insert the cannula up to the liver, and ligate. Briefly dip the liver tissue in 70% ethanol for 1 min, followed by a rinse with 10 ml PBS and 10 ml Liver Tissue Washing Medium. Initiate the perfusion with 100 ml Liver Tissue Washing Medium, followed by 100 ml of Liver OptiTDS™ Digestion Buffer, and finally by 300 ml 100 ml of Liver OptiTDS™ Digestion working solution (see below for details).

2.3 Tissue Preparation and Dissociation

2.3.1 Mouse Liver OptiTDS™

Several important factors can affect yield and viability of primary cell culture, including tissue type, origin of species, tissue age, enzymes, culture medium and growth supplements. The Liver Tissue Dissociation System, OptiTDS™, is suited for optimal dissociation of whole liver or a biopsy of adult mouse liver to yield the maximum number of single primary hepatocytes.

2.3.2 Enzyme Compositions

- Collagenase: from *Clostridium Histolyticum*
- Collagenase I: from *Clostridium Histolyticum*

- Collagenase II: from *Clostridium Histolyticum*
- Hyaluronidase I: from *Bovine Testes*
- Dispase: from *Bacillus polymyxa*

2.3.3 System Components

- Liver Tissue Dissociation System, OptiTDS™, (2 x 1.0 ml)
- Liver OptiTDS™ Digestion Buffer, (4 x 100 ml)

2.3.4 Procedures For Tissue Preparation and Dissociation

1. Warm the 200 ml Liver Tissue Washing Medium and 400 ml Liver OptiTDS™ Digestion Buffer in a water bath (approximately 38-39℃ to achieve 37℃ in the liver) for 10 min. Oxygenation is not necessary. Combine 300 ml Liver OptiTDS™ Digestion Buffer and 1 ml Liver Tissue Dissociation System, OptiTDS™, mix well and allow to incubate in a water bath until use. The remaining 100 ml Liver OptiTDS™ Digestion Buffer will be used for flushing the portal vein described in step 5 below.
2. Set the pump flow rate at 30 ml/min.
3. Anesthetize the mouse (180-200 g) by intraperitoneal injection of Nembutal (100 µl/100 g), and inject heparin into the femoral vein (1,000 IU).
4. Open the abdomen, place a loosely tied ligature around the portal vein (approximately 5 mm from the liver), insert the cannula up to the liver, and ligate.
5. Rapidly incise the sub-hepatic vessels to avoid excess pressure, and start the perfusion with 100 ml Liver Tissue Washing Medium followed by 100 ml of Liver OptiTDS™ Digestion Buffer (without adding tissue dissociation enzymes) at a flow rate of 30 ml/min; verify that the liver whitens within a few seconds.
6. Perfuse 300 ml of the Liver Tissue Dissociation System, OptiTDS™ working solution prepared in step 1at a flow rate of 15 ml/min for 30 min. The liver should swell.
7. Remove the liver and wash it with 100 ml Liver Tissue Washing Medium; after disrupting the Glisson capsule, disperse the cells in 100 ml of complete Mouse Hepatocytes PrimaCell™ Medium (see preparation below in 2.4.1).

2.3.5 Storage:

The tissue dissociation solutions should be reconstituted just before use and can only be stored for 2-4 days at 4 ^{o}C. For long-term use, solutions should be aliquoted before reconstitution and stored at -20 ^{o}C. Avoid repeated freeze-thaw cycles.

2.4 Culture of primary Mouse Liver cells

2.4.1 Medium Preparation.

Thaw out the Hepatocytes PrimaCell™ Medium Supplements with Serum and Liver Fibroblast Growth Inhibitors, Liver FibrOut™ on ice. To every 100 ml Mouse Hepatocytes PrimaCell™ Basal Culture Medium, add 1 ml of Mouse Hepatocytes PrimaCell™ Medium Supplements with Serum; and 1 ml of Liver Fibroblast Growth Inhibitors, Liver FibrOut™, mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use. (**Important:** *Long term use of FibrOut™ in the culture medium may result in loss of targeted primary cells, therefore, we recommend using FibrOut™ for 3-5 cycles of changing culture medium or until an acceptable level of fibroblasts is reached*).

2.4.2 Treatment of Culture Dishes.

To enhance cell attachment to the culture dishes, collagen I-coated plates (Corning, NY) MUST be pre-treated with the complete Mouse Hepatocytes PrimaCell™ Culture Medium (enough to cover the whole cell-growth area) and incubated at 37^{o} C for 5 min. Aspirate the medium and allow the dishes to air-dry in a ventilated cell culture hood for 5-10 min.

2.4.3 Standard primary culture conditions.

8. Filter the suspension through two-layers of gauze or 60-80-μm nylon mesh, allow the viable cells to sediment for 20 min (usually at room temperature), and discard the supernatant (60 ml) containing debris and dead cells.
9. Wash the cells twice with 1 x PBS and once with complete Mouse Hepatocytes PrimaCell™ Medium by slow centrifugations (50 g for 40 s) to remove tissue dissociation enzymes, damaged cells, and non-parenchymal cells.
10. Collect the hepatocytes in 10 ml complete Mouse Hepatocytes PrimaCell™ Medium.
11. Count the cells and seed 5-7 x 10^5 cells/100-mm dishes. Isolated Hepatocytes can be growing for 4-6 days and undergo 1-3 rounds of cell division on collagen I-coated culture dish without obvious differentiation. Freshly isolated mouse hepatocytes were seeded at a density of 2×10^5 to 3×10^5 cells/ml medium on collagen-coated plates. Confluence after plating was 80–90%, and hepatocyte viability greater 90% was reached as assessed by Trypan blue exclusion. After one round of plating, hepatocytes were maintained in the complete culture medium without addition of Liver FibrOut™. Medium was changed daily.

2.4.4 Alternative Primary Culture conditions.

Hepatocyte attachment during primary culture and subculture is more reproducible and efficient when cells are inoculated onto collagen-coated flasks. When short-term (4-6 hours) growth of cell is acceptable for a particular experiment, plain culture dish without a biomatrix coating can be used.

2.4.5 Subculture and Propagation

Most primary cultures cannot be passaged presently using routine trypsin / EDTA procedures. Disaggregation to single cells of the cultured liver cells with 0.1% trypsin in 0.25 mM (0.1%) EDTA will result in extremely poor or no growth. To avoid these consequences Dispase is used. An advantage with this procedure is that dispase can only efficiently detach epithelial cell but not fibroblast, thereby, increasing the purity of the target cell population.

12. Add 0.5% Dispase (Sigma, w/v) to the cell monolayer, just enough to cover the cells (~2.5 ml/25-cm^2 flask), and leave the solution to stand for 40-60 min for primary cultures and 20-40 min for cell lines.
13. Once the epithelial layers begin to detach (they do so as sheets rather than single cells), pipette to help detachment and dis-aggregation into smaller clumps.
14. Wash and replate the cells under standard culture conditions. It may take several days for clumps to attach, so replace the medium carefully when feeding.

III Fibroblast Contamination

There are several techniques have been published in the literature to deal with fibroblast contamination during primary cell culture. These include: (1) Physically remove a well-isolated fibroblast Livery by scraping it with a sterile blunt instrument (e.g., a cell scraper). Care has to be taken to wash the culture up to six times to remove any fibroblasts that have detached in order to prevent them from reseeding and reattaching to the flask. (2) Differential trypsinization can be attempted with the carcinomas. (3) Dispase preferentially (but not exclusively) removes the epithelium during passaging and leaves behind most of the fibroblastic cells attached to the culture vessel. During subculture, cells that have been removed with dispase can be preincubated in plastic Petri dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the epithelium. Clumps of epithelial cells still floating can be transferred to new flasks under standard culture conditions. This technique takes advantage of the fact that fibroblasts in general attach more quickly to plastic than do clumps of epithelial cells, so that a partial purification step is possible. (4) Reduce the concentration of serum to about 2.5-5%, if there are heavy concentrations of fibroblasts. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and using any or all of the techniques mentioned above will eventually push the cells through so many divisions that the fibroblasts will eventually senesce.

Mouse Liver PrimaCell™ includes a fibroblast elimination system, the Mouse Liver Fibroblast Growth Inhibitors, Liver FibrOut™. It contains a mixture of D-valine, collagenase, and formulated serum substitutes. The FibrOut™ kills contaminating fibroblasts, but shows no signs of toxicity toward the target cells, whether derived from an adenoma, a carcinoma or normal liver tissues.

IV References:

1. Li JX, Shi Q, Xiong QB, Prasain JK, Tezuka Y, Hareyama T, Wang ZT, Tanaka K, Namba T, Kadota S. *Tribulusamide A and B, new hepatoprotective lignanamides from the fruits of Tribulus terrestris: indications of cytoprotective activity in murine hepatocyte culture.* Planta Med. 1998 Oct;64(7):628-31.
2. Bhatia SN, Balis UJ, Yarmush ML, Toner M. *Microfabrication of hepatocyte/fibroblast co-cultures: role of homotypic cell interactions.* Biotechnol Prog. 1998 May-Jun;14(3):378-87.
3. Nagasaki T, Lieberman MA. *Liver contains heparin-binding growth factors as the major growth factor for cultured fibroblasts.* Hepatology. 1991 Jan;13(1):6-14.
4. Holme JA. *Xenobiotic metabolism and toxicity in primary monolayer cultures of hepatocytes.* NIPH Ann. 1985 Dec;8(2):49-63.
5. Fu T, Guo D, Huang X, O'Gorman MR, Huang L, Crawford SE, Soriano HE. *Apoptosis occurs in isolated and banked primary mouse hepatocytes.* Cell Transplant. 2001 Jan-Feb;10(1):59-66.
6. Wang H, Gao X, Fukumoto S, Tademoto S, Sato K, Hirai K. *Differential expression and regulation of chemokines JE, KC, and IP-10 gene in primary cultured murine hepatocytes.* J Cell Physiol. 1999 Nov;181(2):361-70.

Mouse Liver PrimaCell™: Hepatocytes

Mouse Liver Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82014	**Mouse Liver PrimaCell™ system**	**kit**	**$**499
4-22131	Mouse Liver Dissociation System, Muscle OptiTDS™ (for 500 ml medium)	1 ml	$134
9-42013	Mouse Hepatocytes PrimaCell™ Basal Culture Medium	500 ml	$73
9-32131	Mouse Hepatocytes PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
7-62131	Mouse Liver Fibroblast Growth Inhibitors, Liver FibrOut™	ea	$164
9-92013	Mouse Liver Tissue Preparation Buffer Set	ea	$75

Mouse Primary Hepatocytes Epithelium Characterization

6-11511	**Mouse Hepatocyte Primarker™ Kit**	**kit**	**$220**
6-11512	Mouse Hepatocyte Primarker™ antibody set	set	$180
6-11513	Mouse Hepatocyte Primarker™ buffer system	set	$90

Mouse Lung PrimaCell™: Alveolar Epithelial Cell II
(Cat No. 2-84104)

I. General Description

Alveolar epithelial type II (AEC II) is small, cuboidal cells that constitute ~60% of the pulmonary alveolar epithelium. These cells are crucial for repair of the injured alveolus by differentiating into alveolar epithelial type I cells. AEC II cells are a promising source of cells that could be used therapeutically to treat distal lung diseases. While these cells are extremely useful in the labomouseory they are notoriously difficult to isolate and culture. The Mouse Alveolar PrimaCell™ kit (Cat No. 2-84104) is designed for the successful isolation and culture of AEC IIs from mouse tissue allowing reproducible and dependable results.

1.1 Components of the Mouse Alveolar PrimaCell™ System

- **Mouse Alveolar Tissue Dissociation System, alveolar OptiTDS™** (2 × 1 ml) --- *A mixture of collagenase and Mouse Alveolar OptiTDS™ Reconstitution Buffer.*
- **Mouse Alveolar *OptiTDS™ Digestion Buffer,*** (2 × 9 ml)
- **Mouse Alveolar Fibroblast Growth Inhibitors, alveolar FibrOut™---**Alveolar FibrOut™ (5 x 200 µl) --- *A mixture of D-valine, collagenase and gentamycin.*
- **Mouse AEC II PrimaCell™ Basal Culture Medium,** (5 × 100 ml) --- *A Modified Ham F12*.
- **Mouse AEC II PrimaCell™ Medium Supplements,** (5 × 1 ml): *Mouse EGF, Hydrocortisone, Cholera toxin penicillin (100 U/ml), streptomycin and Weymouth medium.*
- **Mouse AEC II PrimaCell™ Serum,** (50 ml): A modified f*etal bovine serum.*
- **Mouse Alveolar Tissue Washing Medium,** (1 × 100 ml): *A modified DMED medium with 10µg/ml amphotericin,* 10*µg/ml gentamycin, and 10% FCS.*

1.2 Required materials but NOT included:

- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or mouse fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination.

2.2 Principle

Separation of the AEC II from the pancreases is accomplished by enzymatic digestion using the Alveolar Tissue Dissociation System supplied in this kit. The Alveolar Tissue Dissociation System contains a mixture of collagenase and other reagents at the optimal concentmouseions to gently isolate alveolars.

2.3 Mouse Alveolar Tissue Preparation

1. Mice were anesthetized with 0.2 ml Nembutal by intraperitoneal injection.
2. The abdominal cavity was opened, and mice were exsanguinated by severing the inferior vena cava and the left renal artery.
3. The trachea was isolated and cannulated with a 20-gauge luer stub adapter. The diaphragm was cut, and the chest plate and thymus were removed.
4. With the use of a 21-gauge needle fitted on a 10-ml syringe, lungs were perfused with 10–20 ml **Mouse alveolar tissue washing Medium** via the pulmonary artery.

2.4 Alveolar Separation and Tissue Dissociation

2.4.1 Mouse Alveolar OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the donor used, enzymes, culture mediums and growth supplements. The Mouse Alveolar Tissue Dissociation System is suited for optimal dissociation of normal mouse alveolar tissues to yield maximum number of single AEC II.

2.4.2 Enzyme Compositions

- Collagenase: from *Clostridium Histolyticum*
- Trypsin: : from *Clostridium Histolyticum*

2.4.3 System Components

- Mouse Alveolar Tissue Dissociation System, Alveolar OptiTDS™, (2 × 1 ml).
- Mouse Alveolar OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

5. Prepare fresh enzyme working solution: Add 1ml of the reconstituted tissue dissociation solution to one vial of Mouse Alveolar OptiTDS™ Digestion Buffer (9 ml). Warm the diluted Mouse Alveolar OptiTDS™ working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 5 ml Mouse Alveolar OptiTDS™ working solutions.
6. 3 ml Mouse Alveolar OptiTDS™ working solution was rapidly instilled through the cannula in the trachea followed by 0.5 ml agarose (45°C). Lungs were immediately covered with ice for 2 min to gel the agarose. (Alternative option: The trachea can be closed with a ligature)
7. After this incubation, lungs were removed from the animals and incubated in 1 ml dispase at 37°C for 45 min. Lungs were subsequently transferred to a 60-mm culture dish containing 7 ml Mouse Alveolar OptiTDS™ working solutions, and lung tissue was gently

teased from the bronchi.

8. Lung tissue was minced in a 100-mm culture dish containing Mouse Alveolar OptiTDS™ working solutions.

2.5 AEC IIs Isolation

Note: Please read section 2.6 for specific information on Cervical Epithelium culture and platting before preceding this section.

9. The cell suspension was filtered through progressively smaller cell strainers (100 and 40 μm) and nylon gauze (20 μm). Cells were collected by centrifugation at 130 g for 8 min (4°C) and placed on prewashed 100-mm tissue culture plates that had been coated for 24–48 h at 4°C with 42 μg CD45 and 16μg CD 32 in PBS.
10. After incubation for 1–2 h at 37°C, type II cells were gently panned from the plate and collected by centrifugation.

2.6 Primary AEC IIs Culture

2.6.1 Medium Preparation.

Thaw out **Mouse AEC II PrimaCell™ Basal Culture Medium**, **Mouse AEC II cell PrimaCell™ Medium Supplements**, and **Mouse AEC II PrimaCell™ Serum** on ice.

Mouse Complete AEC II PrimaCell™ Culture Medium: To every 100 ml **Mouse AEC II PrimaCell™ Basal Culture Medium**, add 10 ml **Mouse AEC II PrimaCell™ Medium Supplements** and 1 ml **Mouse AEC II PrimaCell™ Serum**, mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

Mouse Complete AEC II PrimaCell™ Culture Medium/FibrOut: To every 100 ml **Mouse Alveolar PrimaCell™ Basal Culture Medium**, add 10 ml **Mouse Alveolar PrimaCell™ Medium Supplements,** 1 ml **Mouse Alveolar PrimaCell™ Serum**, and 1 ml **Mouse Alveolar Fibroblast Growth Inhibitors, Alveolar FibrOut™**,mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

2.6.2 Primary Cell Culture

(Important: Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination)

11. Type II cells are resuspended:

(a) in **Mouse Complete AEC II PrimaCell™ Culture Medium/FibrOut™** and seed on Matrigel-rat tail collagen (70:30 vol/vol) coated plates. and the medium was changed after the first day of culture and every 2 d thereafter, or

(b) in **Mouse Complete AEC II PrimaCell™ Culture Medium/FibrOut™** and plated on culture dishes coated with fibronectin and collagen, and the medium was changed after the first day of culture and every 2 d thereafter.

Δ ***Safety Note.*** The rest of the biopsy and all tubes, pipettes, plates, etc., used in the procedure should be treated with hypochlorite before disposal.

2.7 Subculture

Propagating AEC II in culture can be somewhat challenging, especially when AEC IIs are cultured alone on plastic, however the following methods have worked consistently in many labomouseories.

12. Remove culture medium, and wash cells with **Mouse Alveolar Washing Medium.**
13. Incubating cells with PBS containing 1 mg/ml collagenase at 37°C for 60 min.
14. The dispersed alveolars were washed and suspended in **Mouse Complete Alveolar PrimaCell™ Culture Medium**.
15. Count an aliquot and seed the cells at the chosen concentration.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Skeletal muscle cells cultures derived from preconfluent layers.

16. Trypsinize cells as above, and centrifuge at 100 g for 10 min.
17. Resuspend cells in **Mouse Complete Alveolar PrimaCell™ Culture Medium** and count cells.
18. Prepare aliquots of 2×10^6 cells/ml in **Mouse Complete Alveolar PrimaCell™ Culture Medium** and 10% glycerol into cryovials.
19. Equilibmousee at 4℃ for 1-2 h.
20. Freeze cells with a programmed freezing appamouseus (Planer) at a cooling mousee of 1℃ per min.
21. To recover cells:
    a. Thaw cryovials quickly in a 37℃ water bath.
    b. Dilute cells tenfold with appropriate **Mouse Complete Alveolar PrimaCell™ Culture Medium**.
    c. Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentmouseion in **Mouse Complete Alveolar PrimaCell™ Culture Medium.**

IV Characterization

AEC II were identified by the modified Papanicolaou or Nile red staining and immunocytochemistry using AEC II-specific antibodies such as anti-RTII70 and anti-LB180 antibodies. Phosphine 3R is a lipophilic fluorescent dye concentmouseed in lamellar bodies of type II cells and can, therefore, be used to identify type II cells. The purity of type II cell can be assessed by PAP stain, electron microscopy, and immunostaining for SP-C.

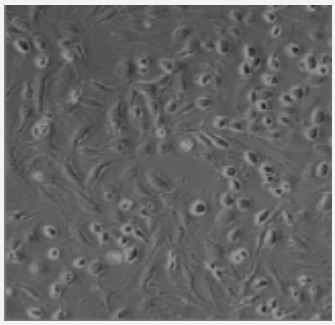


Figure 1. Lung alveolar type II cell observed under inverted microscope images [2].

V References

1. Wang, J., et al., *Pneumocystis stimulates MCP-1 production by alveolar epithelial cells through a JNK-dependent mechanism.* Am J Physiol Lung Cell Mol Physiol, 2007. **292**(6): p. L1495-505.
2. Reddy, N.M., et al., *Deficiency in Nrf2-GSH Signaling Impairs Type II Cell Growth and Enhances Sensitivity to Oxidants.* Am J Respir Cell Mol Biol, 2007. **37**(1): p. 3-8.
3. Chen, J., et al., *Isolation of highly pure alveolar epithelial type I and type II cells from rat lungs.* Lab Invest, 2004. **84**(6): p. 727-35.
4. Jones, G.S., et al., *Ionic content and regulation of cellular volume in rat alveolar type II cells.* J Appl Physiol, 1982. **53**(1): p. 258-66.
5. Rice, W.R., et al., *Maintenance of the mouse type II cell phenotype in vitro.* Am J Physiol Lung Cell Mol Physiol, 2002. **283**(2): p. L256-64.
6. Chen, Z., et al., *Identification of two novel markers for alveolar epithelial type I and II cells.* Biochem Biophys Res Commun, 2004. **319**(3): p. 774-80.
7. Dobbs, L.G., R. Gonzalez, and M.C. Williams, *An improved method for isolating type II cells in high yield and purity.* Am Rev Respir Dis, 1986. **134**(1): p. 141-5.
8. Liu, L., et al., *Involvement of annexin II in exocytosis of lamellar bodies from alveolar epithelial type II cells.* Am J Physiol, 1996. **270**(4 Pt 1): p. L668-76.

Mouse Lung PrimaCell™: Alveolar Epithelial Cell II

Mouse Lung Primary Cell Culture

Cat No.	Description	Qt.	Price
2-84104	**Mouse Lung PrimaCell™ system**	**kit**	**$499**
4-26131	Mouse Lung Tissue Disassociation System, Lung OptiTDS™ (for 500 ml medium)	1 ml	$128
9-48103	Mouse Lung Type II Alveolar Epithelial Cells PrimaCell™ Basal Culture Medium	500 ml	$ 61
9-36131	Mouse Lung Type II Alveolar Epithelial Cells PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$ 140
7-67131	Mouse Lung Fibroblast Growth Inhibitors II, Lung FibrOut™	ea	$146
9-98103	Mouse Lung Tissue Preparation Buffer Set	ea	$75

Mouse Primary Alveolar Epithelial Cell II Characterization

6-12511	**Mouse Alveolar Epithelial Cell II Primarker™ Kit**	**kit**	**$220**
6-12512	Mouse Alveolar Epithelial Cell II Primarker™ antibody set	set	$180
6-12513	Mouse Alveolar Epithelial Cell II Primarker™ buffer system	set	$90

Mouse Muscle PrimaCell™: Skeletal Muscle Cells
(Cat No. 2-82015)

I. General Description

Of all the different tissues and organs in the body, skeletal muscles are the most frequent organ. In adult mouse skeletal muscle the majority of myonuclei are located in syncytial myotubes that were formed by myoblast fusion during fetal and postnatal development. These myonuclei are terminally postmitotic. However, a small fraction of myonuclei is in mononucleate precursor cells called muscle satellite cells which are located between the basal lamina and sarcolemma of myofibers. In healthy adult rodent muscle, satellite cells are mitotically quiescent and do not detectably express MRFs (MyoD family muscle regulatory factors). When stimulated by damage to the muscle or by explant and culture manipulations, some fractions of satellite cells are activated to reenter the cell cycle and/or to express myogenic regulatory factors. The resulting myoblasts subsequently differentiate and fuse to form new replacement myofibers during embryogenesis, mononuclear precursor cells, or myoblasts, fuse to form these huge muscle fibers. Later life during regenerative process subsequent to muscle damage, quiescent mononuclear stem cells (satellite cells) lacated between the sarcolemma and basal lamina of the muscle fiber are activated. These cells begin to proliferate and either fuse with each other into novel myotubes, or they fuse with damaged muscle fibers. Both myoblasts and satellite cells can be isolated from the body and grown in tissue culture, and in optimal culture media they will fuse and differentiate into mature, spontaneously-contracting myotubes. Culture derived from normal and diseased mouse skeletal muscle cells therefore provides an excellent model to study several aspects of early muscle development under normal and pathological conditions. While these cells are extremely useful in the laboratory they are notoriously difficult to isolate and culture.

The Mouse Muscle PrimaCell™ kit (Cat No. 2-82015) is designed for the successful isolation and culture of skeletal muscle cells from mouse muscle allowing reproducible and dependable results.

1.1 Components of the Mouse Muscle PrimaCell™ System

- **Mouse Muscle Tissue Dissociation System , Muscle OptiTDS™** (2 × 1 ml) --- *A mixture of collagenase, collagenase I, collagenase IV, Elastase, Hyaluronidase I, Pronase, trypsin and Muscle OptiTDS™ Reconstitution Buffer*.
- **Muscle Tissue Washing Medium**, (2 × 1 m)
- **Mouse Muscle OptiTDS™ Digestion Buffer,** (1 × 100ml) --- *A modified Ham's F12 without NaHCO3, with 20 mM HEPES, 200 U/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Mouse Muscle Fibroblast Growth Inhibitors, Muscle FibrOut™** ---Muscle FibrOut™ (5 × 200 μl) --- *A mixture of D-valine, collagenase, and gentamycin.*
- **Mouse Skeletal Muscle Cells PrimaCell™ Basal Culture Medium** (5 × 100 ml) --- *A modified Ham's F12*.
- **Mouse Skeletal Muscle Cells PrimaCell™ Medium Supplements with Serum** (10 × 10 ml): *Highly purified and special-treated fetal bovine serum, penicillin, streptomycin.*

1.2 Required materials but NOT included:

- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or mouse fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, fresh gelatin-coated plate or culture dishes are recommended (see below for treatment of culture dishes).

2.2 Principle

Culture myogenic cells from adult mouse skeletal muscle of several species under conditions in which the cells continue to express at least some of their differentiated traits is possible. A cell type called *satellite cells*, the myogenic cells partially mimic the first a few steps of skeletal muscle differentiation. They proliferate and migrate randomly on the substratum and then align and finally form multinucleated myotubes. Although three to four passages can be performed by means of trypsinization, subculture is no longer possible once differentiation (i.e., fusion) is inititated. For the same reason, proliferation is very difficult to estimate once some cells have started to fuse. The procedure described here is an enzymatic method of digesting muscle skeletal tissues. Primary cultures from mouse healthy muscle tissues s are highly enriched in myogenic cells, as evidenced by at least 85-90% positively to desmin by immunostaining at day 10 after seeding. Primary cultures can be grown easily in **Mouse Complete Skeletal Muscle PrimaCell™ Culture Medium**. Without modifying the culture conditions, these cells proliferate and differentiate by fusing to form multinucleated myotubes, confirming the myogenicity of the cultivated cells.

2.3 Mouse Muscle Tissue Preparation

Mouse Muscle: Skeletal muscle cells are usually taken from fresh non-fixed and non-frozen muscle specimens such as thigh muscle.

1. The muscle specimens for culture were immediately placed in a 100-mm tissue culture dish containing 5-10 ml of **Mouse Muscle Tissue Washing Medium**.
2. Trim off nonmuscle tissue from the specimens with a scalpel, and rinse in **Mouse Muscle Tissue Washing Medium**.
3. Cut the muscle tissue into fragments parallel to the fibers and wash in **Mouse Muscle Tissue Washing Medium** prior to weighing the biopsy.
4. Place the fragments parallel to each other in the lid of a Petri dish, cut the fragments into thinner cylinders and then, finally into 1-mm^3 pieces, without crushing the tissue. The final cutting can be done in a tube with long scissors, again avoiding crushing.
5. Discard the **Mouse Muscle Tissue Washing Medium** and incubate tissue specimens with 5-

10 ml 70% ethanol for 1 min, followed by inoculating with 5-10 ml fresh **Mouse Muscle Tissue Washing Medium** for 5 min.

2.4 Skeletal muscle cell Separation and Tissue Dissociation

2.4.1 Mouse Muscle OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Mouse Muscle Tissue Dissociation System is suited for optimal dissociation of normal newborn mouse muscle tissues to yield maximum number of single skeletal muscle cell.

2.4.2 Enzyme Compositions

- Trypsin: from *Bovine Pancreas*
- Collagenase: from *Clostridium Histolyticum*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*
- Elastase
- Hyaluronidase I
- Pronase

2.4.3 System Components

- Mouse Muscle Tissue Dissociation System, Muscle OptiTDS™, (2 × 1 ml).
- Mouse Muscle OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

6. Prepare fresh enzyme working solution: Add 1.0 ml of **Mouse Muscle Tissue Dissociation System, Bone OptiTDS™** to one vial of **Mouse Muscle OptiTDS™ Digestion Buffer** (9 ml). Warm the diluted enzyme working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 mg tissue samples per 10 ml diluted enzyme working solutions.
7. Rinse muscle tissue with **Mouse Muscle Tissue Washing Medium** and let the pieces settles; discard the supernatant.
8. Dissociation can be achieved in either of the following two methods

   (a) Rapid dissociation: Float muscle on Mouse Muscle Tissue Dissociation System working solution for 2-3 hrs at 37 ℃. This works particularly well with full-thickness muscle. Monitor the separation of the muscle carefully when using the rapid dissociation method.

   (b) Slow dissociation: Float the samples on ice-cold Mouse Muscle Tissue Dissociation System working solution at 4℃ for 15-24 h. This is particularly convenient for flexible scheduling of skeletal muscle cells preps.

2.5 Primary skeletal muscle cells Culture

2.5.1 Medium Preparation.

Thaw out **Mouse Skeletal Muscle PrimaCell™ Basal Culture Medium**, **Mouse Skeletal Muscle PrimaCell™ Medium Supplements With Serum** on ice.

Mouse Complete Skeletal Muscle PrimaCell™ Culture Medium: To every 100 ml **Mouse Skeletal Muscle PrimaCell™ Basal Culture Medium**, add 10 ml **Mouse Skeletal Muscle PrimaCell™ Medium Supplements With Serum**, mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

Mouse Complete Skeletal Muscle PrimaCell™ Culture Medium/FibrOut: To every 100 ml **Mouse Skeletal Muscle PrimaCell™ Basal Culture Medium**, add 10 ml **Mouse Skeletal Muscle PrimaCell™ Medium Supplements With Serum**, one vial of **Mouse Muscle Fibroblast Growth Inhibitors, Bone FibrOut™**, mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

2.5.2 Primary Cell Culture

(**Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*)

9. Triturate the culture with a pipette after incubation. The medium should become increasingly opaque as more and more cells are released.
10. Let the fragments settle to the bottom by gravity, forming pellet P1 and supernatant S1.
11. Filter S1 through a 100-µm nylon mesh and into a 20-ml centrifuge tube. Shake or pipette the supernatant gently to resuspend the cells.
12. Centrifuge the tube 8-10 min at 350 g. Discard the supernatant by aspiration.
13. Resuspend the pellet very, very gently by means of a rubber-bulb pipette in precisely 10 ml of **Mouse Complete Skeletal Muscle PrimaCell™ Culture Medium/FibrOut**, and count the cells with a hemocytometer.
14. Dilute the suspension in growth medium to seed culture flasks with about 1.5×10^4 cells/ml. About $1 – 2 \times 10^5$ cells/g are obtained from healthy donor biopsies.
15. Add 15 ml of digestion medium to P1, and incubate the fragments for 30 min in a water bath at 37°C, with periodic shaking.
16. Pipette the suspension to disaggregate the cells and then filter the suspension through nylon mesh. Rinse the filter with 20 ml of **Mouse Complete Skeletal Muscle PrimaCell™ Culture Medium/FibrOut**.
17. Centrifuge the suspension for 8 – 10 min at 350 g, count the cells, and seed as before.
18. Transfer the flasks to a 37°C humidified incubator with 5% CO_2.
19. Maintenance of Cultures: Change the **Mouse Complete Skeletal Muscle PrimaCell™ Culture Medium/FibrOut** very gently 24 h after seeding and then every 3-4 d. The development of these cultures is mainly towards differentiation. The timing of the three phases for mouse muscle cells is about 4-6 d for peak proliferation; then the cells align at about day 8, and around day 10 to 12 an increase in cell fusion and the formation of myotubes are observed. Nevertheless, one must keep in mind that some cells may differentiate earlier and that others will still proliferate when the majority of the culture is undergoing differentiation. Change culture medium to **Mouse Complete Skeletal Muscle PrimaCell™ Culture Medium** after 3-5 cycles or an acceptable level of fibroblast cell contamination is observed.

Δ ***Safety Note.*** The rest of the biopsy and all tubes, pipettes, plates, etc., used in the procedure should be treated with hypochlorite before disposal.

2.6 Subculture

20. Add a small volume of EDTA gently to the cells and remove it immediately.
21. Add sufficient trypsin solution (0.25%) to form a thin layer over the cells.
22. When cells detach, add 5 to 10 ml of **Mouse Complete Skeletal Muscle PrimaCell™ Culture Medium**, pass the culture very gently in and out of a pipette, and then centrifuge the cells for 10 min at 350 g.
23. Count an aliquot and seed the cells at the chosen concentration.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Skeletal muscle cells cultures derived from preconfluent layers.

24. Trypsinize cells as above, and centrifuge at 100 g for 10 min.
25. Resuspend cells in complete **Mouse Complete Skeletal Muscle PrimaCell™ Culture Medium** and count cells.
26. Prepare aliquots of 2×10^6 cells/ml in **Mouse Complete Skeletal Muscle PrimaCell™ Culture Medium** and 10% glycerol into cryovials.
27. Equilibrate at 4℃ for 1-2 h.
28. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
29. To recover cells:
    a. Thaw cryovials quickly in a 37℃ water bath.
    b. Dilute cells tenfold with appropriate **Mouse Complete Skeletal Muscle PrimaCell™ Culture Medium**.
    c. Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in **Mouse Complete Skeletal Muscle PrimaCell™ Culture Medium**.

IV Characterization

The growth curves of mouse myogenic cell cultures obtained in Mouse Complete Skeletal Muscle PrimaCell™ Culture Medium show the three traditional phases: the lag phase, the exponential phase, and the plateau, which corresponds to the onset of fusion. The last, evaluated in terms of the number of nuclei incorporated into myobubes or in terms of a fusion index (the percentage of nuclei incorporated into myotubes relative to the total number of nuclei), commences usually around day 8 after plating and rises dramatically around day 10. According to the sample, this chronology can gain or lose one day. Hence, differentiation, expressed as the number of nuclei per myotube/cm^2, may be observed morphologically. But the differentiation process can also be monitored by the use of biochemical markers (such as the sarcomeric proteins), enzymes involved in differentiation (e.g., creatine phosphokinase and its time-dependent muscle-specific isoform shift), or the appearance of α-actin. Expression of the c-met receptor tyrosine kinase can serve as an effective molecular marker for quiescent or activated satellite cells. A family of genes, such as myogenin, MyoD1 and desmin, the best known of which is MyoD, was shown to activate muscle-specific gene expression in myogenic progenitors. Myogenic cell differentiation involves either the activation of a variety of other genes with concurrent changes in cell surface adhesive properties or the recently shown requirement of cell surface plasminogen activator urokinase and its receptor.

V References

1. Cornelison, D.D. and B.J. Wold, *Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells.* Dev Biol, 1997. **191**(2): p. 270-83.
2. Grounds, M.D., et al., *Identification of skeletal muscle precursor cells in vivo by use of MyoD1 and myogenin probes.* Cell Tissue Res, 1992. **267**(1): p. 99-104.
3. Koller, M.R.P., B. O.; Masters, J. R. W., *Human Cell Culture: Primary Mesenchymal Cells* Vol. 5. 2001: Springer.
4. Bischoff, R., *Proliferation of muscle satellite cells on intact myofibers in culture.* Dev Biol, 1986. **115**(1): p. 129-39.
5. Lawson-Smith, M.J. and J.K. McGeachie, *The identification of myogenic cells in skeletal muscle, with emphasis on the use of tritiated thymidine autoradiography and desmin antibodies.* J Anat, 1998. **192 (Pt 2)**: p. 161-71.
6. Metzinger, L., P. Poindron, and A.C. Passaquin, *A rapid preparation of primary cultures of mouse skeletal muscle cells.* Cytotechnology, 1993. **13**(1): p. 55-60.
7. Sejersen, T., et al., *Rat skeletal myoblasts and arterial smooth muscle cells express the gene for the A chain but not the gene for the B chain (c-sis) of platelet-derived growth factor (PDGF) and produce a PDGF-like protein.* Proc Natl Acad Sci U S A, 1986. **83**(18): p. 6844-8.
8. Askanas, V., A. Bornemann, and W.K. Engel, *Immunocytochemical localization of desmin at human neuromuscular junctions.* Neurology, 1990. **40**(6): p. 949-53.
9. Buckingham, M., *Making muscle in mammals.* Trends Genet, 1992. **8**(4): p. 144-8.
10. Dodson, M.V., B.A. Mathison, and B.D. Mathison, *Effects of medium and substratum on ovine satellite cell attachment, proliferation and differentiation in vitro.* Cell Differ Dev, 1990. **29**(1): p. 59-66.
11. Pegolo, G., V. Askanas, and W.K. Engel, *Expression of muscle-specific isozymes of phosphorylase and creatine kinase in human muscle fibers cultured aneurally in serum-free, hormonally/chemically enriched medium.* Int J Dev Neurosci, 1990. **8**(3): p. 299-308.

Mouse Muscle PrimaCell™: Skeletal Muscle Cells

Mouse Muscle Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82015	**Mouse Muscle PrimaCell™ system**	**kit**	**$499**
4-20141	Mouse Muscle Tissue Dissociation System, Muscle OptiTDS™ (for 500 ml medium)	1 ml	$134
9-42014	Mouse Skeletal Muscle Cells PrimaCell™ Basal Culture Medium	500 ml	$61
9-32141	Mouse Skeletal Muscle Cells PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
7-62141	Mouse Muscle Fibroblast Growth Inhibitors, Muscle FibrOut™	ea	$152
9-92014	Mouse Muscle Tissue Preparation Buffer Set	ea	$75

Mouse Primary Skeletal Muscle Cells Characterization

6-11611	**Mouse Skeletal Muscle Cell Primarker™ Kit**	**kit**	**$220**
6-11612	Mouse Skeletal Muscle Cell Primarker™ antibody set	set	$180
6-11613	Mouse Skeletal Muscle Cell Primarker™ buffer system	set	$90

Mouse Pancreas PrimaCell™ I: Pancreatic Epithelium

(Cat No. 2-82016)

I. General Description:

This protocol is developed for attachment and growth of normal Mouse Pancreatic epithelial cells from mouse pancreas with Mouse Pancreatic PrimaCell™ system (Cat No. 2-82016). This system provides an optimal condition of tissue dissociation system, Pancreatic OptiTDS™ that yields 2-3 times of single cells more than most of the tissue dissociation protocols published in the literature. In addition, this system ensures a high viability of the target cells with improved gradient contained in the culture medium. With CHI's proprietary fibroblast inhibitory system, Pancreatic FibrOut™, cells are growing with contamination of minimized amount of the non-epithelial cells.

The preparation of tissue specimens for cell culture is usually started within 1-2 h of removal from the patient. If this is impossible, fine cutting of the tissue into small pieces (1-2 mm) with scalpels and storage overnight at 4℃ in washing medium (see below) can also prove successful.

Mouse Pancreatic PrimaCell™ system applies to all types of normal adult Mouse biopsies samples. Biopsies samples contain pathological organism (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Mouse Pancreatic PrimaCell™ System

- **Mouse Pancreatic Tissue Dissociation System, Pancreatic OptiTDS™,** (2 aliquots) --- *A mixture of collagenase I, collagenase III, collagenase IV, collagenase, and trypsin*.
- **Mouse Pancreatic OptiTDS™ Reconstitution Buffer**, (2 x 1 ml).
- **Mouse Pancreatic OptiTDS™ Digestion Buffer**, (2 x 10 ml).
- **Mouse Pancreatic Tissue Washing Medium**, (5 x 10 ml) --- *Basal Pancreatic PrimaCell™ Culture Medium with 5% FBS, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Mouse Pancreatic Fibroblast Growth Inhibitors, Pancreatic FibrOut™** (5 x 200 μl) --- *A mixture of* anti-Thy-1 *monoclonal antibody, toxin ricin, and formulated serum substitutes*.
- **Mouse Pancreatic PrimaCell™ Basal Culture Medium**, (5 x100 ml) --- *Modified formulation based on NCTC 168 and Weymouth medium*.
- **Mouse Pancreatic PrimaCell™ Medium Supplements**, (5 x 1.0 ml) --- A mixture of *ethanolamine, phosphoethanolamine, hydrocortisone, ascorbic acid, transferring, insulin, epidermal growth factor, pentagastrin, and deoxycholic acid*.
- **Mouse Pancreatic PrimaCell™ Serum**, (1 x 50 ml) --- H*eat-inactivated and special-treated Fetal-bovine serum.*
- **Coating Solution**, (5 x 10 ml) --- *Basal growth medium containing 10 μg/ml BSA.*

1.2 Required Materials but not provided

- Hank's balanced salt solution (HBSS)
- Dispase (Sigma)

- Pasteur pipettes
- Collagen I-coated Culture dishes
- Scalpels, scissors, and forceps
- Pasteur pipettes and 10-ml pipettes
- Test tubes, 12 and 50 ml

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, collagen I-coated plate (Corning, NY) MUST be pre-treated with the provided BSA by adding 5 ml 10 μg/ml BSA in growth medium and incubate for 5 min. Aspirate the BSA solution; let the dishes be air-dry in the ventilated cell culture hood for 5-10 min.

2.2 Surgical specimens

Remove mouse pancreas with autoclaved scalpels, scissors, and forceps, carefully remove muscle and fat from tissues followed by washing procedures. Place specimens in a 10 ml falcon tube contain 5 ml Pancreatic Tissue Washing Medium followed by inoculating for 10 min at the room temperature. For large tissue specimens, 50 ml falcon tube and more wash medium is needed to ensure thoroughly washing. Aspirate the washing medium and repeat the washing procedures with fresh washing medium two more time. Washing tissue specimens sequentially in 70% ethanol for 1 min at the room temperature, in PBS for 5 min, and in fresh Pancreatic Tissue Washing Medium for 5 min. Collecting tissue specimen by centrifugation prior to tissue dissociation procedures (see below).

2.3 Tissue Preparation and Dissociation

2.3.1 Mouse Pancreatic OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Pancreatic Tissue Dissociation System, OptiTDS™, is suited for optimal dissociation of normal adult Mouse biopsies samples to yield maximum number of single primary cells of Pancreatic tissues.

2.3.2 Enzyme Compositions

- Collagenase I: from *Clostridium Histolyticum*
- Collagenase III: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*
- Collagenase: from *Clostridium Histolyticum*
- Trypsin: from *Bovine Pancreas*

2.3.3 System Components

- Pancreatic Tissue Dissociation System, OptiTDS™, 2 vials.

- Pancreatic OptiTDS™ Reconstitution Buffer, (2 x 1 ml).
- Pancreatic OptiTDS™ Digestion Buffer, (2 x 9 ml).

2.3.4 Procedures For Tissue Preparation and Dissociation

1. Prepare fresh enzyme working solutions: to each vial of Pancreatic Tissue Dissociation System, OptiTDS™, add 1.0 ml of the Pancreatic OptiTDS™ Reconstitution Buffer, Mix well.
2. Add 1.0 ml of the fresh enzyme working solution to one vial of Pancreatic OptiTDS™ Digestion Buffer (9.0 ml). Warm the diluted enzyme working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 4-5 mg tissue samples per 10 ml diluted enzyme working solutions.
3. Mince pre-washed tissue into pieces approximately 0.2-0.5 mm^2 in diameter with two scalpel and forceps.
4. Incubate minced tissues with the diluted enzyme working solutions by incubating minced tissue samples (up to 5 mg) in 10 ml diluted enzyme working solutions with slow magnetic stirring for 30 min at 37 ℃.
5. At the end of tissue dissociation period, gentle triturating tissue with a 10 ml pipette, constitutes filling the empty the barrel at a rate of 2-3 ml per second. Repeat this procedure for 5-6 times.
6. Collecting cells by filtration the mixture through a cell strainer followed by centrifugation at 1 x 100 g. Carefully remove the medium and resuspend the cell pellet with 1.0 ml complete culture medium.
7. Count the cells and seed cells in 3-4 T-25 collagen I-coated flasks (**Important:** pre-treat the flask with the provided BSA containing growth medium, see below) upon the density of viable cells (2.5-5 x 10^5 Cells/Dish).

2.3.5 Storage:

Reconstituted tissue dissociation systems should occur before use and can only be stored for 2-4 days at 4 ^oC. For long-term use, it should be aliquoted and stored at -20 ^oC. Avoid repeated freeze-thaw cycles.

2.4 Culture of primary Mouse Pancreatic cells

2.4.1 Medium Preparation.

Thaw out the Mouse Pancreatic PrimaCell™ Medium Supplements and Mouse Pancreatic PrimaCell™ Serum on ice. To every 100 ml Mouse Pancreatic PrimaCell™ Basal Culture Medium, add one vial of Mouse Pancreatic PrimaCell™ Medium Supplements; 10 ml Mouse Pancreatic PrimaCell™ Serum; and one vial of Mouse Pancreatic Fibroblast Growth Inhibitors, Pancreatic FibrOut™, mix thoroughly and warm the complete medium at a 37^oC water bath for 10 min prior use.

2.4.2 Treatment of Culture Dishes.

To enhance cell attachment to the culture dishes, collagen I-coated plate (Corning, NY) MUST be pre-treated with the provided Coating Solution (Basal growth medium containing 10 µg/ml BSA) by adding appropriate volume of the Coating Solution (enough to cover the whole cell-growth area) and incubate for 5 min. Aspirate the BSA solution; let the dishes be air-dry in the ventilated cell culture hood for 5-10 min.

.

2.4.3 Standard primary culture conditions.

Inoculate epithelial tubules and clumps of cells derived from tissue specimens into T-25 flasks coated with collagen type I with pre-treatment of coating solution at 37℃ in a 5%-CO_2 incubator with 4 ml of complete culture medium. Change the culture medium twice weekly. The tubules and cells start to attach to the substratum, and epithelial cells migrate out within 1-2 d. Most of the tubules and small clumps of epithelium attach within 7 d, but the larger organoids can take up to 6 weeks to attach, although they will remain viable all that time.

2.4.4 Alternative Primary Culture conditions.

The attachment of epithelium during primary culture and subculture is more reproducible and efficient when cells are inoculated onto collagen-coated flasks, and significantly better growth is obtained with 3T3 feeders than without. When the epithelial Breasties expand to several hundred cells per Breasty, they become less dependent on 3T3 feeders, and no further addition of feeders is necessary. All medium and solutions described in the standard culture condition are applicable in this culture method.

2.4.5 Subculture and Propagation

Most pancreatic primary cultures cannot at present be passaged by routine trypsin / EDTA procedures. Disaggregation to single cells of the cultured Pancreatic cells with 0.1% trypsin in 0.25 mM (0.1%) EDTA will result in extremely poor or even zero growth, so Dispase is used instead. One of the advantage using dispase is that dispase can only detach epithelial cell but not the fibroblast, thus increase the purity of epithelium.

8. Add 0.5% Dispase (Sigma, w/v) to the cell monolayer, just enough to cover the cells (~2.5 ml/25-cm^2 flask), and leave the solution to stand for 40-60 min for primary cultures and 20-40 min for cell lines.
9. Once the epithelial layers begin to detach (they do so as sheets rather than single cells), pipette to help detachment and disaggregation into smaller clumps.
10. Wash and replate the cells under standard culture conditions. It may take several days for clumps to attach, so replace the medium carefully when feeding.

III Fibroblast Contamination

There are several techniques have been published in the literature to deal with fibroblast contamination during colorectal primary cell culture. These include: (1) Physically remove a well-isolated fibroblast Breasty by scraping it with a sterile blunt instrument (e.g., a cell scraper). Care has to be taken to wash the culture up to six times to remove any fibroblasts that have detached in order to prevent them from reseeding and reattaching to the flask. (2) Differential trypsinization can be attempted with the carcinomas. (3) Dispase preferentially (but not exclusively) removes the epithelium during passaging and leaves behind most of the fibroblastic cells attached to the culture vessel. During subculture, cells that have been removed with dispase can be preincubated in plastic Petri dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the epithelium. Clumps of epithelial cells still floating can be transferred to new flasks under standard culture conditions. This technique takes advantage of the fact that fibroblasts in general attach much more quickly to plastic than do clumps of epithelial cells, so that a partial purification step is possible. (4) Reduce the

concentration of serum to about 2.5-5% if there are heavy concentrations of fibroblastic cells. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and that using any or all of the preceding techniques will eventually push the cells through so many divisions that any fibroblasts will senesce.

Mouse Pancreatic PrimaCell™ includes a fibroblast elimination system, the Mouse Pancreatic Fibroblast Growth Inhibitors, Pancreatic FibrOut™. It contains a mixture of anti-Thy-1 monoclonal antibody, toxin ricin and formulated serum substitutes. Thy-1 antigen is present on colorectal fibroblasts, but not colorectal epithelial cells; therefore, the conjugate kills contaminating fibroblasts, but shows no signs of toxicity toward the epithelium, whether derived from an adenoma, a carcinoma or normal Pancreatic tissues.

IV References:

1. Youngman KR, Simon PL, West GA, Cominelli F, Rachmilewitz D, Klein JS, Fiocchi C: Localisation of intestinal interleukin 1 activity and protein and gene expression to lamina propria cells. Gastroenterology 1993, 104:749-758.
2. Gibson PR, van de Pol E, Maxwell LE, Gabriel A, Doe WF: Isolation of Pancreatic crypts that maintain structural and metabolic viability in vitro. Gastroenterology 1989, 96:283-291.
3. Whitehead RH, Brown A, Bhathel PS: A method for the isolation and culture of Mouse Pancreatic crypts in collagen gels. In Vitro 1986, 23:436-442.
4. Knoll N, Weise A, Claussen U, Sendt W, Marian B, Glei M, Pool-Zobel BL. 2-Dodecylcyclobutanone, a radiolytic product of palmitic acid, is genotoxic in primary Mouse Pancreatic cells and in cells from preneoplastic lesions.
5. Buset M, Winawer S, Friedman E. Defining conditions to promote the attachment of adult Mouse Pancreatic epithelial cells.

Mouse Pancreas PrimaCell™: Pancreatic Epithelium

Mouse Pancreas Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82016	**Mouse Pancreas PrimaCell™ system**	**kit**	**$499**
4-22161	Mouse Pancreas Tissue Dissociation System, Pancreas OptiTDS™ (for 500 ml medium)	1 ml	$ 128
9-42016	Mouse Pancreatic Epithelium PrimaCell™ Basal Culture Medium	500 ml	$ 73
9-32161	Mouse Pancreatic Epithelium PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
7-62161	Mouse Pancreas Fibroblast Growth Inhibitors, Pancreas FibrOut™	ea	$ 134
9-92016	Mouse Pancreas Tissue I Preparation Buffer Set	ea	$75

Mouse Primary Pancreatic Epithelium Characterization

6-11711	**Mouse Pancreatic Epithelium Primarker™ Kit**	**kit**	**$220**
6-11712	Mouse Pancreatic Epithelium Primarker™ antibody set	set	$180
6-11713	Mouse Pancreatic Epithelium Primarker™ buffer system	set	$90

Mouse Pancreas PrimaCell™ II: Islet Cells
(Cat No. 2-84504)

I. General Description

The endocrine cells of the pancreas are contained in the islets of Langerhans. Each islet with 50-500 um in diameter contains approximately one thousand cells. Hormones produced in the Islets of Langerhans are secreted directly into the blood flow by islet cells. While these cells are extremely useful in the laboratory they are very difficult to isolate and culture. The Mouse Islet PrimaCell™ kit (Cat No. 2-84504) is designed for the successful isolation and culture of islet cells from mouse tissue allowing reproducible and dependable results.

1.1 Components of the Mouse Islet PrimaCell™ System

- **Mouse Islet Tissue Dissociation System, Islet OptiTDS™** (2 × 1 ml) --- *A mixture of collagenase and Mouse Islet OptiTDS™ Reconstitution Buffer.*
- **Mouse *Islet OptiTDS™ Digestion Buffer*,** (2 × 9 ml)
- **Mouse Islet Fibroblast Growth Inhibitors, Islet FibrOut™**---Islet FibrOut™ (5 x 200 µl) --- *A mixture of D-valine, collagenase and gentamycin.*
- **Mouse Islet cell PrimaCell™ Basal Culture Medium,** (5 × 100 ml) --- *A Modified Ham F12*.
- **Mouse Islet cell PrimaCell™ Medium Supplements,** (5 × 1 ml): *Mouse EGF, Hydrocortisone, Cholera toxin penicillin (100 U/ml), streptomycin and Weymouth medium.*
- **Mouse Islet PrimaCell™ Serum,** (50 ml): A modified f*etal bovine serum.*
- **Mouse Islet Tissue Washing Medium,** (1 × 100 ml): *A modified DMED medium with 10µg/ml amphotericin,* 10*µg/ml gentamycin, and 10% FCS.*

1.2 Required materials but NOT included:

- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or mouse fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination.

2.2 Principle

Separation of the islet cell from the pancreases is accomplished by enzymatic digestion using the Islet Tissue Dissociation System supplied in this kit. The Islet Tissue Dissociation System contains a mixture of collagenase and other reagents at the optimal concentrations to gently isolate islets.

2.3 Mouse Islet Tissue Preparation

Mouse Islets: Most islets are obtained from pregnant mice at 20 days of gestation (The day of mating is counted as day 0).

1. Pregnant mice were killed by cervical dislocation and fetuses were rapidly removed. All the procedures were performed under sterile conditions.
2. The fetal pancreases were removed aseptically, placed into culture dish with cold **Mouse Islet Washing Medium**.
3. Tissues were finely minced, and washed with **Mouse Islet Washing Medium**.

2.4 Islet Separation and Tissue Dissociation

2.4.1 Mouse Islet OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the donor used, enzymes, culture mediums and growth supplements. The Mouse Islet Tissue Dissociation System is suited for optimal dissociation of normal mouse islet tissues to yield maximum number of single islet cell.

2.4.2 Enzyme Compositions

- Collagenase: from *Clostridium Histolyticum*
- Trypsin

2.4.3 System Components

- Mouse Islet Tissue Dissociation System, Islet OptiTDS™, (2 × 1 ml).
- Mouse Islet OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

4. Prepare fresh enzyme working solution: Add 1ml of the reconstituted tissue dissociation solution to one vial of Mouse Islet OptiTDS™ Digestion Buffer (9 ml). Warm the diluted Mouse Islet OptiTDS™ working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 5 ml Mouse Islet OptiTDS™ working solutions.
5. Discard the **Mouse Islet Washing Medium,** and float samples on 5 ml of Mouse Islet Tissue Dissociation System working solution (prewarmed to 37℃) and transfer the minced tissue to a sterile centrifuge tube (10–12 pancreases each).
6. The tissue was digested in a shaking water bath at 37 C for 10-30 min.

2.5 Islet cells Isolation

Note: Please read section 2.6 for specific information on Cervical Epithelium culture and platting before preceding this section.

7. The resulting digests were washed three times with cold **Mouse Islet Washing Medium.**
8. The pellets were resuspended in **Mouse Complete Islet PrimaCell™ Culture Medium/FibrOut™** and distributed in 50 mm plastic culture dishes.
9. The islets were cultured for 5 days in 5 ml **Mouse Complete Islet PrimaCell™ Culture Medium/FibrOut** at 37 C in a humidified atmosphere of 5% CO2.

2.6 Primary Islet cells Culture

2.6.1 Medium Preparation.

Thaw out **Mouse Islet PrimaCell™ Basal Culture Medium**, **Mouse Islet cell PrimaCell™ Medium Supplements**, and **Mouse Islet PrimaCell™ Serum** on ice.

Mouse Complete Islet PrimaCell™ Culture Medium: To every 100 ml **Mouse Islet PrimaCell™ Basal Culture Medium**, add 10 ml **Mouse Islet PrimaCell™ Medium Supplements** and 1 ml **Mouse Islet PrimaCell™ Serum**, mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use.

Mouse Complete Islet PrimaCell™ Culture Medium/FibrOut: To every 100 ml **Mouse Islet PrimaCell™ Basal Culture Medium**, add 10 ml **Mouse Islet PrimaCell™ Medium Supplements,** 1 ml **Mouse Islet PrimaCell™ Serum**, and 1 ml **Mouse Islet Fibroblast Growth Inhibitors, Islet FibrOut™**, mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use.

2.6.2 Primary Cell Culture

(**Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*)

10. The medium was changed every day. At the end of the preculture period, the islets attached to the bottom of the culture dishes were gently blown free using a sterilized Pasteur pipette under a stereomicroscope. The fibroblast layer remaining on the bottom of the culture dishes
11. The detached islets were cultured free-floating in 50 mm Petri dishes in Mouse Complete Islet PrimaCell™ Culture Medium/FibrOut changed every other day.

Δ ***Safety Note.*** The rest of the biopsy and all tubes, pipettes, plates, etc., used in the procedure should be treated with hypochlorite before disposal.

2.7 Subculture

Propagating Islet cells in culture can be somewhat challenging, especially when Islet cells are cultured alone on plastic, however the following methods have worked consistently in many laboratories.

12. Remove culture medium, and wash cells with Mouse Islet Washing Medium.
13. Add a small volume of HBSS containing trypsin (0·05%) and EDTA (0·02%) for 5 min Add an equal volume of culture medium and mechanically dispersed the remaining intact islets by mild trituration.

14. The dispersed islets were washed and suspended in Mouse Complete Islet PrimaCell™ Culture Medium.
15. Count an aliquot and seed the cells at the chosen concentration.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Skeletal muscle cells cultures derived from preconfluent layers.

16. Trypsinize cells as above, and centrifuge at 100 g for 10 min.
17. Resuspend cells in **Mouse Complete Islet PrimaCell™ Culture Medium** and count cells.
18. Prepare aliquots of 2×10^6 cells/ml in **Mouse Complete Islet PrimaCell™ Culture Medium** and 10% glycerol into cryovials.
19. Equilibrate at 4℃ for 1-2 h.
20. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
21. To recover cells:
    a. Thaw cryovials quickly in a 37℃ water bath.
    b. Dilute cells tenfold with appropriate **Mouse Complete Islet PrimaCell™ Culture Medium**.
    c. Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in **Mouse Complete Islet PrimaCell™ Culture Medium.**

IV Characterization

Adenylylcyclase can be used as a marker to assess islet cell viability as well as differences in preservation media and may predict islet cell transplant success. Besides, CGRP (Calcitonin gene-related peptide)-containing cells were found primarily in the peripheral portion of the pancreatic islets. Neuropilin-2 is a novel marker expressed in pancreatic islet cells and endocrine pancreatic tumours.

V References

1. Cohen, T., et al., *Neuropilin-2 is a novel marker expressed in pancreatic islet cells and endocrine pancreatic tumours.* J Pathol, 2002. **198**(1): p. 77-82.
2. Navarro-Tableros, V., et al., *Autocrine regulation of single pancreatic beta-cell survival.* Diabetes, 2004. **53**(8): p. 2018-23.
3. Fragner, P., S.L. Lee, and S. Aratan de Leon, *Differential regulation of the TRH gene promoter by triiodothyronine and dexamethasone in pancreatic islets.* J Endocrinol, 2001. **170**(1): p. 91-8.
4. Sjoholm, A., *Differential effects of cytokines on long-term mitogenic and secretory responses of fetal rat pancreatic beta-cells.* Am J Physiol, 1992. **263**(1 Pt 1): p. C114-20.
5. Islam, M.S., A. Sjoholm, and V. Emilsson, *Fetal pancreatic islets express functional leptin receptors and leptin stimulates proliferation of fetal islet cells.* Int J Obes Relat Metab Disord, 2000. **24**(10): p. 1246-53.
6. Lu, M., J. Seufert, and J.F. Habener, *Pancreatic beta-cell-specific repression of insulin gene transcription by CCAAT/enhancer-binding protein beta. Inhibitory interactions with basic helix-loop-helix transcription factor E47.* J Biol Chem, 1997. **272**(45): p. 28349-59.

7. Rosenbaum, T., M.C. Sanchez-Soto, and M. Hiriart, *Nerve growth factor increases insulin secretion and barium current in pancreatic beta-cells.* Diabetes, 2001. **50**(8): p. 1755-62.
8. Cabrera-Valladares, G., et al., *Effect of retinoic acid on glucokinase activity and gene expression and on insulin secretion in primary cultures of pancreatic islets.* Endocrinology, 1999. **140**(7): p. 3091-6.

Mouse Pancreas PrimaCell™ II: Islet Cells

Mouse Pancreas Primary Cell II Culture

Cat No.	Description	Qt.	Price
2-84504	**Mouse Pancreas PrimaCell™ II system**	**kit**	**$499**
4-26531	Mouse Pancreas Tissue Dissociation System II, Pancreas OptiTDS™ (for 500 ml medium)	1 ml	$128
9-48503	Mouse Pancreatic Islets PrimaCell™ II Basal Culture Medium	500 ml	$61
9-37531	Mouse Pancreatic Islets PrimaCell™ II Medium Supplements with Serum (for 500 ml medium)	set	$140
7-67531	Mouse Pancreas Fibroblast Growth Inhibitors II, Pancreas FibrOut™	ea	$146
9-98503	Mouse Pancreas Tissue II Preparation Buffer Set	ea	$75

Mouse Primary Islet Cells Characterization

6-11811	**Mouse Islet Cell Primarker™ Kit**	**kit**	**$220**
6-11812	Mouse Islet Cell Primarker™ antibody set	set	$180
6-11813	Mouse Islet Cell Primarker™ buffer system	set	$90

Mouse Prostate PrimaCell™: Prostate Epithelium
(Cat No. 2-82017)

I. General Description:

This protocol is developed for attachment and growth of normal Mouse prostate epithelial cells from newborn or adult Mouse Prostate with Mouse Prostate PrimaCell™ system (Cat No. 2-82017). This system provides an optimal condition of tissue dissociation system, Prostate OptiTDS™ that yields 4-7 times of single cells more than most of the tissue dissociation protocols published in the literature. In addition, this system ensures a high viability of the target cells with improved gradient contained in the culture medium. With CHI's proprietary fibroblast inhibitory system, FibrOut™, cells are growing with contamination of minimized amount of the non-epithelial cells.

This procedure involves explanting fragments of large Prostate tissue in a serum-free medium (LHC-9) in order to initiate and subsequently propagate fibroblast-free outgrowths of NHBE cells; four subculturings and 30 population doublings are routine.

Mouse Prostate PrimaCell™ system applies to all type tissue samples from Mouse at all age though younger tissue samples are recommended for yielding maximum amount of viable target cells. However, tissue samples contain pathological organism (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Mouse Prostate PrimaCell™ System

- **Prostate Tissue Dissociation System, Prostate OptiTDS™,** (2 aliquots) --- *A mixture of collagenase I, collagenase II, collagenase IV, dispase and trypsin*.
- **Prostate OptiTDS™ Reconstitution Buffer**, (2 x 1 ml).
- **Prostate OptiTDS™ Digestion Buffer**, (2 x 10 ml).
- **Coating Solution:** *A Mixure of Mouse fibronectin, collagen, and crystallized bovine serum albumin (BSA), in basal culture medium.*
- **Prostate Tissue Washing Medium**, (1 x 100 ml) --- *Basal Prostate PrimaCell™ Culture Medium with 5% FBS, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Prostate Tissue Healing Medium,** (1 x 100 ml) --- *A mixture of basal culture medium containing insulin; hydrocortisone; β-retinyl acetate; glutamine; penicillin; stretomycin (50 ug/ml); gentamucin; fungison; FBS.*
- **Mouse Prostate Fibroblast Growth Inhibitors, Prostate FibrOut™** (5 x 200 µl) --- *A mixture of cis-OH-proline, collagenase, D-valine, and formulated serum substitutes*.
- **Mouse Prostate PrimaCell™ Basal Culture Medium**, (5 x100 ml) --- *Modified formulation based on medium 199 and Weymouth medium*.
- **Mouse Prostate PrimaCell™ Medium Supplements**, (5 x 1.0 ml) --- A mixture of *of Mouse fibronectin, collagen, and crystallized bovine serum albumin (BSA)*.
- **Mouse Prostate PrimaCell™ Serum**, (1 x 50 ml) --- *Highly purified special-treated Fetal-bovine serum.*

1.2 Required Materials but not provided

- Plastic tissue culture dishes (60 and 100 mm)
- Scalpels No. 1621 (Becton Dickinson)
- Surgical scissors
- Half-curved microdissecting forceps
- Pipettes (10 and 25 ml)
- Trypsin (Cooper Biomedical), 0.02%, EGTA (Sigma), 0.5 mM, and polyvinylpyrrolidine (USB), 1% solution
- High-O2 gas mixture (50% O2, 45% N2, 5% CO2)
- Gloves sterilized with autoclave (Mouse tissue can be contaminated with biologically hazardous agents)
- Controlled atmosphere chamber
- Rocker platform
- Phase-contrast inverted microscope

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, culture plate (Corning, NY) must be coated with the provided coating solution.

2.2 Surgical specimens

1. Prostate tissue from autopsy of noncancerous donors is recommended for yielding a large number of cells with a 30-40% plating efficiency.
2. Incubate prostate tissue biopsies for up to 10 min in Prostate Tissue Washing Medium to prevent infection. (This procedure should not affect cell viability.)
3. Incubate Prostate biopsies in 10 ml 70% sterile ethanol for 2 min.
4. Rinse tissue twice in Prostate Tissue Washing Medium for 10 min each and kept on ice.

2.3 Tissue PrepaMouseion and Tissue Dissociation

2.3.1 Mouse Prostate OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the donor used, enzymes, culture mediums and growth supplements. The Mouse Prostate Tissue Dissociation System is suited for optimal dissociation of normal Mouse islet tissues to yield maximum number of single islet cell.

2.3.2 Enzyme Compositions

- Collagenase: from *Clostridium Histolyticum*
- Trypsin: from *Clostridium Histolyticum*

2.3.3 System Components

- Mouse Prostate Tissue Dissociation System, Prostate OptiTDS™, (2 × 1 ml).
- Mouse Prostate OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.3.4 Procedures For Tissue PrepaMouseion and Dissociation

4 Prepare fresh enzyme working solution: Add 1ml of the reconstituted tissue dissociation solution to one vial of Mouse Prostate OptiTDS™ Digestion Buffer (9 ml). Warm the diluted Mouse Prostate OptiTDS™ working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 5 ml Mouse Prostate OptiTDS™ working solutions.
5 Discard the **Mouse Prostate Washing Medium,** and float samples on 5 ml of Mouse Prostate Tissue Dissociation System working solution (prewarmed to 37℃) and transfer the minced tissue to a sterile centrifuge tube (10–12 pancreases each).
6 The tissue was digested in a shaking water bath at 37 C for 10-30 min.

2.4 Prostate Epithelial cells Isolation

Note: Please read section 2.5 for specific information on Cervical Epithelium culture and platting before preceding this section.

7 The resulting digests were washed three times with cold **Mouse Prostate Washing Medium.**
8 The pellets were resuspended in **Mouse Complete Prostate PrimaCell™ Culture Medium/FibrOut™** and distributed in 50 mm plastic culture dishes.
9 The Prostates were cultured for 5 days in 5 ml **Mouse Complete Prostate PrimaCell™ Culture Medium/FibrOut™** at 37 ^{o}C in a humidified atmosphere of 5% CO2.

2.5 Primary Prostate Epithelial Cells Culture

2.5.1 Medium PrepaMouseion.

Thaw out **Mouse Prostate PrimaCell™ Basal Culture Medium**, **Mouse Prostate cell PrimaCell™ Medium Supplements**, and **Mouse Prostate PrimaCell™ Serum** on ice.

Mouse Complete Prostate PrimaCell™ Culture Medium: To every 100 ml **Mouse Prostate PrimaCell™ Basal Culture Medium**, add 10 ml **Mouse Prostate PrimaCell™ Medium Supplements** and 1 ml **Mouse Prostate PrimaCell™ Serum** mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use.

Mouse Complete Prostate PrimaCell™ Culture Medium/FibrOut: To every 100 ml **Mouse Prostate PrimaCell™ Basal Culture Medium**, add 10 ml **Mouse Prostate PrimaCell™ Medium Supplements,** 1 ml **Mouse Prostate PrimaCell™ Serum**, and 1 ml **Mouse Prostate Fibroblast Growth Inhibitors, Prostate FibrOut™**,mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use. (**Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*)

2.5.2 Primary Cell Culture

10 The medium was changed every day. At the end of the preculture period, the Prostates attached to the bottom of the culture dishes were gently blown free using a sterilized Pasteur pipette under a stereomicroscope. The fibroblast layer remaining on the bottom of the culture dishes
11 The detached Prostates were cultured free-floating in 50 mm Petri dishes in **Mouse Complete Prostate PrimaCell™ Culture Medium/FibrOut™** changed every other day.

Δ ***Safety Note.*** The rest of the biopsy and all tubes, pipettes, plates, etc., used in the procedure should be treated with hypochlorite before disposal.

2.5.3 Subculture and Propagation

12. Gently rinse the culture dish twice with 0.02% (0.7 mM) EDTA.
13. Add 3 ml of 0.25% trypsin/0.1% (2.5 mM) EDTA, and incubate at 37℃. Examine the dish under phase microscopy every 5 min to detect cell detachment.
14. When most cells have detached, add 12 ml complete melanocyte growth medium to inactive the trypsin activity.
15. Pipette the contents of the dish to ensure complete melanocyte melanocyte detachment.
16. AspiMousee and centrifuge the cells for 5 min at 350 g.
17. AspiMousee the supernatant, resuspend the cells in a complete growth medium, and replate at 2-4 × 10^4 cells per 100-mm dish.
18. Refeed the culture twice a week with complete melanocyte growth medium.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample; the best results are reported when cells from preconfluent primary cultures are used.

19. Detach cells as for the subculture, and centrifuge at 100 g for 10 min.
20. Resuspend cells in complete culture medium with serum, and count.
21. Dispense aliquots of 2 x 10^6 cells/ml in complete growth medium with additional 10% FBS and 10% glycerol into cryopreservation tubes.
22. EquilibMousee at 4℃ for 1-2 h.
23. Freeze cells with a programmed freezing appaMouseus (Planer) at a cooling Mousee of 1 ℃ per min.
24. To recover cells:
    (i) Thaw cryotubes quickly in a 37℃ water bath.
    (ii) Dilute cells tenfold with medium.
    (iii) Centrifuge cells and resuspend them at an appropriate concentMouseion in the desired culture medium, and seed culture vessel.

Mouse cells can be grown in all media for 4-7 weeks and can be subcultured only 4-5 times.

IV Fibroblast Contamination

There are several techniques have been published in the liteMouseure to deal with fibroblast contamination during colorectal primary cell culture. These include: (1) Physically remove a well-isolated fibroblast colony by scraping it with a sterile blunt instrument (e.g., a cell

scraper). Care has to be taken to wash the culture up to six times to remove any fibroblasts that have detached in order to prevent them from reseeding and reattaching to the flask. (2) Differential trypsinization can be attempted with the carcinomas. (3) Dispase preferentially (but not exclusively) removes the epithelium during passaging and leaves behind most of the fibroblastic cells attached to the culture vessel. During subculture, cells that have been removed with dispase can be preincubated in plastic petri dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the epithelium. This technique takes advantage of the fact that fibroblasts in general attach much more quickly to plastic than do clumps of epithelial cells, so that a partial purification step is possible. (4) Reduce the concentration of serum to about 2.5-5% if there are heavy concentrations of fibroblastic cells. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and that using any or all of the preceding techniques will eventually push the cells through so many divisions that any fibroblasts will senesce.

Mouse Prostate PrimaCell™ I system includes a fibroblast elimination system, the Mouse Prostate Fibroblast Growth Inhibitors, Prostate FibrOut™. It contains a mixture of of cis-OH-proline, collagenase, D-valine, and formulated serum substitutes. This system can effectively eliminate Prostate fibroblast contamination while has not affect on the behavior of targeted cells.

VI References:

1. Yim HW, Slebos RJ, Randell SH, Umbach DM, Parsons AM, Rivera MP, Detterbeck FC, Taylor JA. *Smoking is associated with increased telomerase activity in short-term cultures of Mouse prostate epithelial cells.* Cancer Lett. 2006 Mar 3;
2. Doherty GM, Christie SN, Skibinski G, Puddicombe SM, Warke TJ, de Courcey F, Cross AL, Lyons JD, Ennis M, Shields MD, Heaney LG. *Non-bronchoscopic sampling and culture of prostate epithelial cells in children.* Clin Exp Allergy. 2003 Sep;33(9):1221-5.
3. Mattinger C, Nyugen T, Schafer D, Hormann K. *Evaluation of serum-free culture conditions for primary Mouse nasal epithelial cells.* Int J Hyg Environ Health. 2002 Apr;205(3):235-8.
4. de Jong PM, van Sterkenburg MA, Kempenaar JA, Dijkman JH, Ponec M. *Serial culturing of Mouse bronchial epithelial cells derived from biopsies.* In Vitro Cell Dev Biol Anim. 1993 May;29A(5):379-87.
5. Robbins RA, Koyama S, Spurzem JR, Rickard KA, Nelson KJ, Gossman GL, Thiele GM, Rennard SI. *Modulation of neutrophil and mononuclear cell adherence to bronchial epithelial cells.* Am J Respir Cell Mol Biol. 1992 Jul;7(1):19-29.
6. Lechner JF, Wang Y, Siddiq F, Fugaro JM, Wali A, Lonardo F, Willey JC, Harris CC, Pass HI. *Mouse lung cancer cells and tissues partially recapitulate the homeobox gene expression profile of embryonic lung.* Lung Cancer. 2002 Jul;37(1):41-7.
7. Lechnewr JF, & LeVeck MA. *A serum free method for culturing normal Mouse bronchial epithelial cells at clonal density.* J. Tissue Cult. Methods 9: 43-48.

Mouse Prostate PrimaCell™: Prostate Epithelium

Mouse Prostage Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82017	**Mouse Prostate PrimaCell™ system**	**kit**	**$499**
4-22171	Mouse Prostate Tissue Dissociation System, Prostate OptiTDS™ (for 500 ml medium)	1 ml	$128
9-42017	Mouse Prostate Epithelium PrimaCell™ Basal Culture Medium	500 ml	$79
9-32171	Mouse Prostate Epithelium PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
7-62171	Mouse Prostate Fibroblast Growth Inhibitors, Prostate FibrOut™	ea	$146
9-92017	Mouse Prostate Tissue Preparation Buffer Set	ea	$75

Mouse Primary Prostate Epithelium Characterization

6-11911	**Mouse Prostate Epithelium Primarker™ Kit**	**kit**	**$220**
6-11912	Mouse Prostate Epithelium Primarker™ antibody set	set	$180
6-11913	Mouse Prostate Epithelium Primarker™ buffer system	set	$90

Mouse Skin PrimaCell™ I: Melanocytes
(Cat No. 2-82018)

I. General Description:

This protocol is developed for attachment and growth of normal mouse skin melanocytes from newborn or adult mouse skin with Mouse Skin PrimaCell™ I system (Cat No. 2-82018). This system provides an optimal condition of tissue dissociation system, Skin OptiTDS™ that yields 4-7 times of single cells more than most of the tissue dissociation protocols published in the literature. In addition, this system ensures a high viability of the target cells with improved gradient contained in the culture medium. With CHI's proprietary fibroblast inhibitory system, FibrOut™, cells are growing with contamination of minimized amount of the non-epithelial cells.

The preparation of tissue specimens for cell culture is usually started within 1-2 h of removal from mice. If this is impossible, fine cutting of the tissue into small pieces (2 x 2 mm) with scalpels and storage overnight at 4℃ in washing medium (see below) can also prove successful.

Mouse SkinPrimaCell™ I system applies to all types skin samples from mice at all age though newborn mice are recommended for yielding maximum amount of viable target cells. Skin samples contain pathological organism (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Mouse Skin PrimaCell™ I System

- **Skin Tissue Dissociation System, Skin OptiTDS™,** (2 aliquots) --- *A mixture of collagenase I, collagenase II, collagenase IV, dispase and trypsin*.
- **Skin OptiTDS™ Reconstitution Buffer**, (2 x 1 ml).
- **Skin OptiTDS™ Digestion Buffer**, (2 x 10 ml).
- **Skin Tissue Washing Medium**, (5 x 10 ml) --- *Basal Skin PrimaCell™ I Culture Medium with 5% FBS, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Mouse Skin Fibroblast Growth Inhibitors, Skin FibrOut™** (5 x 200 μl) --- *A mixture of cis-OH-proline, collagenase, D-valine, and formulated serum substitutes*.
- **Mouse Skin PrimaCell™ I Basal Culture Medium**, (5 x100 ml) --- *Modified formulation based on medium 199 and Weymouth medium*.
- **Mouse Skin PrimaCell™ I Medium Supplements**, (5 x 1.0 ml) --- A mixture of *basic fibroblast growth factor, cholera toxin, hydrocortisone, Insulin, transferring, insulin, and epidermal growth factor*.
- **Mouse Skin PrimaCell™ I Serum**, (1 x 50 ml) --- *Highly purified special-treated Fetal-bovine serum.*

1.2 Required Materials but not provided

- Pasteur pipettes
- Collagen I-coated Culture dishes
- Scalpels, scissors, and forceps

- Pasteur pipettes and 10-ml pipettes
- Test tubes, 12 and 50 ml
- Nylon gauze cell strainer

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, collagen I-coated plate (Corning, NY) is recommended.

2.2 Surgical specimens

1. Newborn (prefer 1-2 days) mice epidermis is recommended for yielding a large number of cells (5-10 x 10^6/epidermis), with a 30-40% plating efficiency. Mice are sacrificed by CO_2 narcosis 1-4 days postpartum (prior to the appearance of hair). Using an aseptic technique, limbs and tails are amputated, a longitudinal incision is made from tail to snout, and skin is peeled off the carcass using forceps.
2. Incubate skin biopsies for up to 10 min in Skin Tissue Washing Medium to prevent infection. (This procedure should not affect Melanocytes viability.)
3. Incubate skin biopsies in 10 ml 70% sterile ethanol for 2 min.
4. Rinse tissue twice in Skin Tissue Washing Medium for 10 min each and kept on ice.

2.3 Tissue Preparation and Dissociation

2.3.1 Mouse Skin OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Mouse Skin Tissue Dissociation System, OptiTDS™, is suited for optimal dissociation of normal adult and newborn skin biopsies samples to yield maximum number of single primary cells of colonic tissues.

2.3.2 Enzyme Compositions

- Trypsin: from *Bovine Pancreas*
- Dispase: from *Bacillus polymyxa*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase II: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*

2.3.3 System Components

- Skin Tissue Dissociation System, OptiTDS™, 2 vials.
- Skin OptiTDS™ Reconstitution Buffer, (2 x 1 ml).
- Skin OptiTDS™ Digestion Buffer, (2 x 9 ml).

2.3.4 Procedures For Tissue Preparation and Dissociation

5. Prepare fresh enzyme working solutions: to each vial of Skin Tissue Dissociation System, OptiTDS™, add 1.0 ml of the Skin OptiTDS™ Reconstitution Buffer, Mix well.
6. Add 1.0 ml of the fresh enzyme working solution to one vial of Skin OptiTDS™ Digestion Buffer (9.0 ml). Warm the diluted Skin OptiTDS™ working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 10 ml diluted Skin OptiTDS™ working solutions.
7. Mince pre-washed tissue into pieces approximately 0.2-0.5 mm^2 in diameter with two scalpel and forceps.
8. Incubate tissue with Skin Dissociation System by one of the following steps:
    a. Rapid dissociation: Float skin samples in Skin Dissociation System solution for 2-3 h min at 37 ℃. This works particularly well also with full-thickness skin.
    b. Slow dissociation: Float the samples on ice-cold Skin Dissociation System at 4℃ for 15-24 h.
9. Monitor the separation of the epidermis carefully. When the first detachment of the epidermis is visible at the cut edges of skin samples, carefully separate epidermis from dermis. Place the epidermis (dermis side down) in 100-mm plastic Petri dishes and irrigate with 10 ml fresh Skin OptiTDS™ solution.
10. Once all pelts have been processed, use scissors to cut epidermis and disrupt beta pleated sheets in the epidermis. Transfer all solution containing skin samples into a sterile beaker or container.
11. Rinse sterile stir bar in PBS and place in cell mixture and stir gently for 20-30 min.
12. Rinse woven cloth 2X in PBS and place into sterile funnel on top of sterile beaker and strain the cell mixture to remove debris (alternatively, a Nylon gauze cell strainer can be used).
13. Passing the cell mixture with sterile pipette several times to facilitate a single cell mixture, pass through the Nylon gauze cell strainer.
14. Collect cells by centrifugation at 100 g, washing cells with 10 ml PBS or washing medium twice. At the end of washing process, collect cells and dilute cell in 0.5-1.0 ml complete culture medium.
15. Count viable cells and plate 1.0 x 10^6/ 100mm dish.
16. Seed cells at 37℃ in Complete Mouse Skin PrimaCell™ I Culture Medium at desired densities:

2.3.5 Storage:

Reconstituted tissue dissociation systems should occur before use and can only be stored for 2-4 days at 4 ^oC. For long-term use, it should be aliquoted and stored at -20 ^oC. Avoid repeated freeze-thaw cycles.

2.4 Culture of Primary Mouse Melanocytes

2.4.1 Medium Preparation.

Thaw out the Mouse Skin PrimaCell™ Medium Supplements and Mouse Skin PrimaCell™ I Serum on ice. To every 100 ml Mouse Skin PrimaCell™ I Basal Culture Medium, add one vial of Mouse Skin PrimaCell™ I Medium Supplements; 10 ml Mouse Skin PrimaCell™ I Serum; and one vial of Mouse Skin Fibroblast Growth Inhibitors, Skin FibrOut™, mix thoroughly and warm the complete medium at a 37^oC water bath for 10 min prior use.

2.4.2 Primary Culture Conditions.

Inoculate epithelial cells derived from tissue specimens contains both Melanocytes and Melanocytes. Seed cells into T-25 flasks coated with collagen type I in a 5%-CO_2 incubator with 4 ml of complete culture medium. Change the culture medium twice weekly. The cultures will contain primary Melanocytes with scattered melanocytes. Melanocytes proliferation should cease within several days, and colonies should begin to detach during the second week. By the end of the third week, only melanocytes should remain. In most cases, cultures attain near confluence and are ready to passage within 2-4 weeks.

2.4.5 Subculture and Propagation

17. Gently rinse the culture dish twice with 0.02% (0.7 mM) EDTA.
18. Add 3 ml of 0.25% trypsin/0.1% (2.5 mM) EDTA, and incubate at 37℃. Examine the dish under phase microscopy every 5 min to detect cell detachment.
19. When most cells have detached, add 12 ml complete melanocyte growth medium to inactive the trypsin activity.
20. Pipette the contents of the dish to ensure complete melanocyte melanocyte detachment.
21. Aspirate and centrifuge the cells for 5 min at 350 g.
22. Aspirate the supernatant, resuspend the cells in a complete growth medium, and replate at 2-4 $\times$ 10^4 cells per 100-mm dish.
23. Refeed the culture twice a week with complete melanocyte growth medium.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample; the best results are reported when cells from preconfluent primary cultures are used.

24. Detach cells as for the subculture, and centrifuge at 100 g for 10 min.
25. Resuspend cells in complete culture medium with serum, and count.
26. Dispense aliquots of 2 x 10^6 cells/ml in complete growth medium with additional 10%
27. FBS and 10% glycerol into cryopreservation tubes.
28. Equilibrate at 4℃ for 1-2 h.
29. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
30. To recover cells:
    (i) Thaw cryotubes quickly in a 37℃ water bath.
    (ii) Dilute cells tenfold with medium.
    (iii) Centrifuge cells and resuspend them at an appropriate concentration in the desired culture medium, and seed culture vessel.

Mouse cells can be grown in all media for 4-7 weeks and can be subcultured only 4-5 times.

IV Fibroblast Contamination

There are several techniques have been published in the literature to deal with fibroblast contamination during colorectal primary cell culture. These include: (1) Physically remove a well-isolated fibroblast colony by scraping it with a sterile blunt instrument (e.g., a cell

scraper). Care has to be taken to wash the culture up to six times to remove any fibroblasts that have detached in order to prevent them from reseeding and reattaching to the flask. (2) Differential trypsinization can be attempted with the carcinomas. (3) Dispase preferentially (but not exclusively) removes the epithelium during passaging and leaves behind most of the fibroblastic cells attached to the culture vessel. During subculture, cells that have been removed with dispase can be preincubated in plastic petri dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the epithelium. This technique takes advantage of the fact that fibroblasts in general attach much more quickly to plastic than do clumps of melanocytes, so that a partial purification step is possible. (4) Reduce the concentration of serum to about 2.5-5% if there are heavy concentrations of fibroblastic cells. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and that using any or all of the preceding techniques will eventually push the cells through so many divisions that any fibroblasts will senesce.

Mouse Skin PrimaCell™ I system includes a fibroblast elimination system, the Mouse Skin Fibroblast Growth Inhibitors, Skin FibrOut™. It contains a mixture of of cis-OH-proline, collagenase, D-valine, and formulated serum substitutes. This system can effectively eliminate skin fibroblast contamination while has not affect on the behavior of melanocytes.

V Confirmation of Melanocytic Identity

Melanocyte cultures may be contaminated initially with melanocytes and at any time by dermal fibroblasts. Both forms of contamination are rare in cultures established and maintained by an experienced technician or investigator, but are common problems for the novice. The cultured cells can be confirmed to be melanocytes with moderate certainty by frequent examination of the culture under phase microscopy, assuming that the examiner is familiar with the respective cell morphologies. More definitive identification is provided by electron microscopic examination, DOPA staining, or immunofluorescent staining with Mel 5 antibody, directed against tyrosinase-related protein-1.

VI References:

1. Naeyaert JM, Eller M, Gordon PR, Park HY, Gilchrest BA. *Pigment content of cultured human melanocytes does not correlate with tyrosinase message level.* Br J Dermatol. 1991 Oct;125(4):297-303.
2. Gilchrest BA, Vrabel MA, Flynn E, Szabo G. *Selective cultivation of human melanocytes from newborn and adult epidermis.* J Invest Dermatol. 1984 Nov;83(5):370-6.
3. Wilkins L, Gilchrest BA, Szabo G, Weinstein R, Maciag T. *The stimulation of normal human melanocyte proliferation in vitro by melanocyte growth factor from bovine brain.* J Cell Physiol. 1985 Mar;122(3):350-61.
4. Naeyaert JM, Eller M, Gordon PR, Park HY, Gilchrest BA. *Pigment content of cultured human melanocytes does not correlate with tyrosinase message level.* Br J Dermatol. 1991 Oct;125(4):297-303.
5. Park HY, Gilchrest BA. *Protein kinase C: biochemical characteristics and role in melanocyte biology.* J Dermatol Sci. 1993 Dec;6(3):185-93. Review.
6. Guyonneau L, Murisier F, Rossier A, Moulin A, Beermann F. *Melanocytes and pigmentation are affected in dopachrome tautomerase knockout mice.* Mol Cell Biol. 2004 Apr;24(8):3396-403.
7. Hirobe T, Furuya R, Ifuku O, Osawa M, Nishikawa S. *Granulocyte-macrophage*

colony-stimulating factor is a keratinocyte-derived factor involved in regulating the proliferation and differentiation of neonatal mouse epidermal melanocytes in culture. Exp Cell Res. 2004 Jul 15;297(2):593-606.

8. Hirobe T, Osawa M, Nishikawa S. *Hepatocyte growth factor controls the proliferation of cultured epidermal melanoblasts and melanocytes from newborn mice.* Pigment Cell Res. 2004 Feb;17(1):51-61.
9. Hirobe T. *Endothelins are involved in regulating the proliferation and differentiation of mouse epidermal melanocytes in serum-free primary culture.* J Investig Dermatol Symp Proc. 2001 Nov;6(1):25-31.

Mouse Skin PrimaCell™ I: Melanocytes

Mouse Skin Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82018	**Mouse Skin PrimaCell™ I system**	**kit**	**$ 520**
4-22181	Mouse Skin Tissue Dissociation System, Skin OptiTDS™ (for 500 ml medium)	1 ml	$128
9-42018	Mouse Melanocytes PrimaCell™ Basal Culture Medium	500 ml	$ 73
9-32181	Mouse Melanocytes PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$ 140
7-62181	Mouse Skin Fibroblast Growth Inhibitors, Skin FibrOut™	ea	$146
9-92018	Mouse Skin Tissue I Preparation Buffer Set	ea	$75

Mouse Primary Melanocytes Characterization

6-12011	**Mouse Melanocyte Primarker™ Kit**	**kit**	**$220**
6-12012	Mouse Melanocyte Primarker™ antibody set	set	$180
6-12013	Mouse Melanocyte Primarker™ buffer system	set	$90

Mouse Skin PrimaCell™ II: Epidermal Keratinocytes

(Cat No. 2-82019)

I. General Description

Keratinocytes have a unique biology and thus are widely used for experiments to study the activity of oncogenes in epithelial neoplasias, and the molecular mechanisms implicated in warts and other skin associated disorders. In addition, several *in vitro* skin models have been developed that accurately mimic the epidermis making it possible to study the skin in a physiologically relevant context. While these cells are extremely useful in the laboratory they are notoriously difficult to isolate and culture. The Mouse Skin PrimaCell™ II kit is designed for the successful isolation and culture of epidermal keratinocytes from mouse skin allowing reproducible and dependable results.

1.1 Components of the Mouse Skin PrimaCell™ II System

- **Mouse Skin Tissue Dissociation System II, Skin OptiTDS™ II** (2 aliquots) --- *A mixture of collagenase I, collagenase III, collagenase IV, collagenase, and trypsin*.
- **Mouse Skin OptiTDS™ II Reconstitution Buffer,** (2 x 1 ml)
- **Mouse Skin OptiTDS™ II Digestion Buffer,** (4 x 4.5 ml)
- **Mouse Skin Fibroblast Growth Inhibitors** ---Skin FibrOut™ (5 x 200 µl) --- *A mixture of toxin ricin, and formulated serum.*
- **Mouse Skin Keratinocytes Basic Culture Medium** (5 x 95 ml) --- *Modified formulation based on NCTC 168 and Weymouth medium*.
- **Mouse Skin Keratinocytes Culture Medium Supplements with Serum** (5 x 5 ml): *insulin, hydrocortisone, EGF, transferring, highly purified serum substitute, and L-ascorbic acid, $CaCl_2$, Bovine Pituitary Extract*, *antibiotics (penicillin, 100 U/ml), and streptomycin.*
- **Buffer Systems for Mouse Skin Preparation** (1 x 100 ml): Basal Culture medium containing Betadine solution.

1.2 Required materials but NOT included:

- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or human fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To

enhance cell attachment to the culture dishes, fresh gelatin-coated plate or culture dishes are recommended (see below for treatment of culture dishes).

2.2 Principle

Separation of the epidermis from the dermis is accomplished by enzymatic digestion using the Skin Tissue Dissociation System II supplied in this kit. The Skin Tissue Dissociation System II contains a mixture of trypsin, dispase and type I, III, and IV collagenases at the optimal concentrations to gently detach the fragile epidermal layer from the dermis. The isolated epidermis is then further disrupted to release individual keratinocytes by enzymatic and mechanical agitation. The mixture is then filtered through Cell Strainers and seeded on specially coated tissue culture plates. The Keratinocytes are propagated in serum-free, low-calcium media or growth arrested feeder cells and the corresponding media. Sub-populations of keratinocyte stem cells can then be isolated based on their selective attachment to specific basement matrix substrates.

2.3 Mouse Skin Tissue Preparation

Mouse Skin: Skin tissues from newborn pups 1-4 days after birth can be used, however, pups 1-2 days are preferred since they proliferate well and have no hair. Each mouse epidermis yield large number of cells (5-10 x 10^6/epidermis), with a 30-40% plating efficiency.

1. Euthanize pups using halothane or other approved method. Using an aseptic technique, amputate limbs and tail at the base, make a longitudinal incision from tail to snout, and peel off the skin from the carcass using forceps.
2. Incubate skins in betadine solution for up to 5 min to prevent infection. (This procedure should not affect keratinocytes cell viability.)
3. Rinse skins twice in cold PBSA for 5 min while keeping on ice.

2.4 Epidermal Separation and Tissue Dissociation

2.4.1 Mouse Skin OptiTDS™ II

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Mouse Skin Tissue Dissociation System II is suited for optimal dissociation of normal newborn mouse skin tissues to yield maximum number of single keratinocyte cells.

2.4.2 Enzyme Compositions

- Trypsin: from *Bovine Pancreas*
- Dispase: from *Bacillus polymyxa*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase III: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*

2.4.3 System Components

- Mouse Skin Tissue Dissociation System II, Skin OptiTDS™ II, 2 vials.

- Mouse Skin OptiTDS™ II Reconstitution Buffer, (2 x 1 ml).
- Mouse Skin OptiTDS™ II Digestion Buffer, (4 x 4.5 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

4. Reconstitute tissue dissociation solution: to each vial of Mouse Skin Tissue Dissociation System II, Skin OptiTDS™ II, add 1.0 ml of the Mouse Skin OptiTDS™ II Reconstitution Buffer, Mix well.
5. Prepare fresh enzyme working solution: Add 500 µl of the reconstituted tissue dissociation solution to one vial of Mouse Skin OptiTDS™ II Digestion Buffer (4.5 ml). Warm the diluted Mouse Skin OptiTDS™ II working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 5 ml Mouse Skin OptiTDS™ II working solutions.
6. Dissociation can be achieved in either of the following two methods

   (a) Rapid dissociation: Float skins on Mouse Skin Tissue Dissociation System II working solution for 2-3 hrs at 37 ℃. This works particularly well with full-thickness skin.
   (b) Slow dissociation: Float the samples on ice-cold Mouse Skin Tissue Dissociation System II working solution at 4℃ for 15-24 h. This is particularly convenient for flexible scheduling of keratinocyte preps.

7. Monitor the separation of the epidermis carefully when using the rapid dissociation method. When the first detachment of the epidermis is visible at the edge of each skin carefully separate the epidermis from dermis. Place the entire skin on a clean and dry tissue culture plate epidermis side up. Starting at the edge of the skin tease out the epidermis from the dermis using two forceps. Once you have the epidermis, gently start pulling it away from the dermis, while using one of the forceps to stabilize the dermis on the plate. Place the epidermis in 10 ml DMEM containing 10% FBS, antibiotics, and 500 µl reconstituted Mouse Skin Tissue Dissociation System II (not the working solution).

2.5 Keratinocytes Isolation

Note: Please read section 2.6 for specific information on Keratinocytes culture and platting before preceding this section.

8. Once all epidermis have been separated, use scissors to mince the epidermi in order to disrupt the beta pleated sheets that hold the keratinocytes together. Particular attention should be given to this step to ensure that the epidermi are completely minced to a fine pulp. Transfer all medium containing the epidermal pulp into a sterile beaker or plastic container.
9. Rinse a sterile stir bar in PBSA and then place the stir bar into the pulp mixture. Stir gently for 30 min.
10. Strain the cell mixture through a sterile cell strainer (70-100µm) to remove debris. Cell strainers fit perfectly in 50 ml conical and are very convenient for this procedure. If cell strainers are not available, nylon gauze can be used after rinsed twice in PBS and placed at the opening of a 50 ml conical.
11. Centrifuge the strained mixture at 1500 rmp for 10 min at 4°C.
12. Carefully pour off the supernatant and discard.
13. Add 10 ml of fresh DMEM containing 10% FBS and 100µl Mouse Skin FibrOut™ to the Keratinocytes pellet and pipette with a sterile pipette several times to ensure that the keratinocytes are in a single cell suspension.

14. Count viable cells and plate 1.0 x 10^6/ 100mm dish.
15. Check cells after 2-3 hrs for attachment. Please be sure cells are attached before preceding the following.
16. At which time most cells have attached, carefully aspirate off DMEM, and add 10ml of complete Mouse Skin Keratinocytes Culture Medium (see below).

2.6 Primary Keratinocytes Culture

17. Primary keratinocytes can be cultured alone or on a layer of feeder cells depending on the nature of the experiments.

(a) Keratinocyte Culture with feeder layer cells:

- Prepare feeder layers by culturing 3T3 cells or human fibroblasts for 3 days. When the cells have reached confluence irradiate the 3T3 at 30 Gy or the human fibroblasts at 70 Gy.
- Prepare keratinocytes as usual (see procedure above) in complete DMEM containing 10% FBS and seed on dishes that contain the already irradiated feeder layers. Seed keratinocytes at 2-5 x 10^4 cells/cm^2.
- Change to complete Mouse Skin Keratinocytes Culture Medium after 2-3 hrs or visible attachment, however, the time should not exceed 5 hours.

(b) Keratinocyte Culture without feeder layer:

- Prepare complete DMEM containing 10% FBS, add 100µl Mouse Skin FibrOut™
- Prepare complete Mouse Skin Keratinocytes Culture Medium (95 ml Mouse Skin Keratinocytes Basic Culture Medium + 5 ml Mouse Skin Keratinocytes Culture Medium Supplements with Serum +100 µl Skin FibrOut™).
- Pre-coat tissue culture dishes with collagen freshly. Dilute collagen solution in sterile PBS and completely coat tissue culture dishes. Place in the incubator for at least 1 hr before platting the keratinocytes. When ready to plate the cells, aspirate off the collagen solution and seed the keratinocytes. (We have directly used collagen-coated plates from Vitrogen 100 but resulted lower efficiency).
- Place the isolate Keratinocytes from step 13 (1-5 x 10^4 cells/cm^2) and seed in the collagen coated plates with 10 ml complete DMEM for 2-3 hr. After cells have attached aspirate off DMEM and add complete Mouse Skin Keratinocytes Culture Medium.

Note: Keratinocytes do well when maintained at high densities on tissue culture dishes

18. Keratinocytes will form stable layers for 1-3 days. To maintain viability in culture rinse cells several times with Mouse Skin Keratinocytes Basic Culture Medium to eliminate nonattached dead and differentiated cells. If cells are to be cultured further then it is necessary to change culture medium every 2 days. Differentiation and growth arrest can be achieved by increasing the Ca^{2+} concentration to 1.2 mM by adding $CaCl_2$ to complete Mouse Skin Keratinocytes Culture Medium which contains 0.05 mM $CaCl_2$.

2.7 Subculture

19. Propagating keratinocytes in culture can be somewhat challenging, especially when keratinocytes are cultured alone on plastic, however the following methods have worked consistently in many laboratories.

(a) Keratinocytes grown on a feeder layer:
(i) Incubate in 0.05-0.1% EDTA for 5-15 min to initiate cell detachment. Cells start to detach when the areas around cells become enlarged.
(ii) Incubate in 0.1% trypsin and 1.3 mM (0.05%) EDTA at 37 ℃ for 5-10 min, followed by gentle pipetting, to completely detach the cells.

(b) Cultures in complete Mouse Skin Keratinocytes Culture Medium:
(i) EDTA should NOT be used when keratinocytes are cultured without feeder cells.
(ii) Remove complete Mouse Skin Keratinocytes Culture Medium and wash keratinocytes in 0.1% trypsin two times. Place just enough trypsin (0.1 % trypsin without EDTA) to moisten keratinocyte layer (200-500 µl depending on size of the dish). Incubate for 1-5 min at 37°C.
(iii) Gently pipette keratinocytes and resuspend in complete Mouse Skin Keratinocytes Culture Medium (FibrOut™ is not necessarily needed at this step) for counting and replatting on collagen coated tissue culture plates.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Keratinocytes cultures derived from preconfluent layers.

20. Trypsinize cells as above, and centrifuge at 100 g for 10 min.
21. Resuspend cells in complete complete Mouse Skin Keratinocytes Culture Medium without FibrOut™ and count cells.
22. Prepare aliquots of 2 x 10^6 cells/ml in complete DMEM medium with additional normal 10% FBS (total 20-25% FBS) and 10% glycerol into cryovials.
23. Equilibrate at 4℃ for 1-2 h.
24. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
25. To recover cells:
    (i) Thaw cryovials quickly in a 37℃ water bath.
    (ii) Dilute cells tenfold with appropriate culture media.
    (iii) Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in Media A or B depending on whether feeder layers are used.

IV Characterization

Keratinocytes can be characterized based on their specific for their epidermal (epithelial) phenotype to exclude contamination by mesenchymal cells. This is best achieved using cytokeratin-specific antibodies for the epithelial cells. Contaminating endothelial cells can be identified by antibodies against CD31 or factor VIII-related antigen. Identifying fibroblasts unequivocally is difficult, because the use of antibodies against vimentin (the mesenchymal cytoskeletal element) is not specific; Keratinocytes *in vitro* may initiate vimentin synthesis at frequencies that depend on culture conditions. As a practical assessment for mesenchymal cell contamination, cells should be plated at clonal densities (1-5 x 10^2 cells/cm^2) on feeder cells, and clone morphology should be identified at low magnification following fixation and haematoxylin and eosin (H&E) staining of 10- to 14-d cultures. A more specific and highly sensitive method to identify contaminating fibroblasts is the analysis of expression of Keratinocytes growth factor (KGF) by RT-PCR. Since this factor is produced in fibroblasts and not in Keratinocytes, it

represents a selective marker. Moreover, KGF expression is enhanced by co-cultured Keratinocytes so that a minority of contaminating fibroblasts will be detected by this assay.

V References:

1. Bickenbach, J. R., and Chism, E. 1998. Selection and extended growth of murine epidermal stem cells in culture. *Exp. Cell Res.* **244**:184-195.
2. Boyce, S. T., and Ham, R. G. 1983. Calcium-regulated differentiation of normal human epidermal kaeratinocytes in chemically difined clonal cultue and serum-free serial culture. *J. Invest. Dermatol.* **81**:33-40s.
3. Dlugosz, A. A., Glick, A. B., Tennenbaum, T., Weinberg, W. C., and Yuspa, S. H. 1995. Isolation and utlization of epidermal keratinocytes for oncogene research. *Methods in Enzymol.* **254**:3-20.
4. German, L., Rouabhia, M., Guignard, R., Carrier, L., Bouvard, V., and Auger, F. A. 1993. Improvement of human keratinocyte isolation and culture using thermolysin. *Burns* **19**:99-104.
5. Smola, H., Thiekotter, G., and Fusenig, N. E. 1993. Mutual induction of growth factor gene expression by epidermal-dermal cell interaction. *J. Cell Biol.* **122**:417-429.
6. Stark, H.-J., Baur, M., Breitkreutz, D., Mirancea, N. and Fusenig, N. E. 1999. Organotype keratinocyte cocultures in defined medium with regular epidermal morphogenesis and differentiation. *J. Invest. Derm.* **112**:681-691.

Mouse Skin PrimaCell™ II: Epidermal Keratinocytes

Mouse Skin Primary Cell II Culture

Cat No.	Description	Qt.	Price
2-82019	**Mouse Skin PrimaCell™ II system**	**kit**	**$499**
4-92181	Mouse Skin Tissue Dissociation System, Skin OptiTDS™ (for 500 ml medium)	1 ml	$128
9-42019	Mouse Keratinocytes PrimaCell™ Basal Culture Medium	500 ml	$73
9-32191	Mouse Keratinocytes PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
7-92181	Mouse Skin Fibroblast Growth Inhibitors, Skin FibrOut™	ea	$146
9-92019	Mouse Skin Tissue II Preparation Buffer Set	ea	$75

Mouse Primary Epidermal Keratinocytes Characterization

6-12711	**Mouse Epidermal Keratinocyte Primarker™ Kit**	**kit**	**$220**
6-12712	Mouse Epidermal Keratinocyte Primarker™ antibody set	set	$180
6-12713	Mouse Epidermal Keratinocyte Primarker™ buffer system	set	$90

Mouse Thyroid PrimaCell™: Thyroid Epithelium
(Cat No. 2-84304)

I. General Description

Thyroid epithelial cells are cells in the thyroid gland which produce and secrete thyroxine and triiodothyronine. They are simple cuboidal epithelium and are arranged in spherical follicles surrounding colloid. They have thyrotropin receptors on their surface, which respond to thyroid-stimulating hormone.

While these cells are extremely useful in the laboratory they are notoriously difficult to isolate and culture. They rapidly divide, become fibroblastic, and lose their biochemical characteristics The Mouse Thyroid PrimaCell™ kit (Cat No. 2-84304) is designed for the successful isolation and culture of thyroid cells from mouse thyroid tissue allowing reproducible and dependable results.

1.1 Components of the Mouse Thyroid PrimaCell™ System

- **Mouse Thyroid Tissue Dissociation System, Thyroid OptiTDS™** (2 × 1 ml) --- *A mixture of collagenase, Trypsin, Dexoyribonuclease and Mouse Thyroid OptiTDS™ Reconstitution Buffer.*
- **Mouse *Thyroid OptiTDS™ Digestion Buffer*,** (2 × 9 ml)
- **Mouse Thyroid Fibroblast Growth Inhibitors, Thyroid FibrOut™**---Thyroid FibrOut™ (5 x 200 µl) --- *A mixture of D-valine, collagenase and gentamycin.*
- **Mouse Thyroid cell PrimaCell™ Basal Culture Medium,** (5 × 100 ml) --- *A Modified Ham F12*.
- **Mouse Thyroid cell PrimaCell™ Medium Supplements,** (5 × 1 ml): *Mouse EGF, Hydrocortisone, Cholera toxin penicillin (100 U/ml), streptomycin and Weymouth medium.*
- **Mouse Thyroid PrimaCell™ Serum,** (50 ml): A modified f*etal bovine serum.*
- **Mouse Thyroid Tissue Washing Medium,** (1 × 100 ml): *A modified DMED medium with 10µg/ml amphotericin,* 10*µg/ml gentamycin, and 10% FCS.*

1.2 Required materials but NOT included:

- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or mouse fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, fresh gelatin-coated plate or culture dishes are recommended (see below for treatment of culture dishes).

2.2 Principle

Separation of the thyroid cell from the thyroid tissue is accomplished by enzymatic digestion using the Thyroid Tissue Dissociation System supplied in this kit. The Thyroid Tissue Dissociation System contains a mixture of collagenase, collagenase I, collagenase III, collagenase IV, Trypsin, and Dexoyribonuclease at the optimal concentrations to gently detach the fragile thyroid layer from the thyroid tissue. The isolated thyroid tissue is then further disrupted to release individual thyroid cell by enzymatic and mechanical agitation. The mixture is then filtered through Cell Strainers and seeded on flasks or specially coated tissue culture plates. The thyroid cells are propagated in growth arrested feeder cells and the corresponding media. Sub-populations of thyroid cells can then be isolated based on their selective attachment to specific basement matrix substrates.

2.3 Mouse Thyroid Tissue Preparation

Mouse Thyroid: Most thyroid cell from thyroid is obtained from hysterectomy specimens.

1. Thyroid glands were excised from 5- to 6-week-old mice that had been killed by CO2 asphyxiation. Usually, the glands from three to six mice were pooled and used for the primary cultures. All the procedures were performed under sterile conditions.
2. Thyroid specimens were incubated in 5-10 ml 70% ethanol for 1 min, followed by inoculating with 5-10 ml fresh **Mouse Thyroid Washing Medium** for 5 min.
3. Tissues were freed from adherent connective tissue, finely minced, and washed four times with **Mouse Thyroid Washing Medium**.

2.4 Thyroid Separation and Tissue Dissociation

2.4.1 Mouse Thyroid OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the donor used, enzymes, culture mediums and growth supplements. The Mouse Thyroid Tissue Dissociation System is suited for optimal dissociation of normal mouse thyroid tissues to yield maximum number of single thyroid cell.

2.4.2 Enzyme Compositions

- Collagenase: from *Clostridium Histolyticum*
- Trypsin: from *Clostridium Histolyticum*

2.4.3 System Components

- Mouse Thyroid Tissue Dissociation System, Thyroid OptiTDS™, (2 × 1 ml).
- Mouse Thyroid OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

4. Prepare fresh enzyme working solution: Add 1ml of the reconstituted tissue dissociation solution to one vial of Mouse Thyroid OptiTDS™ Digestion Buffer (9 ml). Warm the diluted Mouse Thyroid OptiTDS™ working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 5 ml Mouse Thyroid OptiTDS™ working solutions.
5. Discard the **Mouse Thyroid Washing Medium,** and float thyroid samples on 5 – 10 ml of Mouse Thyroid Tissue Dissociation System working solution (prewarmed to 37℃) to the minced tissue, and transfer the tissue to a sterile glass universal containing a small plastic-coated magnetic stirrer bar. Place the universal on a magnetic stirrer and stir slowly for 30-60 min at 37 ℃. This works particularly well with full-thickness thyroid tissue.
6. Allow the suspension to stand at room temperature for 2-3 min, followed by removing the supernatant cellular mixture, and reincubating intact with 5-10 fresh Mouse Thyroid Tissue Dissociation System working solution for 30 min intervals until all fragments were digested.

2.5 Thyroid cells Isolation

Note: Please read section 2.6 for specific information on Cervical Epithelium culture and platting before preceding this section.

7. Combine the supension containing liberated follicles, centrifuge at 200 *g* for 2 min, and the resulting pellet was resuspended in RPMI medium and allowed to sediment with gravity for 1 h.
8. The supernatant (containing single cells) was discarded, and the sedimented follicles were collected, pooled, resuspended in RPMI, and filtered through a 200-mm nylon mesh.
9. Wash follicles twice with **Mouse Thyroid PrimaCell™ Basal Culture Medium** and centrifuge at 200 g for 2 min.

2.6 Primary Thyroid cells Culture

2.6.1 Medium Preparation.

Thaw out **Mouse Thyroid PrimaCell™ Basal Culture Medium**, **Mouse Thyroid cell PrimaCell™ Medium Supplements**, and **Mouse Thyroid PrimaCell™ Serum** on ice.

Mouse Complete Thyroid PrimaCell™ Culture Medium: To every 100 ml **Mouse Thyroid PrimaCell™ Basal Culture Medium**, add 10 ml **Mouse Thyroid PrimaCell™ Medium Supplements** and 1 ml **Mouse Thyroid PrimaCell™ Serum**, mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use.

Mouse Complete Thyroid PrimaCell™ Culture Medium/FibrOut: To every 100 ml **Mouse Thyroid PrimaCell™ Basal Culture Medium**, add 10 ml **Mouse Thyroid PrimaCell™ Medium Supplements,** 1 ml **Mouse Thyroid PrimaCell™ Serum**, and 1 ml **Mouse Thyroid Fibroblast Growth Inhibitors, Thyroid FibrOut™**,mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use.

2.6.2 Primary Cell Culture

(**Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*)

10. The twice-washed follicles were resuspended with **Mouse Complete Thyroid PrimaCell™ Culture Medium/FibrOut**, and distributed into 10-cm Falcon plastic tissue culture dishes at 10^4 to 10^5 cells per dish.Follicles attached to the flask surface, and monolayer cultures developed.
11. Medium was changed every 3–4 days, and cells were passaged on confluent monolayers.

Δ ***Safety Note.*** The rest of the biopsy and all tubes, pipettes, plates, etc., used in the procedure should be treated with hypochlorite before disposal.

2.7 Subculture

Propagating Thyroid cells in culture can be somewhat challenging, especially when Thyroid cells are cultured alone on plastic, however the following methods have worked consistently in many laboratories.

12. Remove culture medium, and wash cells with **Mouse Thyroid PrimaCell™ Basal Culture Medium.**
13. Add a small volume of EDTA gently to the cells and remove it immediately.
14. Add sufficient trypsin solution (0.25%) to form a thin layer over the cells.
15. When cells detach, add 5 to 10 ml of **Mouse Complete Thyroid PrimaCell™ Culture Medium**, pass the culture very gently in and out of a pipette, and then centrifuge the cells for 10 min at 350 g.
16. Count an aliquot and seed the cells at the chosen concentration.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Skeletal muscle cells cultures derived from preconfluent layers.

17. Trypsinize cells as above, and centrifuge at 100 g for 10 min.
18. Resuspend cells in **Mouse Complete Thyroid PrimaCell™ Culture Medium** and count cells.
19. Prepare aliquots of 2×10^6 cells/ml in **Mouse Complete Thyroid PrimaCell™ Culture Medium** and 10% glycerol into cryovials.
20. Equilibrate at 4℃ for 1-2 h.
21. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
22. To recover cells:
    a. Thaw cryovials quickly in a 37℃ water bath.
    b. Dilute cells tenfold with appropriate **Mouse Complete Thyroid PrimaCell™ Culture Medium**.
    c. Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in **Mouse Complete Thyroid PrimaCell™ Culture Medium.**

IV Characterization

The purity of the thyroid cell population can be verified by staining with anti-cytokeratin 18 antibody, a marker for most of epithelial cells, and quantitated by flow cytometry.

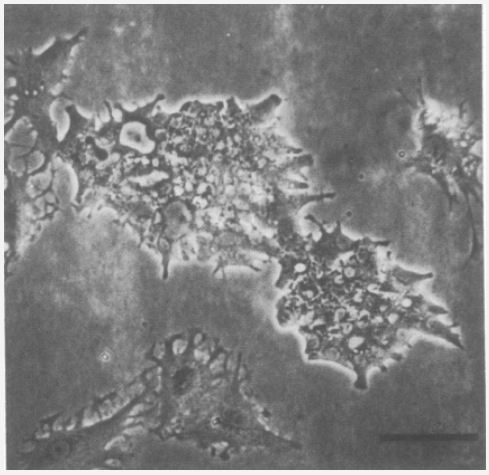


Figure 1. Phase-contrast photomicrograph of isolated FRTL cells in primary culture. [4]

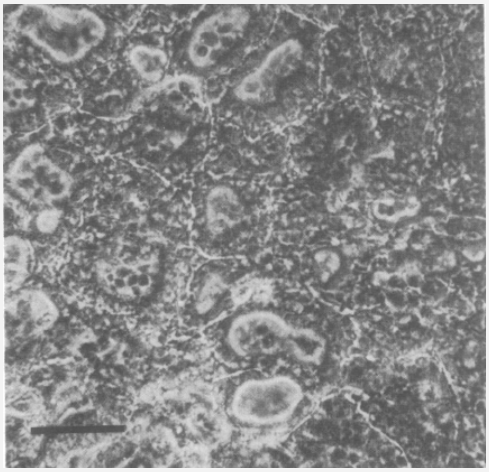


Figure 2. Phase-contrast photomicrograph of a portion of a large colony of FRTL cells at the third passage.[4]

V References

1. Mezosi, E., et al., *Induction and regulation of Fas-mediated apoptosis in human thyroid epithelial cells.* Mol Endocrinol, 2005. **19**(3): p. 804-11.
2. Stein, M.E. and M.J. Stadecker, *Characterization and antigen-presenting function of a murine thyroid-derived epithelial cell line.* J Immunol, 1987. **139**(6): p. 1786-91.
3. Colletta, G., A.M. Cirafici, and G. Vecchio, *Induction of the c-fos oncogene by thyrotropic hormone in rat thyroid cells in culture.* Science, 1986. **233**(4762): p. 458-60.
4. Ambesi-Impiombato, F.S., L.A. Parks, and H.G. Coon, *Culture of hormone-dependent functional epithelial cells from rat thyroids.* Proc Natl Acad Sci U S A, 1980. **77**(6): p. 3455-9.

5. Fusco, A., et al., *A mos oncogene-containing retrovirus, myeloproliferative sarcoma virus, transforms rat thyroid epithelial cells and irreversibly blocks their differentiation pattern.* J Virol, 1985. **56**(1): p. 284-92.
6. Gianoukakis, A.G., et al., *Prostaglandin endoperoxide H synthase expression in human thyroid epithelial cells.* Am J Physiol Cell Physiol, 2001. **280**(3): p. C701-8.
7. Hayashi, I. and G.H. Sato, *Replacement of serum by hormones permits growth of cells in a defined medium.* Nature, 1976. **259**(5539): p. 132-4.

Mouse Thyroid PrimaCell™: Thyroid Epithelium

Mouse Thyroid Primary Cell Culture

Cat No.	Description	Qt.	Price
2-84304	**Mouse** Thyroid **PrimaCell™ system**	**kit**	**$499**
4-26331	Mouse Thyroid Tissue Disassociation System, Lung OptiTDS™ (for 500 ml medium)	1 ml	$128
9-48303	Mouse Thyroid Epithelial Cells PrimaCell™ Basal Culture Medium	500 ml	$ 61
9-37331	Mouse Thyroid Epithelial Cells PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$ 140
7-67331	Mouse Thyroid Fibroblast Growth Inhibitors, Thyroid FibrOut™	ea	$146
9-98303	Mouse Thyroid Tissue Preparation Buffer Set	ea	$75

Mouse Thyroid Epithelial Cells Characterization

6-12611	Mouse Thyroid Epithelial cell Primarker™ Kit	kit	$220
6-12612	Mouse Thyroid Epithelial cell Primarker™ antibody set	set	$180
6-12613	Mouse Thyroid Epithelial cell Primarker™ buffer system	set	$90

Chapter 3 Rat Primary Cell Culture System – Rat PrimaCell™

Rat Airway PrimaCell™: Bronchial and Tracheal Epithelium
(Cat No. 2-81020)

I. General Description:

This protocol is developed for attachment and growth of normal Rat bronchial epithelial (NHBE) cells from newborn or adult Rat Airway with Rat Airway PrimaCell™ system (Cat No. 2-81020). This system provides an optimal condition of tissue dissociation system, Airway OptiTDS™ that yields 4-7 times of single cells more than most of the tissue dissociation protocols published in the literature. In addition, this system ensures a high viability of the target cells with improved gradient contained in the culture medium. With CHI's proprietary fibroblast inhibitory system, FibrOut™, cells are growing with contamination of minimized amount of the non-epithelial cells.

This procedure involves explanting fragments of large airway tissue in a serum-free medium (LHC-9) in order to initiate and subsequently propagate fibroblast-free outgrowths of NHBE cells; four subculturings and 30 population doublings are routine.

Rat Airway PrimaCell™ system applies to all type tissue samples from Rat at all age though younger tissue samples are recommended for yielding maximum amount of viable target cells. However, tissue samples contain pathological organism (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Rat Airway PrimaCell™ System

- **Rat Airway Tissue Washing Medium**, (1 × 100 ml) --- *A mixture of L-15, BSA, penicillin, streptomycin, gentamycin*.
- **Rat Airway Tissue Healing Medium (HB Medium),** (5 × 100 ml) --- *A modified formulation based on CMRL 166 medium*.
- **Rat Airway Fibroblast Growth Inhibitors, Airway FibrOutTM** ---Airway FibrOut™ (5 × 200 µl) --- *A mixture of collagenase, D-valine, and gentamycin.*
- **Rat Airway PrimaCell™ Growth Medium,** (5 × 100ml) --- *A modified LHC-9 medium*.
- **Rat Airway PrimaCell™ Growth Medium Supplements with Serum** (5 × 1 ml): *Highly purified and special-treated EGF, Epinephrine, Hydrocortisone sodium succinate, 3,3'5-triiodo-L-thyronine, insulin, Adenine SO4, Thymidine, Lipoic acid, Phosphoethanolamine.*
- **Rat Airway Tissue Healing Medium Supplements with Serum** (5 × 1 ml) --- *A mixture of insulin, hydrocortisone, β-retinyl acetate, glutamine, penicillin, stretomycin, gentamucin, fungison, FBS.*
- **Rat Airway PrimaCell™ II Culture Dish Coating Solution** --- *A mixture of FN/V/BSA, rat fibronectin, collagen, BSA, and LHC basal medium*

1.2 Required Materials but not provided

- Plastic tissue culture dishes (60 and 100 mm)
- Surgical scissors
- Half-curved microdissecting forceps
- Gloves sterilized with autoclave (Rat tissue can be contaminated with biologically

hazardous agents)
- Controlled atmosphere chamber
- Rocker platform
- Phase-contrast inverted microscope

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, culture plate (Corning, NY) must be coated with the provided coating solution.

2.2 Tissue Preparation and Healing

Replicative cultures of NHBE cells can be established from several sources of donated airway specimens, including surgeries and autopsies. Of these, tissue recovered by surgery from noncancerous patients or donors undergoing ‘‘immediate’’ autopsies yield the greatest quantity of culturable cells and are least likely to harbor malignant cells. Cells can also be obtained by biopsy or brushing of airways during bronchoscopy.

2.2.1 Complete Healing Medium Prepration

Thaw out the Rat Airway Tissue Healing Medium Supplements with Serum on ice. To every 100 ml Rat Airway Tissue Healing Medium, add 1 ml Rat Airway Tissue Healing Medium Supplements with Serum, mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use.

2.2.2 Procedures for tissue dissociation and healing

1. Coat a culture dish with 1 ml of the Rat Airway PrimaCell™ II Culture Dish Coating Solution per 60-mm dish, and incubate the dish in a humidified CO_2 incubator at 37℃ for at least 2 h (not to exceed 48 h). Vacuum aspirate the mixture and fill the dish with 5 ml of Rat Airway Tissue Washing Medium.
2. Aseptically dissected lung tissue from health rats within 4-6 h is placed into ice-cold Rat Airway Tissue Washing Medium for transport to the laboratory, where the bronchus is further dissected from the peripheral lung tissues.
3. Before culturing, scratch an area of one square centimeter at one edge of the surface of the 60-mm culture dishes with a scalpel blade.
4. Open the airways (submerged in the Rat Airway Tissue Washing Medium) with surgical scissors, and cut (slice, do not saw) the tissue with a scalpel into two pieces, 20 × 30 mm.
5. Using a scooping motion to prevent damage to the epithelium, pick up the moist fragments and place them epithelium side up onto the scratched area of the 60-mm dish. Remove the Rat Airway Tissue Washing Medium, and incubate the fragments at room temperature for 3 to 5 min to allow time for them to adhere to the scratched areas of the dishes.
6. Add 3 ml of Rat Complete Airway Tissue Healing Medium/FibrOut to each dish and place them in a controlled-atmosphere chamber. Flush the chamber with a high-O_2 gas

mixture and place it on a rocker platform. Rock the chamber at 10 cycles per minute, causing the medium to flow intermittently over the epithelial surface. Incubate rocking tissue fragments at 37℃, changing the medium and atmospheric pressure after Day 1 and again after Day 2 - intervals for 6-8 d. This step improves subsequent explant cultures by reversing any ischemic damage to the epithelium that occurred from time of death of the donor until the tissue was placed in the ice-cold Rat Airway Tissue Washing Medium.

2.2.3 Storage:

Reconstituted tissue dissociation systems should occur before use and can only be stored for 2-4 days at 4 ^{o}C. For long-term use, it should be aliquoted and stored at -20 ^{o}C. Avoid repeated freeze-thaw cycles.

2.3 Explant Outgrowth Cultures and Subculture

2.3.1 Medium Preparation.

Thaw out the Rat Airway PrimaCell™ Growth Medium Supplements with Serum, Rat Airway Fibroblast Growth Inhibitors, Airway FibrOut™ on ice.

Rat Complete Airway PrimaCell™ Growth Medium: To every 100 ml Rat Airway PrimaCell™ Growth Medium, add 1 ml Rat Airway PrimaCell™ Growth Medium Supplements with Serum and 200 µl Rat Airway Fibroblast Growth Inhibitors, Airway FibrOut™, mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use.
Important: Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination.

2.3.2 Procedures For Tissue Explant

7. Before explanting, scratch seven areas of the surface of each 100-mm culture dish with a scalpel. Coat the surfaces of the scratched culture dishes with the Rat Airway PrimaCell™ Culture Dish Coating Solution, and aspirate the surplus solution as before.
8. Cut the moist ischemia-reversed fragments into 7 × 7-mm pieces, and explant the pieces epithelium side up on the scratched areas. Incubate the pieces at room temperature without medium for 3-5 min, as before.
9. Add 10 ml of Rat Complete Airway PrimaCell™ Growth Medium containg FibrOut™ to each dish, and incubate explants at 37℃ in a humidified 5% air/CO_2 incubator. Replace spent medium with fresh medium every 3 to 4 d.

After 8 to 11 d of incubation, when epithelial cell outgrowths radiate from the tissue explants more than 0.5 cm, transfer the explants to new culture dishes scratched and coated with Rat Airway PrimaCell™ Culture Dish Coating Solution to produce new outgrowths of epithelial cells. This step can be repeated up to seven times with high yields of NHBE cells.

2.3.3 Procedures for Dissociation and Subculture of Bronchial Epithelium

10. Incubate the postexplant outgrowth cultures in Rat Complete Airway PrimaCell™ Growth Medium for an additional 2 to 4 d before trypsinizing (with the trypsin/EGTA/PVP solution) for subculture or for experimental use.
11. Aspirate the medium and bathe the culture two times with Rat Airway Tissue Washing

Medium.

12. Remove the Rat Airway Tissue Washing Medium and incubate the culture at room temperature in 0.5 M urea for 5 min.
13. Remove the urea solution by aspiration and bathe the cells in a minimal volume of 0.05mg/mL Tripsin and 0.5mM/ml EGTA, and incubate at room temperature until the cells float free (usually 5–10 min).
14. Resuspend the cells with Rat Airway Tissue Washing Medium and pellet by centrifugation (125 g for 5 min).

Resuspend the cells with Rat Complete Airway PrimaCell™ Growth Medium, enumerate the cells and reinoculate at the desired cell density into culture dishes that have been coated with Rat Airway PrimaCell™ Culture Dish Coating Solution.

III Cryopreservation

Both dissociated NHBE cells and bronchial tissue fragments can be cryopreserved with good viability using relatively routine procedures, as follows.

15. Suspend the tissue (0.5-cm^3 fragments) or pelleted normal rat bronchial epithelial (NHBE) cells (2–5 × 10^6) in 0.5 ml of cold 2 × Rat Complete Airway PrimaCell™ Growth Medium in a freezing vial.
16. Add 0.5 ml of DMSO freezing medium to the vial, swirl the mixture, and close the vial.
17. Transfer vial(s) to a controlled-rate freezer and freeze the cells/tissues at 1℃/min according to the manufacturer's directions.
18. Transfer the frozen vials to liquid N2 for storage.
19. Resurrect the cells/tissues by rapidly warming the vial to 37℃.
20. Swab the vial with 70% alcohol, open it, and transfer the cells to 10 ml of Rat Complete Airway PrimaCell™ Growth Medium.
21. Pellet the cells, resuspend in Rat Complete Airway PrimaCell™ Growth Medium, and inoculate into Rat Airway PrimaCell™ Culture Dish Coating Solution-coated culture dishes containing Rat Complete Airway PrimaCell™ Growth Medium.

IV Characterization

NHBE are identified by several criteria based on the characteristic structure and function of normal epithelium. In explant outgrowth cultures, polygon-shaped epithelial cells grow out from the periphery of the explant onto the culture dish before the fusiform fibroblast cells. Cytochemical stains can further distinguish epithelial cells and fibroblasts in primary cultures. Squamous epithelial cells stain positively with the immunoperoxidase method for prekeratin and keratin, whereas fibroblasts stain negatively. In some cultures, epithelial cells will stain positively with alcian blue-PAS before and after treatment with diastase, indicating the production of acidic and neutral mucopolysaccharides, two components of mucus. Most cultures, however, will not exhibit positive staining for mucous substances. Epithelial cells in first-passage cultures are similar in appearance to those in explant outgrowth cultures, and they continue to react positively with the keratin antibodies. Scanning electron microscopy of subcultures shows colonies composed of prolate spherical cells covered with varying numbers of microvilli and apposed cell borders. In addition, rat bronchial epithelial cells do not form colonies in soft agar when plated at a density of 100,000 per 1 ml, and chromosome analysis shows that the cells retain the normal rat karyotype (2N = 46) throughout the replicative phase. Additionally, these cells are metabolically active and capable of converting xenobiotics to DNA adducts.

V References:

1. Yim HW, Slebos RJ, Randell SH, Umbach DM, Parsons AM, Rivera MP, Detterbeck FC, Taylor JA. *Smoking is associated with increased telomerase activity in short-term cultures of Rat bronchial epithelial cells.* Cancer Lett. 2006 Mar 3;
2. Doherty GM, Christie SN, Skibinski G, Puddicombe SM, Warke TJ, de Courcey F, Cross AL, Lyons JD, Ennis M, Shields MD, Heaney LG. *Non-bronchoscopic sampling and culture of bronchial epithelial cells in children.* Clin Exp Allergy. 2003 Sep; 33(9):1221-5.
3. Mattinger C, Nyugen T, Schafer D, Hormann K. *Evaluation of serum-free culture conditions for primary Rat nasal epithelial cells.* Int J Hyg Environ Health. 2002 Apr; 205(3):235-8.
4. de Jong PM, van Sterkenburg MA, Kempenaar JA, Dijkman JH, Ponec M. *Serial culturing of Rat bronchial epithelial cells derived from biopsies.* In Vitro Cell Dev Biol Anim. 1993 May; 29A(5):379-87.
5. Robbins RA, Koyama S, Spurzem JR, Rickard KA, Nelson KJ, Gossman GL, Thiele GM, Rennard SI. *Modulation of neutrophil and mononuclear cell adherence to bronchial epithelial cells.* Am J Respir Cell Mol Biol. 1992 Jul;7(1):19-29.
6. Lechner JF, Wang Y, Siddiq F, Fugaro JM, Wali A, Lonardo F, Willey JC, Harris CC, Pass HI. *Rat lung cancer cells and tissues partially recapitulate the homeobox gene expression profile of embryonic lung.* Lung Cancer. 2002 Jul; 37(1):41-7.
7. Lechnewr JF, & LeVeck MA. *A serum free method for culturing normal Rat bronchial epithelial cells at clonal density.* J. Tissue Cult. Methods 9: 43-48.

Rat Airway PrimaCell™: Bronchial and Tracheal Epithelium

Rat Airway Primary Cell Culture

Cat No.	Description	Qt.	Price
2-81020	**Rat Airway PrimaCell™ System**	**kit**	**$499**
7-61011	Bronchial and Tracheal Fibroblast Growth Inhibitors (for 500 ml medium), Airway FibrOut™, Rat	1 ml	$146
9-41001	Bronchial and Tracheal PrimaCell™ Basal Medium, Rat	500 ml	$61
9-31011	Bronchial and Tracheal PrimaCell™ Growth Medium Supplements (for 500 ml medium), Rat	set	$140
4-21011	Rat Bronchial and Tracheal Tissue Disassociation system	ea	$128
9-91001	Rat Airway Tissue Preparation Buffer Set	ea	$75

Rat Airway Primary Cell Characterization

6-22811	**Rat Bronchial and Tracheal Epithelium Primarker™ Kit**	**kit**	**$220**
6-22812	Rat Bronchial and Tracheal Epithelium Primarker™ antibody set	set	$180
6-22813	Rat Bronchial and Tracheal Epithelium Primarker™ buffer system	set	$90

Rat Bone Marrow PrimaCell™: Hematopoietic Cells

(Cat No. 2-82022)

I. General Description:

This protocol is developed for Hematopoietic Cells from newborn or adult Rat Bone marrow with Rat Bone marrow PrimaCell™ system (Cat No. 2-82022). This system provides an optimal condition of tissue dissociation system, Bone Marrow OptiTDS™ that yields 3-4 times of single cells more than most of the tissue dissociation protocols published in the literature. In addition, this system ensures a high viability of the target cells with improved gradient contained in the culture medium. With CHI's proprietary fibroblast inhibitory system, FibrOut™, cells are growing with contamination of minimized amount of the non- Hematopoietic Cells.

Marrow is aspirated into growth medium and maintained as an adherent cell multilayer for at least 12, and up to 30, weeks. Stem cells and maturing and mature myeloid cells are released from the adherent layer into the growth medium. Granulocyte/macrophage progenitor cells can be assayed in soft gels..

Rat Bone marrow PrimaCell™ system applies to all type tissue samples from rat at all age though younger tissue samples are recommended for yielding maximum amount of viable target cells. However, tissue samples contain pathological organism (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Rat Bone marrow PrimaCell™ System

- **Rat Bone marrow PrimaCell™ system, Bone marrow OptiTDS™,** (2 x 1 ml) --- *A mixture of collagenase, collagenase I, collagenase I and Rat bone marrow OptiTDS™ Reconstitution Buffer.*
- **Rat bone marrow OptiTDS™ Digestion Buffer**, (2 × 9 ml).
- **Rat Bone Marrow Fibroblast Growth Inhibitors, Bone Marrow FibrOut™** (5 × 200 µl) --- *A mixture collagenase and gentamycin*.
- **Rat Bone Marrow Tissue Washing Medium**, (1 × 100 ml) --- *HBSS with 5% BSA, 200 U/ml of penicillin, 200 µg/ml of streptomycin, and 50 µg/ml of gentamycin*.
- **Rat Bone Marrow PrimaCell™ Basal Culture Medium,** (5 × 100 ml) --- *A modified Fischer's Medium.*
- **Rat Bone Marrow PrimaCell™ Medium Supplements**, (5 × 1.0 ml) --- *A mixture of Hydrocortisone, Sodium Succinate, penicillin, streptomycin, and NaHCO3*.
- **Rat Bone Marrow PrimaCell™ Serum**, (10 × 10 ml) --- *Highly purified special-treated Horse Serum.*

1.2 Required Materials but not provided:

- 70% sterile ethanol
- Plastic tissue culture dishes (60 and 100 mm)
- Surgical scissors
- Half-curved microdissecting forceps
- Pipettes (10 and 25 ml)

- Gloves sterilized with autoclave (rat tissue can be contaminated with biologically hazardous agents)
- Rocker platform

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, collagen-coated culture plate (Corning, NY) must be used in this experiment.

2.2 Surgical specimens

Bone Marrow: Hematopoietic cells are from rat bone marrow in femur. One femur contains 2-2.5 $\times 10^7$ nucleated cells.

1. Kill the rat by cervical dislocation or institutional procedures.
2. Wet the fur with 70% alcohol and remove both femurs. Collect femurs in a Petri dish on ice containing Rat Bone Marrow Tissue Washing Medium.
3. In a laminar flow hood:
   (a) Clean off any remaining muscle tissue using gauze swabs.
   (b) Hold the femur with forceps and cut off the knee end. The 21G needle should fit snugly into the bone cavity.
   (c) Cut off the other end of the femur as close to the end as possible.
   (d) Keep the Rat Bone Marrow Tissue Washing Medium soaked femurs in ice till tissue dissociation procedure.

2.3 Tissue Preparation and Dissociation

2.3.1 Rat Bone Marrow OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Rat Bone Marrow Tissue Dissociation System, OptiTDS™, is suited for optimal dissociation of normal adult and newborn Bone marrow samples to yield maximum number of single primary cells of colonic tissues.

2.3.2 Enzyme Compositions

- Collagenase: from *Clostridium Histolyticum*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase II: from *Clostridium Histolyticum*

2.3.3 System Components

- Rat Bone Marrow Tissue Dissociation System, OptiTDS™, 2 x 1.0 ml.
- Rat Bone Marrow OptiTDS™ Digestion Buffer, 2 × 9 ml.

2.3.4 Procedures For Tissue Preparation and Dissociation

4. Add 1.0 ml of Rat Bone Marrow Tissue Dissociation System, Bone OptiTDS™ to one vial of Rat Bone OptiTDS™ Digestion Buffer (9 ml). Warm the diluted enzyme working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 5-7 g tissue samples per 10 ml diluted enzyme working solutions.
5. Insert the tip of the bone into a bottle of 10 ml diluted enzyme working solutions, and aspirate and depress the syringe plunger several times until all the bone marrow is flushed out of the femur.
6. Repeat steps 5 with the other bones.

2.3.5 Storage:

Reconstituted tissue dissociation systems should occur before use and can only be stored for 2-4 days at 4 ^{o}C. For long-term use, it should be aliquoted and stored at -20 ^{o}C. Avoid repeated freeze-thaw cycles.

2.4 Culture of Primary Rat Keratinocytes

2.4.1 Medium Preparation.

Thaw out the Rat Bone marrow PrimaCell™ Medium Supplements and Rat Bone marrow PrimaCell™ I Serum on ice. To prepare complete Rat Bone Marrow Medium, add one vial of Rat Bone marrow PrimaCell™ I Medium Supplements and 10 ml Rat Bone marrow PrimaCell™ I Serum to every 100 ml Rat Bone Marrow PrimaCell™ Basal Culture Medium; adding one vial of Rat Bone marrow Fibroblast Growth Inhibitors, Bone Marrow FibrOut™ to Complete Rat Bone Marrow Medium makes Complete Rat Bone Marrow Medium. Mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use.
Important: Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination.

2.4.2 Primary Cell Culture

7. Disperse the marrow to a suspension by pipetting the large marrow cores through a 10-ml pipette. There is no need to disaggregate small clumps of cells.
8. Centrifuge cells for 2 min at 580 g at room temperature and remove the supernatant.
9. Dispense 10-ml aliquots of the cell suspension into 25-cm^2 tissue culture flasks by using Complete Rat Bone Marrow Medium/FibrOut, swirling the suspension often to ensure an even distribution of the cells in the 10 cultures.
10. Gas the flasks with 5% CO_2 in air and tighten the caps.
11. Incubate the cultures horizontally at 33℃.
12. Feed the cultures weekly:
    a) Agitate the flasks gently to suspend the loosely adherent cells.
    b) Remove 5 ml of growth medium, including the suspension cells; take care not to touch the layer of adherent cells with the pipette.
    c) Add 5 ml of fresh Complete Rat Bone Marrow Medium containing FibrOut™ to each flask; to avoid damage; do not dispense the medium directly onto the adherent layer.
    d) Gas the cultures and replace them in the incubator.

2.4.3 Subculture and Propagation

13. Gently rinse the culture dish twice with 0.02% (0.7 mM) EDTA.
14. Add 3 ml of 0.25% trypsin/0.1% (2.5 mM) EDTA, and incubate at 37℃. Examine the dish under phase microscopy every 5 min to detect cell detachment.
15. When most cells have detached, add 12 ml Complete Rat Bone Marrow Medium to inactive the trypsin activity.
16. Pipette the contents of the dish to ensure complete hematopoietic cells detachment.
17. Aspirate and centrifuge the cells for 5 min at 350 g.
18. Aspirate the supernatant, resuspend the cells in a Complete Rat Bone Marrow Medium, and replate at 2-4 × 10^4 cells per 100-mm dish.
19. Refeed the culture twice a week with Complete Rat Bone Marrow Medium.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample; the best results are reported when cells from preconfluent primary cultures are used.

20. Detach cells as for the subculture, and centrifuge at 100 g for 10 min.
21. Resuspend cells in Complete Rat Bone Marrow Medium and count.
22. Dispense aliquots of 2 × 10^6 cells/ml in Complete Rat Bone Marrow Medium with 10% glycerol into cryopreservation tubes.
23. Equilibrate at 4℃ for 1-2 h.
24. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
25. To recover cells:

d) Thaw cryotubes quickly in a 37℃ water bath.
e) Dilute cells tenfold with medium.
f) Centrifuge cells and resuspend them at an appropriate concentration in the desired Complete Rat Bone Marrow Medium, and seed culture vessel.

Rat cells can be grown in all media for 4-7 weeks and can be subcultured only 4-5 times.

IV Fibroblast Contamination

There are several techniques have been published in the literature to deal with fibroblast contamination during colorectal primary cell culture. These include: (1) Physically remove a well-isolated fibroblast colony by scraping it with a sterile blunt instrument (e.g., a cell scraper). Care has to be taken to wash the culture up to six times to remove any fibroblasts that have detached in order to prevent them from reseeding and reattaching to the flask. (2) Differential trypsinization can be attempted with the carcinomas. (3) Dispase preferentially (but not exclusively) removes the epithelium during passaging and leaves behind most of the fibroblastic cells attached to the culture vessel. During subculture, cells that have been removed with dispase can be preincubated in plastic petri dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the hematopoietic cells. This technique takes advantage of the fact that fibroblasts in general attach much more quickly to plastic than do clumps of melanocytes, so that a partial purification step is possible. (4) Reduce the concentration of serum to about 2.5-5% if there are heavy concentrations of fibroblastic cells. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and that using any or all of the preceding techniques will eventually push the cells through so many divisions that any fibroblasts will senesce.

Rat Bone marrow PrimaCell™ system includes a fibroblast elimination system, the Rat Bone marrow Fibroblast Growth Inhibitors, Bone marrow FibrOut™. It contains a mixture of D-valine, proline, collagenase and gentamycin. This system can effectively eliminate Bone marrow fibroblast contamination while has no affect on the behavior of hematopoietic cells.

V Characterization of Hematopoietic Cells

Because Hematopoietic Cells are very heterogeneous, containing many cell types, many assays have been developed in order to characterize the cell populations that are present in a sample. Upon culture of these cells, the ratio of different populations may change significantly, such that the total cell number generated is not an adequate measure of outcome. Therefore, the use of appropriate assays is critical to determine the success of particular culture technique. Histology is the first method to assay Hematopoietic Cells. These methods utilize spreads of Wright-Giemsa stained cells under oil-immersion microscopy, or automated instruments that have been developed tocarry out these differentials (counting of different cell types). It is most useful for assessing mature cell populations with large numbers and distinctive morphological features. Flow cytometry has been used extensively in the study of Hematopoietic Cells. Antibodies detecting different cell types have been developed. Because of the close relation of many cell types, combinations of antigens are often required to definitively identify a particular cell. In order to deal with rarity of stem and progenitor cells, many in vitro biological function assays have been developed, such as colony-forming unit assay (CFU) and long-term culture- initiating cell assay (LTC-IC), . Most of these assays are performed by culturing cells under defined conditions and examining their progeny, both in number and type.

VI References:

1. Koller, M.R.P., B. O.; Masters, J. R. W., *Human Cell Culture: Primary Hematopoietic Cells*. 1999: Springer.
2. Ivanovic Z, Belloc F, Faucher JL, Cipolleschi MG, Praloran V, Dello Sbarba P. *Hypoxia maintains and interleukin-3 reduces the pre-colony-forming cell potential of dividing CD34(+) murine bone marrow cells.* Exp Hematol. 2002 Jan; 30(1):67-73.
3. Rosler ES, Brandt JE, Chute J, Hoffman R. *An in vivo competitive repopulation assay for various sources of human hematopoietic stem cells.* Blood. 2000 Nov 15; 96(10):3414-21.
4. Okubo T, Matsui N, Yanai N, Obinata M. *Stroma-dependent maintenance of cytokine responsive hematopoietic progenitor cells derived from long-term bone marrow culture.* Cell Struct Funct. 2000 Apr; 25(2):133-9.
5. Ploemacher RE, Engels LJ, Mayer AE, Thies S, Neben S. *Bone morphogenetic protein 9 is a potent synergistic factor for murine hemopoietic progenitor cell generation and colony formation in serum-free cultures.* Leukemia. 1999 Mar; 13(3):428-37.

Rat Bone Marrow PrimaCell™: Bone Marrow Hematopoietic Cell

Rat Bone Marrow Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82022	**Rat Bone Marrow PrimaCell™ System**	**kit**	**$550**
7-61021	Rat Bone Marrow Fibroblast Growth Inhibitors (for 500 ml medium), Bone Marrow FibrOut™	1 ml	$152
9-41002	Rat Bone Marrowl PrimaCell™ Basal Medium	500 ml	$73
9-31021	Rat Bone Marrow Hematopoietic cells PrimaCell™ Growth Medium Supplements and serum (for 500 ml medium)	set	$140
4-21021	Rat Bone Marrow Tissue Disassociation system	ea	$140
9-91002	Rat Bone Marrow Tissue Preparation Buffer Set	ea	$75

Rat Bone Marrow Primary Cell Characterization

6-23011	**Rat Bone Marrow Hematopoietic Cell Primarker™ Kit**	**kit**	**$220**
6-23012	Rat Bone Marrow Hematopoietic Cell Primarker™ antibody set	set	$180
6-23013	Rat Bone Marrow Hematopoietic Cell Primarker™ buffer system	set	$90

Rat Bone PrimaCell™: Osteoblasts
(Cat No. 2-82021)

I. General Description

An osteoblast (from the Greek words for "bone" and "germ" or embryonic) is a mononucleate cell that is responsible for bone formation. Osteoblasts produce osteoid, which is composed mainly of Type I collagen. Osteoblasts are also responsible for mineralization of the osteoid matrix. Primary cultures of osteoblasts are advantageous for studies of bone cell metabolism and differentiation because they retain a normal genotype.

Although bone is mechanically difficult to handle, thin slices treated extensively with Tissue Preparation Buffer subsequently digested in bone tissue dissociation system provide in the kit, give rise to cultures of osteoblasts that have some functional characteristics of the tissue. This protocol is developed for attachment and growth of normal Rat Bone cells from newborn or adult Rat Bone with Rat Bone PrimaCell™ system (Cat No. 2-82002). This system provides an optimal condition of tissue dissociation system, Bone OptiTDS™ that yields 4-5 times of single cells more than most of the tissue dissociation protocols published in the literature. In addition, this system ensures a high viability of the target cells with improved gradient contained in the culture medium. With CHI's proprietary fibroblast inhibitory system, FibrOut™, cells are growing with contamination of minimized amount of the non-epithelial cells.

200

The Rat Bone PrimaCell™ system is suited for culturing Osteoblasts from bone tissues of new born rats (4-7-weeks age).

1.1 The Rat Bone PrimaCell™ system include:

- **Rat Bone Tissue Dissociation System, Bone OptiTDS™** (2 × 1.0 ml) --- *collagenase I with a modified reconstitution buffer*.
- **Rat Bone OptiTDS™ Digestion Buffer,** (2 × 9 ml)
- **Bone Fibroblast Growth Inhibitors, Bone FibrOut™** (5 × 1.0 ml) --- *A mixture of cis-OH-proline, collagenase, Gentamycin.*
- **Rat Bone Tissue Washing Medium**, (1 × 100 ml) --- *A modified HBSS with 5% BSA, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Rat Bone Osteoblasts PrimaCell™ Medium Supplements With Serum** (5 × 1.0 ml): *A mixture of highly purified and special treated serum and proprietary osteoblast supplements.*
- **Basal Bone Osteoblasts PrimaCell™ Basal Culture Medium (**5 × 100 ml) --- A modified Ham's F12 culture medium.
- **Rat Bone Tissue Preparation Buffer** (1 x 100 ml) --- A mixture of EDTA/EGTA solution.

1.2 Required Materials but not provided

- Centrifuge tube, 50 ml
- Nylon gauze cell strainer (BD Bioscience)

- Petri dishes, collagenase -I coated, 100 mm (Corning, NY)
- Scalpels, curved forceps
- 70% ethanol, sterile
- PBSA (PBS containing 10% BSA), sterile.
- 9-cm Petri dishes
- 25- or 75-cm2 flasks (Corning, Falcon, Nunc)

II. Procedures

2.1 Material Preparation

Bone culture suffers from the inherent problem that the hard nature of the tissue makes manipulation difficult. However, conventional primary explant culture or digestion in collagenase I release cells that may be passaged in the usual way. All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, culture plate (Corning, NY) must be coated with collagen-I.

2.2 Surgical specimens

Rat bone: Rat primary osteoblasts is isolated from bone samples from new born rats (4-7-week age) under sterile conditions. Each rat bone yield large number of cells (6-10 × 10^6/1 g tissue specimen), with a 30-40% plating efficiency.

1. The bone specimens for culture should be immediately placed in a 100-mm tissue culture dish containing 5-10 ml of Rat Bone Tissue Washing Medium.
2. Wash trabecular bone specimens repeatedly with Rat Bone Tissue Washing Medium to remove the fat and blood cells. The trabeculae are excised with scalpel and forceps under sterile conditions.
3. After collecting as much bone as possible, wash the remaining blood and fat cells away by rinsing the specimens three times with Rat Bone Tissue Washing Medium and cut into 2-5 mm fragments.
4. Wash the cut trabeculae with Rat Bone Tissue Washing Medium.
5. Discard the Rat Bone Tissue Washing Medium and incubate tissue specimens with 5-10 ml 70% ethanol for 1 min, followed by inoculating with 5-10 ml fresh Rat Bone Tissue Preparation Buffer for 5 min, and finally washing the bone specimens in 5-10 ml fresh Rat Bone Tissue Washing Medium.

2.3 Tissue Preparation and Dissociation

2.3.1 Rat Bone OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Rat Bone Tissue Dissociation System, Bone OptiTDS™, is suited for optimal dissociation of normal adult and newborn Bone biopsies samples to yield maximum number of single primary cells of colonic tissues.

2.3.2 Enzyme Compositions

- Collagenase I: from *Clostridium Histolyticum*

2.3.3 System Components

- Rat Bone Tissue Dissociation System, Bone OptiTDS™ (2 × 1ml)
- Rat Bone OptiTDS™ Digestion Buffer, (2 × 9 ml)

2.3.4 Procedures For Tissue Dissociation

6. Prepare fresh enzyme working solutions: to each vial of Rat Bone Tissue Dissociation System, Bone OptiTDS™, add 1.0 ml of the Rat Bone OptiTDS™ Reconstitution Buffer, Mix well.
7. Add 1.0 ml of the fresh enzyme working solution to one vial of Rat Bone OptiTDS™ Digestion Buffer (9 ml). Warm the diluted enzyme working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 5-7 mg tissue samples per 10 ml diluted enzyme working solutions.
8. Place the pieces of bone in a small sterile bottle with a magnetic stirrer, and add 4 ml of digestion solution. (This amount should cover the bone specimens.)
9. Stir the solution containing bone fragments at room temperature for 45 min.
10. Remove the suspension of released cells and discard it, since these cells are most likely to contain fibroblasts.
11. Add a second aliquot of 4 ml of digestion solution to the bone fragments, and stir the mixture at room temperature for 30 min.
12. If necessary, the disassociation step is repeated several more times. With large amounts of bone, the digestion period can be increased to 1-3 h. .

2.3.5 Storage:

Reconstituted tissue dissociation systems should occur before use and can only be stored for 2-4 days at 4 ^{o}C. For long-term use, it should be aliquoted and stored at -20 ^{o}C. Avoid repeated freeze-thaw cycles.

2.4 Culture of Primary Rat Osteoblasts

2.4.1 Medium Preparation.

Thaw out Rat Bone PrimaCell™ Basal Culture Medium, Rat Bone Osteoblasts PrimaCell™ Medium Supplements With Serum, and Rat Bone Fibroblast Growth Inhibitors, Bone FibrOut™ on ice.

Rat Complete Bone PrimaCell™ Culture Medium: To every 100 ml Rat Bone PrimaCell™ Basal Culture Medium, add 10 ml Rat Bone Osteoblasts PrimaCell™ Medium Supplements With Serum, mix thoroughly and warm the complete medium at a 37 ^{o}C water bath for 10 min prior use.

Rat Complete Bone PrimaCell™ Culture Medium containing FibrOut™: To every 100 ml Rat Bone PrimaCell™ Basal Culture Medium, add 10 ml Rat Bone Osteoblasts PrimaCell™ Medium Supplements With Serum, one vial of Rat Bone Fibroblast Growth Inhibitors, Bone FibrOut™, mix thoroughly and warm the complete medium at a 37 ^{o}C water bath for 10 min prior use.
Important: Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination.

2.4.2 Primary Cell Isolation and Culture

13. Collect the digestion solution from bone fragments, and centrifuge it for 2 min at 580 g at room temperature.
14. After removing the supernatant, suspend the cells in 4 ml of Rat Complete Bone PrimaCell™ Culture Medium containing FibrOut™, and count the cells.
15. Centrifuge the suspension at 580 g for 10 min, and resuspend the cells in 4 ml of Rat Complete Bone PrimaCell™ Culture Medium containing FibrOut™. This suspension will become the inoculums.
16. Preincubate 75-cm^2 flasks for 20 min with 8 ml of Rat Complete Bone PrimaCell™ Culture Medium containing FibrOut™ to equilibrate the tissue for digestion procedures.
17. Remove the preincubation solution and add 2 ml of Rat Complete Bone PrimaCell™ Culture Medium containing FibrOut™.
18. Add 4 ml of medium containing the cell suspension. The inoculums should contain 6,000-10,000 cells per cm^2 of surface area.
19. Finally, add another 6 ml of Rat Complete Bone PrimaCell™ Culture Medium containing FibrOut™, to give a total volume of 12 ml.
20. In the interim, add an additional 4 ml of digestion solution to the remaining pieces of bone, and repeat the digestion for 30 min. The released cells are harvested, and, if necessary, the digestion step is repeated several more times. With large amounts of bone, the digestion period can be increased to 1-3 h. Cell counts are performed after each digestion period, and the released cells are used to inoculate a different flask.

2.4.3 Subculture

21. Remove the pieces of explant.
22. Remove the medium and rinse the cell layer with Rat Bone Tissue Washing Medium, 0.2 ml/ cm^2.
23. Add trypsin to the flask, 0.1 ml/ cm^2, and incubate at 37˚C until the cells have detached and separated from one another. Monitor cell detachment and separation on the microscope. In general, a10-min incubation is sufficient.
24. Transfer the released cells to a centrifuge tube with an equal volume of Rat Complete Bone PrimaCell™ Culture Medium.
25. Centrifuge the cells at 600 g for 5 min.
26. Discard the supernatant, and resuspend the cells in Complete Bone PrimaCell™ Culture Medium by gentle, repeated pipetting.
27. Set one aliquot aside for the determination.
28. Inoculate the remaining cells into culture flasks or wells that have previously been equilibrated with medium. The cells should reattach within 24 h.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample; the best results are reported when cells from preconfluent primary cultures are used.

29. Detach cells as for the subculture, and centrifuge at 100 g for 10 min.
30. Resuspend cells in Rat Complete Bone PrimaCell™ Culture Medium and count.
31. Dispense aliquots of 2×10^6 cells/ml in Rat Complete Bone PrimaCell™ Culture Medium with 10% glycerol into cryopreservation tubes.

32. Equilibrate at 4℃ for 1-2 h.
33. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
34. To recover cells:
    a) Thaw cryotubes quickly in a 37℃ water bath.
    b) Dilute cells tenfold with medium.
    c) Centrifuge cells and resuspend them at an appropriate concentration in the desired Rat Complete Bone PrimaCell™ Culture Medium, and seed culture vessel.

IV Fibroblast Contamination

There are several techniques have been published in the literature to deal with fibroblast contamination during colorectal primary cell culture. These include: (1) Physically remove a well-isolated fibroblast colony by scraping it with a sterile blunt instrument (e.g., a cell scraper). Care has to be taken to wash the culture up to six times to remove any fibroblasts that have detached in order to prevent them from reseeding and reattaching to the flask. (2) Differential trypsinization can be attempted with the carcinomas. (3) Dispase preferentially (but not exclusively) removes the epithelium during passaging and leaves behind most of the fibroblastic cells attached to the culture vessel. During subculture, cells that have been removed with dispase can be preincubated in plastic petri dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the osteoblasts. (4) Reduce the concentration of serum to about 2.5-5% if there are heavy concentrations of fibroblastic cells. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and that using any or all of the preceding techniques will eventually push the cells through so many divisions that any fibroblasts will senesce.

Rat Bone PrimaCell™ system includes a fibroblast elimination system, the Rat Bone Fibroblast Growth Inhibitors, Bone marrow FibrOut™. It contains a mixture of cis-OH-proline, collagenase, and gentamycin. This system can effectively eliminate Bone fibroblast contamination while has not affect on the behavior of osteoblasts.

V Characterization of Osteoblasts

In order to characterize the rat osteoblasts, various analysis need to be performed. Due to presence of large amount of rough endoplasmic reticulum, cytoplasm of osteoblasts appears to be basophilic via normal HE stain. A large Golgi apparatus is also present in the centre, and the nucleus is spherical and large. These include measurement of cell growth, alkaline phosphatase, and response to PTH by measuring cyclic adenosine monophosphate (cAMP) production. Several genes, such as alkaline phosphatase, osteocalcin, and Cbfa1/Osf2, are known to be regulated during osteoblastic differentiation and are commonly used as “osteoblast markers” for *in vitro* or in *vivo* studies. In addition, osteoblasts can be stained for procollagen type 1 (PICP), alpha 1(I) procollagen, Bone Gla Protein (BGP), Bone Sialoprotein (BSP), etc. In some case, one can determine the appearance of osteoblasts by immunohistochemical staining of AP and collagen I (Figure 8 and Figure 9).

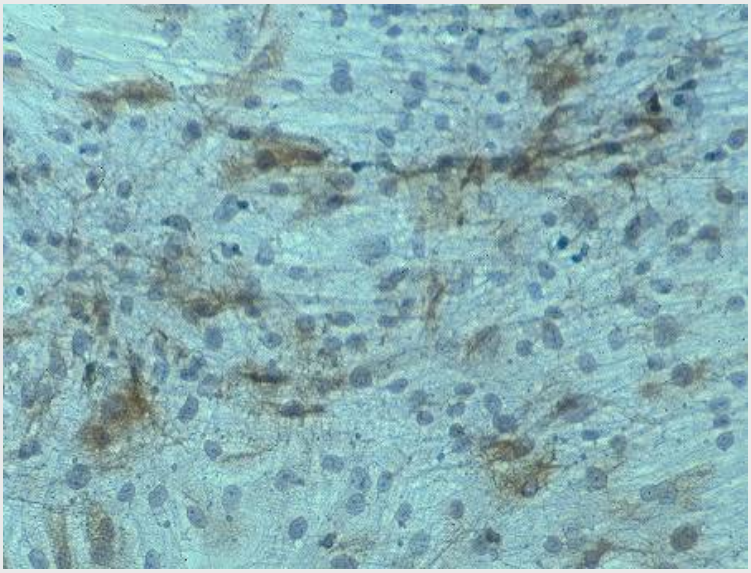


Figure 8: Histochemistry of AP in osteoblast cultures from the maxilla. Shown here are the blue / purple staining of AP positive cells (x300)(Günter Lauer, 2001, ref. 2).

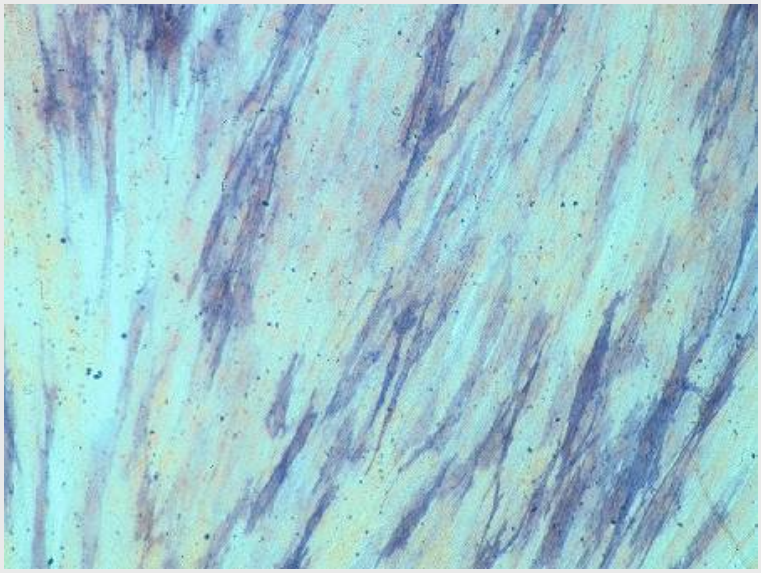


Figure 9: **Immunohistochemistry of collagen I in osteoblast cultures from the maxilla** (× 150)(Günter Lauer, 2001, ref. 2).

VI References:

1. Garcia, T., Roman-Roman, S., Jackson, A., Theilhaber, J., Connolly, T., Spinella-Jaegle, S., Kawai, S., Courtois, B., Bushnell, S., Auberval, M*., et al.* (2002). *Behavior of osteoblast, adipocyte, and myoblast markers in genome-wide expression analysis of rat calvaria primary osteoblasts in vitro.* Bone *31*, 205-211.
2. Günter Lauer, R.S. (2001). *Isolation of human osteoblasts and in vitro amplification for tissue engineering and subsequent bone repair.* Int Poster J Dent Oral Med.
3. Ongphiphadhanakul, B., Jenis, L.G., Braverman, L.E., Alex, S., Stein, G.S., Lian, J.B., and Baran, D.T. (1993). *Etidronate inhibits the thyroid hormone-induced bone loss in rats assessed by bone mineral density and messenger ribonucleic acid markers of osteoblast and osteoclast function.* Endocrinology *133*, 2502-2507.
4. Zhou, H., Choong, P., McCarthy, R., Chou, S.T., Martin, T.J., and Ng, K.W. (1994). *In situ hybridization to show sequential expression of osteoblast gene markers during bone formation in vivo.* J Bone Miner Res *9*, 1489-1499.

Rat Bone PrimaCell™: Osteoblasts

Rat Bone Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82021	**Rat Bone PrimaCell™ System**	**kit**	**$**499
7-61151	Rat Bone Fibroblast Growth Inhibitors, Bone FibrOut™ (for 500 ml medium)	1 ml	$158
9-41015	Rat Bone Osteoblasts PrimaCell™ Basal Culture Medium	500 ml	$73
9-31151	Rat Bone Osteoblasts PrimaCell™ Medium Supplements With Serum (for 500 ml medium)	set	$140
4-21151	Rat Bone Tissue Dissociation System, Bone OptiTDS™	ea	$134
9-91015	Rat Bone Osteoblasts Tissue Preparation Buffer Set	ea	$75

Rat Bone Primary Cell Characterization

6-24011	**Rat Osteoblast Primarker™ Kit**	**kit**	**$220**
6-24012	Rat Osteoblast Primarker™ antibody set	set	$180
6-24013	Rat Osteoblast Primarker™ buffer system	set	$90

Rat Brain PrimaCell™ I: Cerebellar Granule Cells
(Cat No. 2-82024)

I. General Description

Neurons are extremely fastidious in their choice of substrate. Nurons usually grow very poorly on untreated glass or plastic surfaces, but demonstrate neurite outgrowth on collagen and poly-D-lysine. Neurite outgrowth is stimulated by the polypeptide Nerve Growth Factor (NGF) and several other unique growth factors secreted by glial cells. Most tissue culture conditions used for neurons do not support neuronal proliferation, even with neurons isolated from embryonic stages where evidence of mitosis was observed *in vivo*. Recently, however, breakthroughs in techniques have identified methods of propagating neurons in ways which allow proliferation *in vitro* and relocalization of those neurons *in vivo*.

Cerebellar granule cells in culture provide a well characterized neuronal cell population that is well suited for morphological and biochemical studies of cellular and molecular correlates of mechanisms of survival/apoptosis and neurodegeneration/neuroprotection. Cerebellar granule cell is one of the most reliable models for the study of neural development, function and pathology. The Rat Brain PrimaCell kit (Cat No. 2-82024) allows the isolation of cerebellar granule cells from adult rats. Non-neuronal cells are prevented from interfering with the target cells by the addition of Brain Fibroblast Growth Inhibitors, FibrOut™ to the culture medium.

1.1 Components of the Rat Brain PrimaCell™ I System

- **Rat Brain Tissue Dissociation System, Brain OptiTDS™** (2 × 1 ml) --- *A mi×ture of collagenase, collagenase I, Trypsin and Rat Brain OptiTDS™ Reconstitution Buffer.*
- **Rat Brain OptiTDS™ Digestion Buffer,** (2 × 9 ml).
- **Rat Brain Fibroblast Growth Inhibitors, Brain FibrOut™** --- Cervi× FibrOut™ (5 x 200 µl) --- *A mi×ture of cis-OH-proline, collagenase and gentamycin.*
- **Rat Brain Cerebellar Granule Cells PrimaCell™ Basal Culture Medium,** (5 x 100 ml) --- A *Modified DMEM medium*.
- **Rat Brain Cerebellar Granule Cells PrimaCell™ Medium Supplements,** (5 × 1 ml): *Glucose, L-glutamine, KCl, Insulin, P-aminobenzoic acid, and Gentamycin.*
- **Rat Brain PrimaCell™ Serum,** (5 x 10 ml) --- *A modified heat inactivated fetal calf serum.*
- **Rat Brain Tissue Washing Medium,** (1 x 100 ml): *HBSS with 200U/ml penicillin, 200 µg/ml streptomycin, 50 µg/ml gentamycin and 5% BSA.*
- **Brain PrimaCell™ Culture Dish Coating Solution,** (1 x 100 ml) --- *Poly-L-lysine solution.*

1.2 Required materials but NOT included

- PBS
- Water bath
- Tissue culture dishes
- 35-mm tissue culture Petri dishes
- Scalpels, scissors, and forceps
- Pasteur pipettes and 10-ml pipettes

- Test tubes, 12 and 50 ml

1.3 Siliconization of Pasteur pipettes

- Prepare a diluted Apuasil solution provided in this kit.
- Dip the pipettes into the solution and flush out the insides of the pipettes.
- Air-dry the pipettes for 24 h, or dry for several minutes at 100° C.
- Sterilize the pipettes by dry heat.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, fresh **Brain PrimaCell™ Culture Dish Coating Solution**-coated plate or culture dishes are recommended (see below for treatment of culture dishes).

Treatment of Culture Dishes:

.

1. Add 1 ml of **Rat Brain PrimaCell™ Culture Dish Coating Solution** to each of the 35-mm tissue culture dishes.
2. Incubate the tissue culture dishes with the **Rat Brain PrimaCell™ Culture Dish Coating Solution** for a minimum of 2 hrs, and aspirate.
3. Air dry tissue culture plates in a hood.

2.2 Procedure Outline

The cerebella from four to eight neonatal rats are cut into small cubes and incubated with the Rat Brain Tissue Dissociation System, Brain OptiTDS™. The provided Rat Brain OptiTDS™ is diluted 1:10 in the provided Rat Brain OptiTDS™ Digestion Buffer and the diluted Rat Brain OptiTDS™ is incubated with brain tissue at a ratio of 10 ml diluted Rat Brain OptiTDS™ solution per 3-5 g tissue sample for 30 min at 37 °C. The cell suspension is seeded in coated culture dishes or flasks prepared above.

2.3 Rat Cerebella Tissue Preparation

Rat Brain: Brain from 7-8 day rats yield a large number of cells (5-10 × 10^6), with a 30-40% plating efficiency. Rats are sacrificed by CO_2 narcosis or a method that is approved by the home institution. Carefully remove the skin and dissect out the area containing the cerebella.

4 Dissect out the cerebella aseptically and place in Rat Brain Tissue Washing Medium at room temperature.

5 Mince the tissue using scalpels into small cubes measuring approximately 0.5 mm^3.

6 Transfer the minced tissue into 15 ml conical tubes and wash the tissue three times with Rat Brain Tissue Washing Medium. Allow the tissue to settle to the bottom of the tubes between each washing.

2.4 Cerebella Separation and Tissue Dissociation

2.4.1 Rat Brain OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. Rat Brain Tissue Dissociation System is suited for optimal dissociation of normal rat cerebella tissues to yield maximum number of single Cerebellar Granule Cell (CGC).

2.4.2 Brain OptiTDS™ Compositions

- Collagenase: from *Clostridium Histolyticum*
- Collagenase I: from *Clostridium Histolyticum*
- Trypsin

2.4.3 System Components

- Rat Brain Tissue Dissociation System, Brain OptiTDS™, (2 × 1 ml).
- Rat Brain OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

7 Reconstitute the Rat Brain Tissue Dissociation System stock solution by adding 1 ml Rat Brain OptiTDS™ to 9 ml of Rat Brain OptiTDS™ Digestion Buffer, mix well. We recommend using 10 ml diluted Brain OptiTDS™ solution per 3-5 g of brain tissue and follow by incubating in a water bath for 30 min at 37° C.

8 Disaggregate the tissue by trituration through a siliconized Pasteur pipette - until a single-cell suspension is obtained.

2.7 Cerebellar Granule Cell Isolation and Culture

2.6.1 Medium Preparation

Thaw out Rat Brain Cerebellar Granule Cells PrimaCell™ Medium Supplements, Rat Brain PrimaCell™ Serum, and Rat Brain Fibroblast Growth Inhibitors, Brain FibrOut™ on ice.

Rat Complete Brain PrimaCell™ I Culture Medium: To every 100 ml Rat Brain PrimaCell™ Basal Culture Medium, add 10 ml Rat Brain Cerebellar Granule Cells PrimaCell™ Medium Supplements, 10 ml Rat Brain PrimaCell™ Serum, mix thoroughly and warm the complete medium at a 37 °C water bath for 10 min prior use.

Rat Complete Brain PrimaCell™ I Culture Medium containing FibrOut™: To every 100 ml Rat Brain PrimaCell™ Basal Culture Medium, add 10 ml Rat Brain Cerebellar Granule Cells PrimaCell™ Medium Supplements, 10 ml Rat Brain PrimaCell™ Serum, and one vial of Rat Brain Fibroblast Growth Inhibitors, Brain FibrOut™, mix thoroughly and warm the complete medium at a 37 °C water bath for 10 min prior use. **Important:** Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination.

9 Once all cells have been separated, allow the cell suspension to remain in the 50 ml conical for 3-5 min, allowing the small clumps of tissue to settle to the bottom of the tube. Carefully remove these clumps with a Pasteur pipette.
10 Centrifuge the single-cell suspension at 200 g for 5 min, and carefully aspirate off the supernatant.
11 Re-suspend the pellet in Complete Brain Medium (as described in step 5), and seed the cells at a concentration of 2.5-3.0 × 10^6 cell/dish on previously treated culture plates.
12 After 2-4 days (best results are usually obtained after 2 days), continue to culture the cells with Complete Media containing the Rat Brain Fibroblast Growth Inhibitors, Brain FibrOut™ for 24 h. FibrOut™ is used at 1:500 dilution in Complete Media.
13 After 24-48 hrs or culturing, switch to Complete Brain Medium without FibrOut™.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample; the best results are reported when cells from preconfluent primary cultures are used.
14 Detach cells as for the subculture, and centrifuge at 100 g for 10 min.
15 Resuspend cells in Rat Complete Brain PrimaCell™ I Culture Medium and count.
16 Dispense aliquots of 2 × 10^6 cells/ml in Rat Complete Brain PrimaCell™ I Culture Medium with 10% glycerol into cryopreservation tubes.
17 Equilibrate at 4℃ for 1-2 h.
18 Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
19 To recover cells:
a) Thaw cryotubes quickly in a 37℃ water bath.
b) Dilute cells tenfold with medium.
c) Centrifuge cells and resuspend them at an appropriate concentration in the desired Rat Complete Brain PrimaCell™ I Culture Medium, and seed culture vessel.

IV Characterization

Neurons can be identified immunologically using neuron-specific enolase antibodies or by using tetanus toxin as a neuronal marker. It is reported that alpha 6 subunit of gamma-aminobutyric type A receptors is used to detect the purity of cerebellar granule cells. Astrocyte contamination can be assessed by staining cultures with glial fibrillary acidic protein as a marker.

V References

1. Drejer J, Larsson OM, Schousboe A. 1983. Characterization of uptake and release processes for D- and L-aspartate in primary cultures of astrocytes and cerebellar granule cells. *Neurochem Res.* 8(2):231-43.
2. Kingsbury AE, Gallo V, Woodhams PL, Balazs R. 1985. Survival, morphology and adhesion properties of cerebellar interneurones cultured in chemically defined and serum-supplemented medium. *Brain Res*. 349(1-2):17-25.
3. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. 1998. Embryonic stem cell lines derived from human blastocysts.. *Science.* 282(5391):1145-7.
4. Levi-Montalcini R, Calissano P. 1979. The nerve-growth factor. *Sci Am.* 240(6):68-77.
5. Marchionni MA, Goodearl AD, Chen MS, Bermingham-McDonogh O, Kirk C, Hendricks M, Danehy F, Misumi D, Sudhalter J, Kobayashi K, et al. 1993. Glial growth factors are

alternatively spliced erbB2 ligands expressed in the nervous system. *Nature.* 362(6418):312-8.

Rat Brain PrimaCell™ I: Cerebellar Granule Cells

Rat Brain Primary Cell Culture I

Cat No.	Description	Qt.	Price
2-82024	**Rat Brain PrimaCell™ I System**	**kit**	**$499**
7-61041	Rat Brain Fibroblast Growth Inhibitors, Brain FibrOut™ (for 500 ml medium)	1 ml	$146
9-41004	Rat Brain Cerebellar Granule Cells PrimaCell™ Basal Culture Medium	500 ml	$85
9-31041	Rat Brain Cerebellar Granule Cells PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
4-21041	Rat Brain Tissue Dissociation System, Brain OptiTDS™	ea	$128
9-91004	Rat Brain Tissue I Preparation Buffer Set	ea	$75

Rat Primary Cerebellar Granule Cells Characterization

6-25011	**Rat Cerebellar Granule Cell Primarker™ Kit**	**kit**	**$220**
6-25012	Rat Cerebellar Granule Cell Primarker™ antibody set	set	$180
6-25013	Rat Cerebellar Granule Cell Primarker™ buffer system	set	$90

Rat Brain PrimaCell™ II: Olfactory Bulb Ensheathing Cells
(Cat No. 2-82025)

I. General Description

Olfactory bulb Ensheathing cells have a unique biology and thus are widely used for experiments to study the activity of oncogenes in epithelial neoplasias, and the molecular mechanisms implicated in warts and other brain associated disorders. In addition, several *in vitro* brain models have been developed that accurately mimic the epidermis making it possible to study the brain in a physiologically relevant context. While these cells are extremely useful in the laboratory they are notoriously difficult to isolate and culture. The Rat Brain PrimaCell™ II kit (Cat No. 2-82025) is designed for the successful isolation and culture of Olfactory bulb Ensheathing cells from rat brain allowing reproducible and dependable results.

1.1 Components of the Rat Brain PrimaCell™ I System

- **Rat Brain Tissue Dissociation System, Brain OptiTDS™** (2 × 1 ml) --- *A mixture of collagenase, collagenase II, Dexoyribonuclease I and Rat Brain OptiTDS™ II Reconstitution Buffer.*
- **Rat Brain OptiTDS™ Digestion Buffer,** (2 × 9 ml)
- **Rat Brain Tissue Washing Medium,** (1 × 100 ml) --- (1 × 100 ml) --- *A modified Leibowitz L-15 with 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin.*
- **Rat Brain Fibroblast Growth Inhibitors, Brain FibrOut™** ---Brain FibrOut™ (5 × 200 μl) --- *A mixture of cis-OH-proline, collagenase and gentamycin.*
- **Rat Brain PrimaCell™ II Basal Culture Medium** (5 × 100 ml) --- A *Modified mixture of DMEM and glucose*.
- **Rat Brain PrimaCell™ II Medium Supplements** (5 × 1 ml) --- *A mixture of gentamycin, BSA Pathocyte, Glutamine, Bovine pancreas insulin, Transferrin, Progesterone, Putrescine, L-thyroxine, Selenium, and 3,3'5-triiodo-L-thyronine.*
- **Rat Brain PrimaCell™ Serum** (5 × 10 ml) --- *A modified Fetal Calf serum.*
- **Rat Brain PrimaCell™ II Culture Dish Coating Solution** (3 × 10 ml): A modified buffer with Poly-L-lysine.

1.2 Required materials but NOT included:

- DMEM (Invitrogen Cat No. 10313-021)
- Fetal Bovine Serum (FBS, Sigma-Aldrich)
- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or rat fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, fresh coated plate or culture dishes are recommended (see below for treatment of culture dishes). Culture plastics and glass coverslips with diluted **Rat Brain PrimaCell™ II Culture Dish Coating Solution** (incubate for at least 1 h to a maximum of 24 h), wash the plastics and coverslips once with PBSA, and air dry them prior to use. A greater yield of cells is obtained from neonatal rat rather than older animal, although it is possible to make preparations from animals of any age.

2.2 Principle

The protocol describes a rapid method for purifying a population of glial cells from the olfactory bulb by using cell adhesion character.

2.3 Rat Brain Tissue Preparation

1. Kill the rats by decapitation.
2. Pin the head dorsal side up onto a dissecting board and spray with 70% ethanol. Dip all dissecting instruments in 70% ethanol prior to use, and shake the instruments dry.
3. Remove the skin from the head by using sharp curved scissors, and make a circular cut to remove the top of the skull, revealing the brain and the two olfactory bulbs at the tip of the nose.
4. Using curved forceps, gently release the olfactory bulbs from the brain, and place them in a Petri dish containing a 5-10 ml 70% ethanol for 1 min, followed by inoculating with 5-10 ml fresh **Rat Brain Tissue Washing Medium** for 5 min.
5. Using a sterile scalpel blade, chop the olfactory bulbs into small pieces.

2.4 Epidermal Separation and Tissue Dissociation

2.4.1 Rat Brain OptiTDS™ II

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Rat Brain Tissue Dissociation System II is suited for optimal dissociation of normal newborn rat brain tissues to yield maximum number of single Olfactory bulb Ensheathing cells cells.

2.4.2 Enzyme Compositions

- Trypsin: from *Bovine Pancreas*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase II: from *Clostridium Histolyticum*
- Dexoyribonuclease I

2.4.3 System Components

- Rat Brain Tissue Dissociation System II, Brain OptiTDS™ II, (2 × 1 ml).
- Rat Brain OptiTDS™ II Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

6. Prepare fresh enzyme working solution: Add 1ml of **Rat Brain Tissue Dissociation System, Brain OptiTDS™** to one vial of **Rat Brain OptiTDS™ Digestion Buffer** (9 ml). Warm the diluted **Rat Brain Tissue Dissociation System** working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 5 ml **Rat Brain Tissue Dissociation System** working solution.
7. Place the pieces into a small vial containing 5 ml of **Rat Brain Tissue Dissociation System** working solution.
8. Incubate the pieces of olfactory bulbs at 37℃ for 30 - 45 min.

2.8 Olfactory bulb Ensheathing Cells Isolation

9. Centrifuge the resultant suspension at 1,000 rpm for 5 min and remove the supernatant.
10. Resuspend the pelleted bulb tissue in 5 ml of **Rat Brain Tissue Washing Medium**.
11. Glial cells are fragile; therefore, to produce a single-cell suspension, the olfactory bulb tissue must be dissociated gently, taking care not to produce air bubbles. Dissociate the tissue through 19G hypodermic needle followed by a 23G hypodermic needle.
12. Add 5 ml of **Rat Brain Tissue Washing Medium**, and strain the cell mixture through a sterile cell strainer (70-100μm) into a centrifuge tube to remove debris.

2.6 Primary Olfactory bulb Ensheathing Cells Culture

2.6.1 Medium Preparation.

Thaw out **Rat Brain PrimaCell™ II Basal Culture Medium**, **Rat Brain PrimaCell™ II Medium Supplements**, and **Rat Brain Serum** on ice.

Rat Complete Brain PrimaCell™ II Culture Medium: To every 100 ml **Rat Brain PrimaCell™ II Basal Culture Medium**, add 1 ml **Rat Brain PrimaCell™ II Medium Supplements** and 10 ml **Rat Brain Serum** mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

Rat Complete Brain PrimaCell™ II Culture Medium containing FibrOut™: To every 100 ml **Rat Brain PrimaCell™ II Basal Culture Medium**, add 1 ml **Rat Brain PrimaCell™ II Medium Supplements** and 10 ml **Rat Brain Serum**, and 200 μl **Rat Brain Fibroblast Growth Inhibitors, Brain FibrOut™** mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use. (Important: *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*)

2.6.1 Primary Cell Culture

13. Spin the suspension again at 1,000 rpm for 10 min.

14. Remove the supernatant and resuspend the cells in **Rat complete Brain PrimaCell™ II Basal Culture Medium/FibrOut** yield a concentration of 8.5×10^5 cells/ml
15. The cell suspension was seeded into uncoated tissue culture plate or flask, and incubated at 37°C and 5% CO2 for 18 hours. Most of the fibroblasts attached during this first incubation period since fibroblasts will settle within one hour of seeding.
16. The supernatants from the step 15 were poured into uncoated tissue culture plate or flask, and incubated at 37°C and 5% CO2 to allow for the attachment of astrocytes. Primary olfactory bulb ensheathing cells do not attach to uncoated slides for 96–120 hours.
17. After 36 hours of incubation, most of the ensheathing cells remained in the supernatant. This supernatant was used to seed poly-L-lysine-coated tissue culture plate or flask. After seeding, the ensheathing cells attached within 48 hours and neurons do not survive this culture environment.
18. Cells were maintained in an incubator (37°C, 5% CO_2) for 8 days, and the **Rat complete Brain PrimaCell™ II Culture Medium/FibrOut** was changed every 2 days.

2.9 Subculture

Propagating Olfactory bulb Ensheathing cells in culture can be somewhat challenging, however the following methods have worked consistently in many laboratories.

19. Remove complete **Rat complete Brain PrimaCell™ II Culture Medium** and wash cells in 0.1% trypsin two times. Place just enough trypsin (0.1 % trypsin without EDTA) to moisten articular carilage layer (200-500 µl depending on size of the dish).
20. Incubate for 1-5 min at 37°C. Gently pipette cells and resuspend in **Rat complete Brain PrimaCell™ II Culture Medium** (FibrOut™ is not necessarily needed at this step) for counting and replatting on collagen coated tissue culture plates.
21. Gently pipette chondrocytes and resuspend in **Rat complete Brain PrimaCell™ II Culture Medium** for counting and replatting on tissue culture plates.

III Cryopreservation

22. Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Olfactory bulb Ensheathing cells cultures derived from preconfluent layers.
    - **(a)** Trypsinize cells as above, and centrifuge at 100 g for 10 min.
    - **(b)** Resuspend cells in **Rat complete Brain PrimaCell™ II Culture Medium** and count cells.
    - **(c)** Prepare aliquots of 2×10^6 cells/ml in **Rat complete Brain PrimaCell™ II Culture Medium** and 10% glycerol into cryovials.
    - **(d)** Equilibrate at 4℃ for 1-2 h.
    - **(e)** Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
    - **(f)** To recover cells:
        - (i) Thaw cryovials quickly in a 37℃ water bath.
        - (ii) Dilute cells tenfold with appropriate **Rat complete Brain PrimaCell™ II Culture Medium**.
        - (iii) Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in **Rat complete Brain PrimaCell™ II Culture Medium**.

IV Characterization

Primary cultures of olfactory bulb ensheathing cells obtained from rat olfactory bulb synthesize carnosine (β-alanyl histidine). Olfactory bulb ensheathing cells can be characterized based on their specific for their glia phenotype to exclude contamination by neuronal cells which were anti-neuron specific enolase (NSE) positive. The source of tissue (embryonic, new-born, adult olfactory bulb) as well as the culture conditions could influence the cell phenotype. The purity is determined by using glial fibrillary acidic protein (GFAP), galactocerebroside (GalC), p75 NGF receptor antibodies or S-100β the for the olfactory bulb ensheathing cells. Contaminating endothelial cells can be identified by antibodies against NSE. Recently, De Mello et al. determined cell purities by immunostaining with a combination of anti-S100, anti-cow S100, anti-p75, anti-GFAP, anti Thy-1, anti-O1 and anti O4.

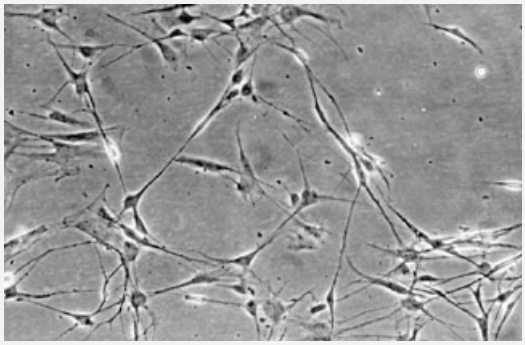


Figure 14. O1-positive staining of olfactory bulb ensheathing cells. (3).

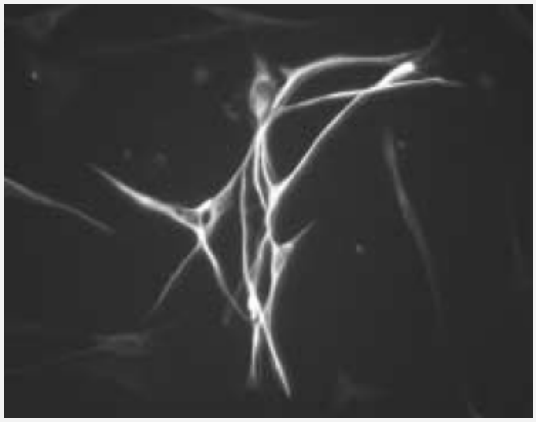


Figure 15. S100-positive staining of olfactory bulb ensheathing cells. (3).

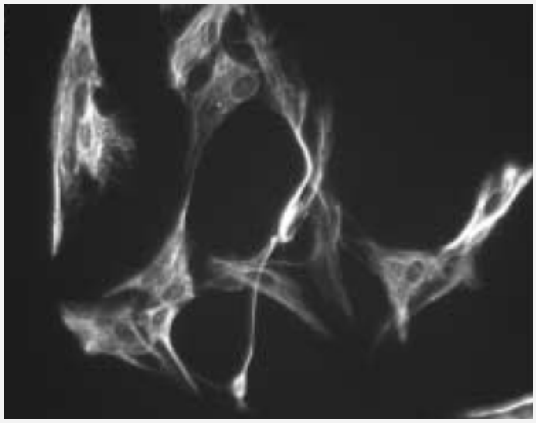


Figure 16. GFAP-positive staining of olfactory bulb ensheathing cells. (2).

V References:

1. Bakardjiev, A., *Biosynthesis of carnosine in primary cultures of rat olfactory bulb.* Neurosci Lett, 1997. **227**(2): p. 115-8.
2. Moreno-Flores, M.T., et al., *Immortalized olfactory ensheathing glia promote axonal regeneration of rat retinal ganglion neurons.* J Neurochem, 2003. **85**(4): p. 861-71.
3. Lipson, A.C., et al., *Neurotrophic properties of olfactory ensheathing glia.* Exp Neurol, 2003. **180**(2): p. 167-71.
4. De Mello, T.R., et al., *Culture conditions affect proliferative responsiveness of olfactory ensheathing glia to neuregulins.* Glia, 2007. **55**(7): p. 734-45.
5. Kumar, R., et al., *Functional differences and interactions between phenotypic subpopulations of olfactory ensheathing cells in promoting CNS axonal regeneration.* Glia, 2005. **50**(1): p. 12-20.
6. Pastrana, E., et al., *Genes associated with adult axon regeneration promoted by olfactory ensheathing cells: a new role for matrix metalloproteinase 2.* J Neurosci, 2006. **26**(20): p.

5347-59.
7. Gudino-Cabrera, G. and M. Nieto-Sampedro, *Schwann-like macroglia in adult rat brain.* Glia, 2000. **30**(1): p. 49-63.
8. Ramon-Cueto, A. and M. Nieto-Sampedro, *Glial cells from adult rat olfactory bulb: immunocytochemical properties of pure cultures of ensheathing cells.* Neuroscience, 1992. **47**(1): p. 213-20.

Rat Brain PrimaCell™ II: Olfactory Bulb Ensheathing Cells

Rat Brain Primary Cell Culture II

Cat No.	Description	Qt.	Price
2-82025	**Rat Brain PrimaCell™ II system**	**kit**	**$520**
4-21051	Rat Brain Tissue Dissociation System, Brain OptiTDS™ (for 500 ml medium)	1 ml	$134
9-41005	Rat Brain PrimaCell™ II Basal Culture Medium	500 ml	$85
9-31051	Rat Brain PrimaCell™ II Medium Supplements with Serum (for 500 ml medium)	set	$140
7-61051	Rat Brain Fibroblast Growth Inhibitors, Brain FibrOut™	ea	$170
9-91005	Rat Brain Tissue II Preparation Buffer Set	ea	$75

Rat Primary Olfactory Bulb Ensheathing Cells Characterization

6-26011	**Rat Olfactory Bulb Ensheathing Cell Primarker™ Kit**	**kit**	**$220**
6-26012	Rat Olfactory Bulb Ensheathing Cell Primarker™ antibody set	set	$180
6-26013	Rat Olfactory Bulb Ensheathing Cell Primarker™ buffer system	set	$90

Rat Breast PrimaCell™: Mammary Epithelium
(Cat No. 2-82023)

I. General Description:

This protocol is developed for attachment and growth of normal Rat Breast epithelial cells from rat mammary gland or breast tissues with Rat Breast PrimaCell™ system (Cat No. 2-96031). This system provides an optimal condition of tissue dissociation system, Breast OptiTDS™ that yields 5-7 times of single cells more than most of the tissue dissociation protocols published in the literature. In addition, this system ensures a high viability of the target cells with improved gradient contained in the culture medium. With CHI's proprietary fibroblast inhibitory system, Breast FibrOut™, cells are growing with contamination of minimized amount of the non-epithelial cells.

The preparation of tissue specimens for cell culture is usually started within 1-2 h of removal from the animal. If this is impossible, fine cutting of the tissue into small pieces (1-2 mm) with scalpels and storage overnight at 4℃ in washing medium (see below) can also prove successful.

Rat Breast PrimaCell™ system applies to all types of normal adult Rat tissue samples. Tissue samples contain pathological organism (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Rat Breast PrimaCell™ System

- **Rat Breast Tissue Dissociation System, Breast OptiTDS™,** (2 x 1 ml) --- *A mixture of collagenase I, collagenase III, collagenase IV, collagenase, and trypsin*.
- **Rat Breast OptiTDS™ Digestion Buffer**, (2 x 9 ml).
- **Rat Breast Tissue Washing Medium**, (1 x 100 ml) --- *Basal Breast PrimaCell™ Culture Medium with 5% FCS, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Rat Breast Fibroblast Growth Inhibitors, Breast FibrOut™** (5 x 200 μl) --- *A mixture of cis-OH-proline, toxin ricin, gentamycin and formulated serum substitutes*.
- **Rat Breast PrimaCell™ Basal Culture Medium**, (5 x100 ml) --- *Modified formulation based on RPMI 1640 and DMEM medium*.
- **Rat Breast PrimaCell™ Medium Supplements**, (5 x 1.0 ml) --- A mixture of *EGF, Insulin, Hydrocortisone, Cholera toxin, penicillin, and streptomycin*.
- **Rat Breast PrimaCell™ Serum**, (1 x 50 ml) --- H*eat-inactivated and special-treated Fetal Calf Serum.*

1.2 Required Materials but not provided

- Pasteur pipettes
- Collagen I-coated Culture dishes
- Scalpels, scissors, and forceps
- Pasteur pipettes and 10-ml pipettes
- Test tubes, 12 and 50 ml

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, collagen I-coated plate (Corning, NY) MUST be pre-treated with 5 ml Coating Solutions for 5 min. Aspirate the coating solutions; let the dishes be air-dry in the ventilated cell culture hood for 5-10 min.

2.2 Surgical specimens

Biopsies of about 3-4 mm3 are taken with biopsy forceps to portions of mammary glands. All surgical specimens should be immediately placed in Rat Breast Tissue Washing Medium, transported on ice to the laboratory within 1 h and worked up immediately. With autoclaved scalpels, scissors, and forceps, carefully remove muscle and fat from specimens followed by washing procedures. Place specimens in a 10 ml falcon tube contain 5-10 ml fresh Breast Tissue Washing Medium followed by inoculating for 10 min at the room temperature. For large tissue specimens, 50 ml falcon tube and more wash medium is needed to ensure thoroughly washing. Aspirate the washing medium and repeat the washing procedures with fresh washing medium two more time. Washing tissue specimens sequentially in 70% ethanol for 1 min at the room temperature, in PBS for 5 min, and in fresh Breast Tissue Washing Medium for 10 min. Collecting tissue specimen by centrifugation prior to tissue dissociation procedures (see below).

2.3 Tissue Preparation and Dissociation

2.3.1 Rat Breast OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Breast Tissue Dissociation System, OptiTDS™, is suited for optimal dissociation of normal adult Rat biopsies samples to yield maximum number of single primary cells of Breast tissues.

2.3.2 Enzyme Compositions

- Collagenase I: from *Clostridium Histolyticum*
- Collagenase III: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*
- Collagenase: from *Clostridium Histolyticum*
- Trypsin: from *Bovine Pancreas*

2.3.3 System Components

- Breast Tissue Dissociation System, OptiTDS™, (2 x 1 ml).
- Breast OptiTDS™ Digestion Buffer, (2 x 9 ml).

2.3.4 Procedures For Tissue Preparation and Dissociation

1 Prepare fresh enzyme working solutions: add 1.0 ml of Breast Tissue Dissociation System, OptiTDS™, to one vial of Breast OptiTDS™ Digestion Buffer (9.0 ml). Warm the diluted enzyme working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 4-5 g tissue samples per 10 ml diluted enzyme working solutions.
2 Mince pre-washed tissue into pieces approximately 0.2-0.5 mm^2 in diameter with two scalpel and forceps.
3 Incubate minced tissues with the enzyme working solutions by incubating minced tissue samples (up to 5 g) in 10 ml fresh enzyme working solutions with slow magnetic stirring for 30 min at 37 ℃.
4 At the end of tissue dissociation period, gentle triturating tissue with a 10 ml pipette, constitutes filling the empty the barrel at a rate of 2-3 ml per second. Repeat this procedure for 5-6 times.
5 Collecting cells by filtration the mixture through a cell strainer to remove fatty layer and larger undigested tissues followed by centrifugation at 1 x 100 g. Carefully collect enzyme working solutions and resuspend the cell pellet with 1.0 ml complete culture medium.
6 Count the cells and seed cells in 3-4 T-25 collagen I-coated flasks (**Important:** pre-treat the flask with the provided Coating Solutions for 5 min and air-dry in hood prior to use) upon the density of viable cells (2.5-5 x 10^5 Cells/flask).
7 The undigested tissues can be repeated for digestion process by inoculating the tissue with the saved enzyme working solutions from step 5 for another 30 min, to obtain additional cells.

2.3.5 Storage:

Reconstituted tissue dissociation systems should occur before use and can only be stored for 2-4 days at 4 ^oC. For long-term use, it should be aliquoted and stored at -20 ^oC. Avoid repeated freeze-thaw cycles.

2.4 Culture of primary Rat Mammary Epithelial cells

2.4.1 Medium Preparation.

Preparation of complete culture medium: thaw out one vial of **Rat Mammary Epithelium PrimaCell™ Medium Supplements** and one vial of **Rat Breast PrimaCell™ Serum**, one vial of **Rat Breast Fibroblast Growth Inhibitors, Breast FibrOut™** on ice. To every 100 ml Rat **Mammary Epithelium PrimaCell™ Basal Culture Medium**, add one vial of Rat Mammary Epithelium PrimaCell™ Medium Supplements; one vial **Rat Breast PrimaCell™ Serum**; and one vial of **Rat Breast Fibroblast Growth Inhibitors, Breast FibrOut™**, mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use. (**Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*).

2.6.1 Treatment of Culture Dishes.

To enhance cell attachment to the culture dishes, collagen I-coated plate (Corning, NY) MUST be freshly pre-treated with the provided Coating Solutions by adding appropriate volume of Coating Solutions (enough to cover the whole cell-growth area) and incubate for 5 min. Aspirate the medium; let the dishes be air-dry in the ventilated cell culture hood for 5-10 min prior to use.

.

2.6.1 Standard primary culture conditions.

Inoculate epithelial tubules and clumps of cells derived from tissue specimens into T-25 flasks coated with collagen type I with pre-treatment of basal culture medium as described above at 37℃ in a 5%-CO_2 incubator with 4 ml of complete culture medium prepared in 2.4.1. Change the culture medium twice weekly. The tubules and cells start to attach to the substratum, and epithelial cells migrate out within 1-2 days. Most of the tubules and small clumps of epithelium attach within 4 days.

2.6.1 Alternative Primary Culture conditions.

The attachment of epithelium during primary culture and subculture is more reproducible and efficient when cells are inoculated onto collagen-coated flasks, and significantly better growth is obtained with 3T3 feeders than without. When the epithelial cells expand several passages, they become less dependent on 3T3 feeders, and no further addition of feeders is necessary. All medium and solutions described in the standard culture condition are applicable in this culture method.

2.4.5 Subculture and Propagation

Most mammary epithelium primary cultures cannot at present be passaged by routine trypsin / EDTA procedures. Disaggregation to single cells of the cultured Breast cells with 0.1% trypsin in 0.25 mM (0.1%) EDTA will result in extremely poor or even zero growth, so Dispase is used instead. One of the advantage using dispase is that dispase can only detach epithelial cell but not the fibroblast, thus increase the purity of epithelium.

8. Add 0.5% Dispase (Sigma, w/v) to the cell monolayer, just enough to cover the cells (~2.5 ml/25-cm^2 flask), and leave the solution to stand for 40-60 min for primary cultures and 20-40 min for cell lines.
9. Once the epithelial layers begin to detach (they do so as sheets rather than single cells), pipette to help detachment and disaggregation into smaller clumps.
10. Wash and replate the cells under standard culture conditions. It may take several days for clumps to attach, so replace the medium carefully when feeding.

III Fibroblast Contamination

There are several techniques have been published in the literature to deal with fibroblast contamination during colorectal primary cell culture. These include: (1) Physically remove a well-isolated fibroblasts by scraping it with a sterile blunt instrument (e.g., a cell scraper). Care has to be taken to wash the culture up to six times to remove any fibroblasts that have detached in order to prevent them from reseeding and reattaching to the flask. (2) Differential trypsinization can be attempted with the carcinomas. (3) Dispase preferentially (but not exclusively) removes the epithelium during passaging and leaves behind most of the fibroblastic cells attached to the culture vessel. During subculture, cells that have been removed with dispase can be preincubated in plastic Petri dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the epithelium. Clumps of epithelial cells still floating can be transferred to new flasks under standard culture conditions. This technique takes advantage of the fact that fibroblasts in general attach much more quickly to plastic than do clumps of epithelial cells, so that a partial purification step is possible. (4) Reduce the concentration of serum to about

2.5-5% if there are heavy concentrations of fibroblastic cells. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and that using any or all of the preceding techniques will eventually push the cells through so many divisions that any fibroblasts will senesce.

Rat Breast PrimaCell™ includes a fibroblast elimination system, the Rat Breast Fibroblast Growth Inhibitors, Breast FibrOut™. It contains a mixture of cis-OH-proline, toxin ricin, gentamycin and formulated serum substitutes. This proprietary mixture kills contaminating fibroblasts, but shows no signs of toxicity toward the epithelium, whether derived from an adenoma, a carcinoma or normal Breast tissues.

IV References:

1. Blatchford DR, Quarrie LH, Tonner E, McCarthy C, Flint DJ, Wilde CJ. *Influence of microenvironment on mammary epithelial cell survival in primary culture.* J Cell Physiol. 1999 Nov; 181(2):304-11.
2. Finch LM, Craig VA, Kind AJ, Schnieke A, Scott A, Wells M, Wilde CJ. *Primary culture of ovine mammary epithelial cells*. Biochem Soc Trans. 1996 Aug; 24(3):369S.
3. Taylor JA, Forsyth IA, Wang MW. *Activity of progesterone and anti-progestins in a rat mammary primary cell culture system.* J Steroid Biochem Mol Biol. 1996 Apr; 58(1):117-21.
4. Rohlfs EM, Louie DS, Zeisel SH. *Lipid synthesis and secretion by primary cultures of rat mammary epithelial cells.* J Cell Physiol. 1993 Dec; 157(3):469-80.
5. Lin TP, Hom YK, Richards J, Nandi S. *Effects of antioxidants and reduced oxygen tension on rat mammary epithelial cells in culture.* In Vitro Cell Dev Biol. 1991 Mar; 27A (3 Pt 1):191-6.
6. Raber JM, D'Ambrosio SM. *Isolation of single cell suspensions from the rat mammary gland: separation, characterization, and primary culture of various cell populations.* In Vitro Cell Dev Biol. 1986 Aug; 22(8):429-39.

Rat Breast PrimaCell™: Mammary Epithelium

Rat Breast Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82023	**Rat Breast PrimaCell™ system**	**kit**	**$499**
4-21031	Rat Breast Tissue Dissociation System, Breast OptiTDS™ (for 500 ml medium)	1 ml	$146
9-41003	Rat Mammary Epithelium PrimaCell™ Basal Culture Medium	500 ml	$ 79
9-31031	Rat Mammary Epithelium PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
7-61031	Rat Breast Fibroblast Growth Inhibitors, Breast FibrOut™	ea	$152
9-91003	Rat Breast Tissue Preparation Buffer Set	ea	$75

Rat Primary Mammary Epithelium Characterization

6-27011	**Rat Mammary Epithelium Primarker™ Kit**	**kit**	**$220**
6-27012	Rat Mammary Epithelium Primarker™ antibody set	set	$180
6-27013	Rat Mammary Epithelium Primarker™ buffer system	set	$90

Rat Cartilage PrimaCell™: Articular Cartilage
(Cat No. 2-82026)

I. General Description

Articular cartilage is a unique tissue where hydrostatic pressure is a significant component of the mechanical loading environment. Articular cartilage is sparsely populated by chondrocytes immobilized in the extracellular matrix. The chondrocytes, or cells of articular cartilage, represent 1% of the matrix volume, are responsible for secreting matrix molecules such as type II collagen and glycosaminoglycan (GAG) to maintain the correct size and mechanical properties of the tissue. Chondrocytes are highly specialized cells of mesenchymal origin that are responsible for synthesis, maintenance, and degradation of the cartilage matrix.Chondrocytes live in an unusual and constantly changing physicochemical environment. They receive signals during the loading of the tissue and produce, through a balance between macromolecular synthesis and degradation, a mechanically resilient extracellular matrix influenced by changes to the intracellular composition, such as cell volume, pH and ionic content. Chondrocytes have a unique biology and thus are widely used for experiments to study the molecular mechanisms implicated in cartilage associated disorders. A great deal of research in the field of rheumatology has been focused on understanding the mechanisms that induce metabolic changes in articular chondrobytes during osteoarthritis and rheumatoid arthritis. While these cells are extremely useful in the laboratory they are notoriously difficult to isolate and culture. They rapidly divide, become fibroblastic, and lose their biochemical characteristics The Rat Cartilage PrimaCell™ kit (Cat No. 2-82026) is designed for the successful isolation and culture of chondrocytes from rat cartilage allowing reproducible and dependable results.

1.1 Components of the Rat Cartilage PrimaCell™ System

- **Rat Cartilage Tissue Dissociation System , Cartilage OptiTDS™** (2 × 1 ml) --- *A mixture of collagenase I, collagenase II, Hyaluronidase I, trypsin and Rat Cartilage OptiTDS™ Reconstitution Buffer,* (2 × 1 ml).
- **Rat Cartilage OptiTDS™ Digestion Buffer,** (2 × 9 ml)
- **Rat Cartilage PrimaCell™ Washing Medium** (1 × 100 ml) --- *A modified Ham's F12 with netilmycin, Vancomycin, ceftazidim, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin.*
- **Rat Cartilage Fibroblast Growth Inhibitors, Cartilage FibrOut™** ---Cartilage FibrOut™ (5 × 200 μl) --- *A mixture of D-valine,collagenase and gentamycin.*
- **Rat Cartilage Carilage Basic Culture Medium** (5 × 100 ml) --- A *Modified formulation based on Ham's F12 medium*.
- **Rat Cartilage PrimaCell™ Medium Supplements** (5 × 1 ml): *gentamycin.*
- **Rat Cartilage PrimaCell™ Washing Medium** (1 × 100 ml) --- *A modified Ham's F12 with netilmycin, Vancomycin, and ceftazidim.*
- **Rat Cartilage PrimaCell™ Serum** (5 × 10 ml) --- *A modified Fetal Calf serum.*
- **Rat Cartilage PrimaCell™ Gelation Solution** (5 × 10 ml) --- *A modified mixture of HEPES and $CaCl_2$.*
- **Rat PrimaCell™ Solubilization Solution** (4 × 10 ml) --- *A modified mixture of HEPES and EDTA.*

- **Rat PrimaCell™ Alginate Solution** (5 × 1 ml) --- *A modified mixture of HEPES Sodium alginateand, and NaCl.*
- **Rat PrimaCell™ Alginate Washing Solution** (5 × 10 ml) --- *NaCl.*

1.2 Required materials but NOT included:

- DMEM (Invitrogen Cat No. 10313-021)
- Fetal Bovine Serum (FBS, Sigma-Aldrich)
- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or rat fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, fresh gelatin-coated plate or culture dishes are recommended (see below for treatment of culture dishes).

2.2 Principle

Among the various methods explored for maintaining the phenotype of chondrocytes, culture in alginate beads appears the most promising, since it has been shown that this culture system leads to the formation of a matrix similar to that of native articular cartilage. The system maintains the expression of the differentiated phenotype and is also able to restore it in dedifferentiated chondrocytes. Another advantage over other three-dimensional methods is that cells can easily be recovered after the culture is completed, allowing protein and gene expression studies.

2.3 Rat Cartilage Tissue Preparation

Rat Cartilage: Prepare cultures from knee, shoulder, and hip joints. Fetal or young donors are preferable to adults, as they provide higher quantities of cells and take longer to senesce.

1. Wash tissue pieces thoroughly with **Rat Cartilage PrimaCell™ Washing Medium** before dissection. Dissection should begin without delay. Remove skin, muscle, and tendons from joints. Carefully take cartilage fragments from articulations that are free of connective tissue.
2. Using crossed scalpels, mince cartilage slices into 1-mm^3 pieces.
3. Transfer cartilage fragments into a 30-ml flat-bottomed vial.
4. Incubate tissue specimens with 5-10 ml 70% ethanol for 1 min, followed by inoculating with 5-10 ml fresh **Rat Cartilage PrimaCell™ Dissection Medium** for 5 min.

2.4 Articular carilage Separation and Tissue Dissociation

2.4.1 Rat Cartilage OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Rat Cartilage Tissue Dissociation System is suited for optimal dissociation of normal newborn rat cartilage tissues to yield maximum number of single chondrocyte.

2.4.2 Enzyme Compositions

- Trypsin: from *Bovine Pancreas*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase II: from *Clostridium Histolyticum*
- Hyaluronidase I

2.4.3 System Components

- Rat Cartilage Tissue Dissociation System, Cartilage OptiTDS™ , (2 × 1 ml).
- Rat Cartilage OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

5. Prepare fresh enzyme working solution: Add 1ml of **Rat Cartilage Tissue Dissociation System, Cartilage OptiTDS™** to one vial of **Rat Cartilage OptiTDS™ Digestion Buffer** (9 ml). Warm the diluted **Rat Cartilage Tissue Dissociation System** working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 5 ml **Rat Cartilage Tissue Dissociation System** working solution.
6. Discard the **Rat Cartilage PrimaCell™ Dissection Medium,** and add 5 – 10 ml of **Rat Cartilage Tissue Dissociation System** working solution (prewarmed to 37℃) and incubate the fragments with moderate magnetic agitation for 30-60 min at 37 ℃ in a sealed vial at room temperature.

2.10 Chondrocytes Isolation

Note: Please read section 2.6 for specific information on chondrocytes culture and platting before preceding this section.

7. Transfer the cell suspension into a 50-ml centrifuge tube and mix on a vortex mixer for a few seconds.
8. Remove residual material left after digestion by passing the digested material through a 70-μm nylon filter.
9. Centrifuge the filtrate at 400 g for 10 min.
10. Resuspend the cell pellet in 20 ml of **Rat Cartilage PrimaCell™ Dissection Medium**, and count the cells with a hemocytometer.

2.11 Primary chondrocytes Culture

2.6.1 Medium Preparation.

Thaw out **Rat Cartilage PrimaCell™ II Basal Culture Medium**, **Rat Cartilage PrimaCell™ II Medium Supplements**, and **Rat Cartilage Serum** on ice.

Rat Complete Cartilage PrimaCell™ II Culture Medium: To every 100 ml **Rat Cartilage PrimaCell™ II Basal Culture Medium**, add 1 ml **Rat Cartilage PrimaCell™ II Medium Supplements** and 10 ml **Rat Cartilage Serum**, mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

Rat Complete Cartilage PrimaCell™ II Culture Medium/FibrOut: To every 100 ml **Rat Cartilage PrimaCell™ II Basal Culture Medium**, add 1 ml **Rat Cartilage PrimaCell™ II Medium Supplements** and 10 ml **Rat Cartilage Serum**,and 200 µl **Rat Cartilage Fibroblast Growth Inhibitors, Cartilage FibrOut™** mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

2.6.1 Primary Cell Culture

(Important: *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*)

11. Centrifuge the cells at 400 g for 10 min.
12. Resuspend the cell pellet in 1 ml of Cartilage PrimaCell™ Alginate Solution, and then dilute the suspension progressively in more Cartilage PrimaCell™ Alginate Solution, until a cellular density of 2×10^6 cells/ml is reached. This progressive dilution is necessary to obtain a homogeneous cell suspension in alginate.
13. Express the cell suspension in drops through a 21 G needle into the Cartilage PrimaCell™ Gelation Solution with moderate magnetic stirring, and allow the alginate to polymerize for 10 min to form beads.
14. Wash the beads 3 times in 5 vol Cartilage PrimaCell™ Alginate Washing Solution.
15. Distribute the beads, 5 ml (1×10^7 cells), into 75-cm^2 flasks containing 20 ml of **Rat Complete Cartilage PrimaCell™ Culture Medium/FibrOut**.
16. Incubate the culture at 37˚C in a humidified atmosphere of 5% CO_2, and 95% air.

2.7 Recovery

17. Discard the medium.
18. Add 2 vol of Cartilage PrimaCell™ Solubilization Solution to the beads.
19. Incubate the culture 15 min at 37˚C.
20. Centrifuge the cells at 400 g for 10 min.
21. Resuspend the cell pellet in **Rat Complete Cartilage PrimaCell™ Culture Medium/FibrOut** containing 0.06% collagenase.
22. Incubate the cells for 30 min at 37˚C in a humidified atmosphere of 5% CO_2, and 95% air.
23. Centrifuge the cells at 400 g for 10 min.
24. Resuspend the cell pellet in **Rat Cartilage PrimaCell™ Basal Culture Medium**, and count the cells with a hemocytometer.
25. Centrifuge the cells at 400 g for 10 min.
26. Repeat steps 28, 29, without counting the cells.

2.8 Subculture

Propagating chondrocytes in culture can be somewhat challenging, especially when chondrocytes are cultured alone on plastic, however the following methods have worked consistently in many laboratories.
Remove complete Rat Chondrocytes Culture Medium and wash chondrocytes in 0.1% trypsin two times. Place just enough trypsin (0.1 % trypsin without EDTA) to moisten articular carilage layer (200-500 µl depending on size of the dish). Incubate for 1-5 min at 37°C.

27. Remove complete Rat Chondrocytes Culture Medium and wash chondrocytes in 0.1% trypsin two times. Place just enough trypsin (0.1 % trypsin without EDTA) to moisten articular carilage layer (200-500 µl depending on size of the dish).
28. Incubate for 1-5 min at 37°C. Gently pipette chondrocytes and resuspend in complete Rat Chondrocytes Culture Medium (FibrOut™ is not necessarily needed at this step) for counting and replatting on collagen coated tissue culture plates.
29. Gently pipette chondrocytes and resuspend in **Rat Complete Cartilage PrimaCell™ Culture Medium** (FibrOut™ is not necessarily needed at this step) for counting and replatting on tissue culture plates.

III Cryopreservation

30. Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Chondrocytes cultures derived from preconfluent layers.
    - **(a)** Trypsinize cells as above, and centrifuge at 100 g for 10 min.
    - **(b)** Resuspend cells in **Rat Complete Cartilage PrimaCell™ Culture Medium** and count cells.
    - **(c)** Prepare aliquots of 2×10^6 cells/ml in **Rat Complete Cartilage PrimaCell™ Culture Medium** and 10% glycerol into cryovials.
    - **(d)** Equilibrate at 4℃ for 1-2 h.
    - **(e)** Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
    - **(f)** To recover cells:
        - (i) Thaw cryovials quickly in a 37℃ water bath.
        - (ii) Dilute cells tenfold with appropriate **Rat Complete Cartilage PrimaCell™ Culture Medium**.
        - (iii) Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in **Rat Complete Cartilage PrimaCell™ Culture Medium**.

IV Characterization

The cells of articular cartilage live in an unusual and constantly changing physicochemical environment. Chondrocytes, the only cells found in cartilage, produce and maintain the cartilaginous matrix. Classic markers of a chondrocytic phenotype are—Sox9, collagen II, and aggrecan (Expression of chondrocyte markers by cells of normal and degenerate intervertebral discs). Sox9 is the major regulator of the chondrocytic phenotype. It is a potent promoter of collagen II gene expression, a molecule which is produced almost exclusively in the chondrocyte. The proteoglycan aggrecan is also a characteristic gene product of chondrocytes. Besides, there are various markers for differentiation of chondrocytes: 11-fibrau, a reliable and sensitive marker of chondrocyte phenotype, and a useful marker to characterize chondrocyte differentiation stage; Annexin VI, a marker of late chondrocyte differentiation;

Cartilage oligomeric matrix protein (COMP), a sensitive marker for the differentiation state of articular primary chondrocytes; Cathepsin B, a marker of the dedifferentiated chondrocyte phenotype; cartilage matrix protein (CMP, or Matrilin-1), a mature chondrocyte marker; Collagen X and Collagen IX; etc.

V References:

1. Ikenoue, T., et al., *Mechanoregulation of human articular chondrocyte aggrecan and type II collagen expression by intermittent hydrostatic pressure in vitro.* J Orthop Res, 2003. **21**(1): p. 110-6.
2. Ho, Y.C., et al., *Highly efficient baculovirus-mediated gene transfer into rat chondrocytes.* Biotechnol Bioeng, 2004. **88**(5): p. 643-51.
3. Hauselmann, H.J., et al., *Phenotypic stability of bovine articular chondrocytes after long-term culture in alginate beads.* J Cell Sci, 1994. **107 (Pt 1)**: p. 17-27.
4. Hall, A.C., E.R. Horwitz, and R.J. Wilkins, *The cellular physiology of articular cartilage.* Exp Physiol, 1996. **81**(3): p. 535-45.
5. Loeser, R.F., et al., *Human chondrocyte expression of growth-arrest-specific gene 6 and the tyrosine kinase receptor axl: potential role in autocrine signaling in cartilage.* Arthritis Rheum, 1997. **40**(8): p. 1455-65.
6. Loeser, R.F., Jr. and R. Wallin, *Vitamin K-dependent carboxylation in articular chondrocytes.* Connect Tissue Res, 1991. **26**(3): p. 135-44.
7. Magne, D., et al., *The new IL-1 family member IL-1F8 stimulates production of inflammatory mediators by synovial fibroblasts and articular chondrocytes.* Arthritis Res Ther, 2006. **8**(3): p. R80.
8. Lotz, M., I. Clark-Lewis, and V. Ganu, *HIV-1 transactivator protein Tat induces proliferation and TGF beta expression in human articular chondrocytes.* J Cell Biol, 1994. **124**(3): p. 365-71.
9. Guicheux, J., et al., *Primary human articular chondrocytes, dedifferentiated chondrocytes, and synoviocytes exhibit differential responsiveness to interleukin-4: correlation with the expression pattern of the common receptor gamma chain.* J Cell Physiol, 2002. **192**(1): p. 93-101.

Rat Cartilage PrimaCell™: Articular Cartilage

Rat Cartilage Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82026	**Rat Cartilage PrimaCell™ system**	**kit**	**$499**
4-21061	Rat Cartilage Tissue Dissociation System, Cartilage OptiTDS™ (for 500 ml medium)	1 ml	$134
9-41006	Rat Cartilage PrimaCell™ Basal Culture Medium	500 ml	$73
9-31061	Rat Cartilage PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
7-61061	Rat Cartilage Fibroblast Growth Inhibitors, Cartilage FibrOut™	ea	$146
9-91006	Rat Cartilage Tissue Preparation Buffer Set	ea	$75

Rat Primary Articular Cartilage Characterization

6-28011	**Rat Articular Cartilage Primarker™ Kit**	**kit**	**$220**
6-28012	Rat Articular Cartilage Primarker™ antibody set	set	$180
6-28013	Rat Articular Cartilage Primarker™ buffer system	set	$90

Rat Cervix PrimaCell™: Cervical Epithelium
(Cat No. 2-82027)

I. General Description

The physiologic and pathologic conditions that affect the uterine cervix are of considerable contemporary interest. In particular, the recognition of the role of genital human papillomaviruses (HPVs) as the etiologic agent in cervical carcinoma has focused attention on the biology of cervical epithelium. Primary cervical epithelia are crucial target for pharmacotherapy. While these cells are extremely useful in the laboratory, they are notoriously difficult to isolate and culture. The Rat Cervix PrimaCell™ kit (Cat No. 2- 82027) is designed for the successful isolation and culture of cervical epithelia from rat cervix tissue allowing reproducible and dependable results.

1.1 Components of the Rat Cervix PrimaCell™ System

- **Rat Cervix Tissue Dissociation System, Cervix OptiTDS™** (2 × 1 ml) --- *A mixture of collagenase, collagenase I, collagenase IV, Trypsin, Dexoyribonuclease and Rat Cervix OptiTDS™ Reconstitution Buffer.*
- **Rat *Cervix OptiTDS™ Digestion Buffer,*** (2 × 9 ml)
- **Rat Cervix Fibroblast Growth Inhibitors, Cervix FibrOut™**---Cervix FibrOut™ (5 x 200 µl) --- *A mixture of D-valine, collagenase and gentamycin.*
- **Rat Cervical Epithelium PrimaCell™ Basal Culture Medium,** (5 × 100 ml) --- *A Modified Weymouth medium*.
- **Rat Cervical Epithelium PrimaCell™ Medium Supplements,** (5 × 1 ml): *Rat EGF, Hydrocortisone, Cholera toxin penicillin (100 U/ml), streptomycin and Weymouth medium.*
- **Rat Cervix PrimaCell™ Serum,** (50 ml): A modified f*etal bovine serum.*
- **Rat Cervix Tissue Washing Medium,** (1 × 100 ml): *A modified DMED medium with 10µg/ml amphotericin,* 10*µg/ml gentamycin, and 10% FCS.*

1.2 Required materials but NOT included:

- DMEM (Invitrogen Cat No. 10313-021)
- Fetal Bovine Serum (FBS, Sigma-Aldrich)
- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or rat fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

1.3 Preparation of Swiss 3T3 Fibroblasts (optional):

(a) A large master stock of cells should be prepared and frozen in individual ampules of 1×10^6 cells. Cells should not be used for more than 20 passages.

(i) Grow 3T3s in DMEM/10% calf serum in 175-cm^2 tissue culture flasks. Inoculate cells at 1.5×10^4 cells/cm^2. Change the medium after 2d. Subculture every 4-5 d.

(ii) To avoid low-level contamination, maintain one master flask of cells on antibiotic-free medium; these cells are then used at each passage to inoculate the flasks required for that week's feeder cells.

(b) Feeder layers are inactivated by irradiation with 60 Gy (6,000 rad), either from an X-ray or 60Co source. Irradiated cells (XR-3T3) may be kept at 4℃ for 3-4 d.

(c) In the absence of a source of irradiation, inactivate feeder cells with mitomycin C.

(i) Expose 3T3 cells growing in monolayer to 400 μg/ml of mitomycin C for 1 h at 37℃.

(ii) Trypsinize the treated cells, resuspend and wash the cell pellet twice with fresh medium with serum, resuspend the cells at a suitable concentration in complete medium, and use.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, fresh gelatin-coated plate or culture dishes are recommended (see below for treatment of culture dishes).

2.2 Principle

Separation of the cervical epithelium from the cervix tissue is accomplished by enzymatic digestion using the Cervix Tissue Dissociation System supplied in this kit. The Cervix Tissue Dissociation System contains a mixture of collagenase, collagenase I, collagenase IV, Trypsin, and Dexoyribonuclease at the optimal concentrations to gently detach the fragile cervix layer from the cervix tissue. The isolated cervix tissue is then further disrupted to release individual cervical epithelium by enzymatic and mechanical agitation. The mixture is then filtered through Cell Strainers and seeded on flasks or specially coated tissue culture plates. The cervical epithelia are propagated in growth arrested feeder cells and the corresponding media. Sub-populations of cervical epithelia can then be isolated based on their selective attachment to specific basement matrix substrates.

2.3 Rat Cervix Tissue Preparation

Rat Cervix: Most cervical epithelium from cervix is obtained from hysterectomy specimens.

1. The female rat is killed by decapitation and the uterine cervix dissected out under a dissecting microscope. Transfer biopsy immediately to Rat Cervix Tissue Washing Medium. Rinse cervical biopsy two to three times with 5 ml of cold **Rat Cervix Tissue Washing Medium**.
2. Place the biopsy, epithelial surface down, on a sterile culture dish.
3. Using surgical blade, cut and scrape away as much of the muscle and stroma as possible, leaving a thin, opaque epithelial strip.
4. Mince the epithelial strip finely with curved iris scissors.
5. Incubate tissue specimens with 5-10 ml 70% ethanol for 1 min, followed by inoculating with 5-10 ml fresh **Rat Cervix Tissue Washing Medium** for 5 min.

2.4 Cervix Separation and Tissue Dissociation

2.4.1 Rat Cervix OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the donor used, enzymes, culture mediums and growth supplements. The Rat Cervix Tissue Dissociation System is suited for optimal dissociation of normal rat cervix tissues to yield maximum number of single cervical epithelium.

2.4.2 Enzyme Compositions

- Collagenase: from *Clostridium Histolyticum*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*
- Trypsin
- Dexoyribonuclease

2.4.3 System Components

- Rat Cervix Tissue Dissociation System, Cervix OptiTDS™, (2 × 1 ml).
- Rat Cervix OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

6. Prepare fresh enzyme working solution: Add 1ml of the reconstituted tissue dissociation solution to one vial of Rat Cervix OptiTDS™ Digestion Buffer (9 ml). Warm the diluted Rat Cervix OptiTDS™ working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 5 ml Rat Cervix OptiTDS™ working solutions.
7. Discard the **Rat Cervix Tissue Washing Medium,** and float cervix samples on 5 – 10 ml of Rat Cervix Tissue Dissociation System working solution (prewarmed to 37℃) to the epithelial mince, and transfer the tissue to a sterile glass universal containing a small plastic-coated magnetic stirrer bar.
8. Place the universal on a magnetic stirrer and stir slowly for 30-60 min at 37 ℃. This works particularly well with full-thickness cervix. Monitor the separation of the cervix carefully when using the rapid dissociation method.
9. Allow the suspension to stand at room temperature for 2-3 min
10. Remove the supernatant containing single cells to a 50-ml centrifuge tube, and spin down the cell mixture at 1000 rmp (800g) for 5 min. Collect the supernatant dissociation system working solution which can be reused, and add 10 ml of **Rat Complete Cervix PrimaCell™ Culture Medium** to resuspend cells.
11. Add a further 5-10 ml of warm Rat Cervix Tissue Dissociation System working solution to the fragments in the universal, and repeat the steps 8-10.

2.6 Cervixal Epithelia Isolation

Note: Please read section 2.6 for specific information on Cervical Epithelium culture and platting before preceding this section.

12. Combine the supension containing single cells, and strain the cell mixture through a sterile cell strainer (70-100μm) into a 50-ml centrifuge tube to remove debris. Cell strainers fit perfectly in 50 ml conical and are very convenient for this procedure. If cell strainers are not available, nylon gauze can be used after rinsed twice in PBS and placed at the opening of a 50 ml

conical.

13. Centrifuge the strained mixture at 1000 rmp (800 g) for 5 min at 4°C.
14. Remove the supernatant; add 10 ml of complete medium to the pellet, resuspend the cells vigorously to give a single-cell suspension, and count the cells with a hemocytometer. Assess cell viability with trypan blue exclusion.

2.6 Primary Cervical Epithelia Culture

2.6.1 Medium Preparation.

Thaw out **Rat Cervix PrimaCell™ Basal Culture Medium**, **Rat Cervical Epithelium PrimaCell™ Medium Supplements**, and **Rat Cervix PrimaCell™ Serum** on ice.

Rat Complete Cervix PrimaCell™ Culture Medium: To every 100 ml **Rat Cervix PrimaCell™ Basal Culture Medium**, add 10 ml **Rat Cervix PrimaCell™ Medium Supplements** and 1 ml **Rat Cervix PrimaCell™ Serum** mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

Rat Complete Cervix PrimaCell™ Culture Medium/FibrOut: To every 100 ml **Rat Cervix PrimaCell™ Basal Culture Medium**, add 10 ml **Rat Cervix PrimaCell™ Medium Supplements,** 1 ml **Rat Cervix PrimaCell™ Serum**, and 1 ml **Rat Cervix Fibroblast Growth Inhibitors, Cervix FibrOut™**,mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

2.6.2 Primary Cell Culture

(Important: Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination)

15. Primary Cervical Epithelia Primary Cervical Epithelia can be cultured alone or on a layer of feeder cells depending on the nature of the experiments.

- Cervical Epithelium Culture with feeder layer cells:
  - Prepare feeder layers by culturing 3T3 cells 3 days.When the cells have reached confluence irradiate the 3T3 at 30 Gy
  - Dilue the cervical cell suspension with **Rat Complete Cervix PrimaCell™ Culture Medium/FibrOut**, and plate cells out at 2×10^4 cells/cm^2 together with 1×10^5 cells/cm^2 of lethally inactivated 3T3 cells (i.e., for 1×10^5 cervical cells, 5×10^5 XR-3T3/50-mm dish).
  - Incubate the cultures at 37℃ in 5% CO_2.
- Cervical Epithelium Culture without feeder layer:
  - Spin the suspension at 1,000 rpm (800 g) for 5 min. Remove the supernatant and resuspend the cells in 10 ml of **Rat Cervix Tissue Washing Medium**.
  - Spin the suspension again and wash the cells once more with **Rat Cervix Tissue Washing Medium**. Resuspend the cells in 10 ml of **Rat Complete Cervix PrimaCell™ Culture Medium/FibrOut**, and seed into culture dishes or flasks at a density of 2×10^5 cells/cm2 (10^6 cells/50-mm Petri dish, 4×10^6 /90-mm Petri dish).
  - Incubate the cultures at 37℃ in 5% CO2

16. Seventy-two hours after the initial platin, replace the medium with a **Rat Complete Cervix PrimaCell™ Culture Medium/FibrOut**. Check the cultures microscopically to ensure that the feeder layer is adequate. Add further feeder cells if necessary. Change the **Rat Complete Cervix PrimaCell™ Culture Medium/FibrOut** twice weekly; keratinocyte colonies become visible on the microscope by days 9-12 and should be visible to the naked eye by days 14-16. Change **Rat Complete Cervix PrimaCell™ Culture Medium/FibrOut** to **Rat Complete Skeletal Muscle PrimaCell™ Culture Medium** after 3-5 cycles or an acceptable level of fibroblast cell contamination is observed. Cultures should be subcultured at this time.

Δ ***Safety Note.*** The rest of the biopsy and all tubes, pipettes, plates, etc., used in the procedure should be treated with hypochlorite before disposal.

2.7 Subculture

17. Propagating Cervical Epithelia in culture can be somewhat challenging, especially when Cervical Epithelia are cultured alone on plastic, however the following methods have worked consistently in many laboratories.

(a) Cervical Epithelia grown on a feeder layer:
- (i) Spin the suspension at 1,000 rpm (80 g) for 5 min. Remove the supernatant and resuspend the cells in 10 ml of **Rat Cervix Tissue Washing Medium**.
- (ii) Spin the suspension again and wash the cells once more with **Rat Cervix Tissue Washing Medium**. Resuspend the cells in **Rat Complete Skeletal Muscle PrimaCell™ Culture Medium** and plate them onto culture dishes at 10^5 cells/cm^2 (5×10^5 cells/50-mm Petri dish, 2 $cm^2 \times 10^6$ cells/90-mm Petri dish).
- (iii) Cellsmay also be frozen at this stage for recovery at a later date.

(b) Cultures in complete Rat Cervical epithelium Culture Medium:
- (i) Remove the medium from the cell layer, and remove the feeders by rinsing rapidly with 0.01% EDTA. Wash twice with **Rat Cervix Tissue Washing Medium.**
- (ii) To each culture dish, add enough prewarmed trypsin/EDTA to cover the cell sheet. Leave the cultures at 37℃ until the keratinocytes have detached; check for detachment with a microscope. Do not leave the cells in trypsin for more than 20 min.
- (iii) Remove the cell suspension from the plate and transfer it to a sterile centrifuge tube.
- (iv) Rinse the growth surface with **Rat Complete Cervix PrimaCell™ Culture Medium** and add to the suspension. Mix and dispense the suspension with a 10-ml pipette.
- (v) Spin the cells at 1,000 rpm for 5 min, remove the supernatant, add 10 ml of **Rat Complete Cervix PrimaCell™ Culture Medium**, and resuspend the cells vigorously with a 10-ml pipette to achieve a single-cell suspension.
- (vi) Count the cells with a hemocytometer.
- (vii) Cells may be replated on inactivated 3T3 cells and grown as just described or frozen for later recovery.

III Cryopreservation

18. Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Cervical Epithelia cultures derived from preconfluent layers.

(a) Trypsinize cells as above, and centrifuge at 100 g for 10 min.
(b) Resuspend cells in **Rat Complete Cervix PrimaCell™ Culture Medium** and count cells.
(c) Prepare aliquots of 2 x 10^6 cells/ml in **Rat Complete Cervix PrimaCell™ Culture Medium** and 10% glycerol into cryovials.
(d) Equilibrate at 4℃ for 1-2 h.
(e) Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
(f) To recover cells:
(i) Thaw cryovials quickly in a 37℃ water bath.
(ii) Dilute cells tenfold with **Rat Complete Cervix PrimaCell™ Culture Medium**.
(iii) Centrifuge cells get rid of the glycerol and resuspend at an appropriate con centration in **Rat Complete Cervix PrimaCell™ Culture Medium**.

IV Characterization

Studies on primary cultures of cervical epithelia have identified differences in morphology and gene expression between cells derived from endocervical and ectocervical regions. However, cervical epithelia have been relatively underinvestigated with regard to their in vitro growth characteristics, immune functions, and susceptibility to toxic agents and mechanisms of infection by pathogens other than HPV. The presence of specific keratins within cells is dependent on both the origin of the epithelial cells as well as the stage of differentiation. Alterations in keratin expression could serve as markers for the differentiation of rat cervical epithelial cells in vitro.

V References

1. Freshney, R.I.F.a.M.G., *CULTURE OF EPITHELIAL CELLS*, ed. 2. Vol. 5. 2002: Wiley-Liss, Inc.
2. Fichorova, R.N., J.G. Rheinwald, and D.J. Anderson, *Generation of papillomavirus-immortalized cell lines from normal human ectocervical, endocervical, and vaginal epithelium that maintain expression of tissue-specific differentiation proteins.* Biol Reprod, 1997. **57**(4): p. 847-55.
3. Wright, T.C., Jr., *Characterization of keratins from rat cervical epithelial cells in vivo and in vitro.* Cancer Res, 1987. **47**(24 Pt 1): p. 6678-85.

Rat Cervix PrimaCell™: Cervical Epithelium

Rat Cervix Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82027	**Rat Cervix PrimaCell™ system**	**kit**	**$499**
4-21071	Rat Cervix Tissue Dissociation System, Cervix OptiTDS™ (for 500 ml medium)	1 ml	$128
9-41007	Rat Cervical Epithelium PrimaCell™ Basal Culture Medium	500 ml	$ 79
9-31071	Rat Cervical Epithelium PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$ 140
7-61071	Rat Cervix Fibroblast Growth Inhibitors, Cervix FibrOut™	ea	$146
9-98007	Rat Cervix Tissue Preparation Buffer Set	ea	$75

Rat Primary Cervical Epithelium Characterization

6-29011	**Rat Cervical Epithelium Primarker™ Kit**	**kit**	**$220**
6-29012	Rat Cervical Epithelium Primarker™ antibody set	set	$180
6-29013	Rat Cervical Epithelium Primarker™ buffer system	set	$90

Rat Colon PrimaCell™: Colorectal Epithelium
(Cat No. 2-82028)

I. General Description:

The Rat Colon PrimaCell™ kit (Cat No. 2-82028) is developed to isolate and encourage the growth of normal rat colonic epithelial cells derived from 1-3 mm of rat colon tissues. This kit includes the Colon OptiTDS™ Tissue Dissociation System that provides the optimal enzymatic conditions for isolating viable cells from whole tissue and has been proven to yield 5-7 times more cells than most of the protocols published in the literature. The Rat Colon PrimaCell™ kit also contains the optimal media and supplements that ensure the isolated colonic epithelial cells remain viable in tissue culture. The specific growth of colonic epithelial cells is further ensured by including Colon FibrOut™, a proprietary blend of enzymes and chemical reagents that inhibits the growth of aberrant fibroblasts that usually overtake primary cell cultures and crowd out the growth of colonic epithelial cells.

The Rat Colon PrimaCell™ kit can be used to isolate primary colonic epithelial cells from normal rat tissue samples. The results obtained from tissue samples that may contain pathological organisms (virus, parasites, etc.) or malignancies may not yield optimal results using this system. Tissue specimens used for colon epithelial cells should ideally be used within 1-2 hrs of biopsy and, therefore, tissue removal from the patient. If this is not feasible, the biopsy sample can be cut into small pieces (1-2 mm) and stored at
4° C in washing media (provided in this kit) overnight as described in the protocol below.

1.1 Components of Rat Colon PrimaCell™ System

- **Colonic Tissue Dissociation System, Colon OptiTDS™,** (2 aliquots) --- *A* proprietary *mixture of collagenase I, collagenase III, collagenase IV, collagenase, and trypsin*.
- **Colon OptiTDS™ Reconstitution Buffer**, (2 x 1 ml).
- **Colon OptiTDS™ Digestion Buffer**, (2 x 9 ml).
- **Colonic Tissue Washing Medium**, (5 x 10 ml) --- *Basal Colon PrimaCell™ Culture Medium with 5% FBS, 200 u/ml of penicillin, 200 µg/ml of streptomycin, and 50 µg/ml of gentamycin*.
- **Rat Colon Fibroblast Growth Inhibitors, Colon FibrOut™** (5 x 200 µl) --- *A* proprietary *mixture of* anti-*Thy-1 monoclonal antibody, D-valine, collagenase, and gentamycin*.
- **Rat Colon PrimaCell™ Basal Culture Medium**, (5 x 100 ml) --- *Modified formulation based on NCTC 168 and Weymouth medium*.
- **Rat Colon PrimaCell™ Medium Supplements**, (5 x 1.0 ml) --- A mixture of *ethanolamine, phosphoethanolamine, hydrocortisone, ascorbic acid, transferring, insulin, epidermal growth factor, pentagastrin, and deoxycholic acid*.
- **Rat Colon PrimaCell™ Serum**, (1 x 50 ml) --- H*eat-inactivated and special-treated Fetal-bovine serum.*
- **Coating Solution**, (5 x 10 ml) --- *Basal growth medium containing 10 µg/ml BSA.*

1.2 Required Materials NOT included in the kit

- 70% sterile ethanol

- Pasteur pipettes, 10 ml pipettes
- Collagen I pre-coated tissue culture dishes
- Scalpels, scissors, and forceps
- Falcon Conicals, 15 and 50 ml

II. Procedures

2.1 Preparation of Tissue Culture Plates

All materials used in this experiment must be sterile to prevent contamination. To enhance cell attachment to tissue culture dishes, collagen I pre-coated plates (Corning, NY) MUST be further pre-treated with the Coating Solution included in this kit for 5 min. Aspirate the Coating Solution and allow the dishes to air-dry in a ventilated cell culture hood for 5-10 min before use.

2.2 Surgical specimens

Once isolated, tissue specimens should be placed immediately in 5-10 ml Colonic Tissue Washing Medium, transported on ice to the laboratory and worked up within 1 hr of isolation. Colon tissue specimens (1-3 mm^3) are further dissected to isolate the mucosal layer away from the muscle layer. With autoclaved scalpels, scissors, and forceps, carefully remove muscle and fat from tissue specimens. Place tissue in a 50 ml conical tube (Falcon) containing 5 ml fresh Colonic Tissue Washing Medium and incubate while shaking at room temperature for 10 min. For large tissue specimens, use a 50 ml conical tube (Falcon) and a larger volume of Colonic Tissue Washing Medium to ensure thorough washing. Aspirate the washing medium and repeat the washing procedures using fresh washing medium two more times. After the initial washes, incubate tissue in 70% ethanol for 1 min at room temperature, followed by incubating in 5-10 ml fresh Colonic Tissue Washing Medium for 5 min. Collect tissue pieces by gentle centrifugation and proceed to dissociation.

2.3 Tissue Preparation and Dissociation

2.3.1 Rat Colon OptiTDS™

The isolation of primary cells is confounded by several important factors that can greatly affect yield and cell viability. The Colon Tissue Dissociation System, Colon OptiTDS™, is developed to produce the optimal conditions that allow for the dissociation of colonic epithelial cells from normal adult rat tissue samples. This system uses a defined proprietary ratio of specific enzymes to yield the maximum number of single primary cells that remain viable in tissue culture.

2.3.2 Enzyme Compositions

- Collagenase I: from *Clostridium Histolyticum*
- Collagenase III: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*
- Hyaluronidase I: from *Bovine Testes*

2.3.3 System Components

- Colonic Tissue Dissociation System, OptiTDS™: 2 vials
- Colon OptiTDS™ Reconstitution Buffer: (2 x 1 ml)

- Colon OptiTDS™ Digestion Buffer: (2 x 9 ml)

2.3.4 Procedures For Tissue Preparation and Dissociation

8. Add 1.0 ml of the Colon OptiTDS™ Reconstitution Buffer to a vial of Colonic Tissue Dissociation System OptiTDS™ and mix well (Do not Vortex) with hand. It is important that the Tissue Dissociation System OptiTDS is freshly prepared and is provided in aliquots for your convenience.
9. Transfer the 1.0 ml of freshly prepared enzyme solution from step 1, to a vial containing 9.0 ml of Colon OptiTDS™ Digestion Buffer. Warm this enzyme solution at 37 ℃ for 10 min, just prior to use. For optimal results, we recommend a ratio of 100-150 mg tissue per 10 ml of enzyme solution.
10. Mince the pre-washed tissue into pieces measuring approximately 0.2-0.5 mm^2 in diameter with scalpel and forceps or scissors.
11. Incubate the minced tissue with the diluted enzyme solution with gentle stirring for 30 min at 37 ℃. Up to 5 mg of minced tissue can be incubated with 10 ml of the diluted enzyme solution.
12. Gently pipette sample with a 10 ml pipette, filling the barrel of the pipette at a rate of 2-3 ml per second. Repeat this for 5-6 times.
13. Filter the cell mixture through a cell strainer (100μM). Centrifuge the strained mixture at 100 g. Carefully remove the supernatant and resuspend the cell pellet with1.0 ml of Complete Colon Culture Medium (See 2.4.1 for preparation).
14. Count and seed the cells in 3 or 4 T-25 collagen I-coated flasks containing 4 ml of Complete Colon Culture Medium (**Important:** Collagen coated plates must be pre-treated with the provided Coating Solution, see section 2.1). This procedure will give you approximately 2.5-5 x 10^5 Cells/per T-25 flask.

2.3.5 Storage of Tissue Dissociation System:

Colonic Tissue Dissociation System, Colon OptiTDS™ should only be reconstituted when needed for cell preparation and can be stored for 2-4 days at 4 ^{o}C. For long-term use, it should be aliquoted and stored at -20 ^{o}C. Avoid repeated freeze-thaw cycles.

2.4 Culture of primary rat colon cells

2.4.1 Medium Preparation

Thaw the Rat Colon PrimaCell™ Medium Supplements and Rat Colon PrimaCell™ Serum on ice. To prepare Complete Colon Culture Media dilute one vial of Rat Colon PrimaCell™ Medium Supplements into 100 ml Rat Colon PrimaCell™ Basal Culture Medium. Add 10 ml Rat Colon PrimaCell™ Serum and one vial of Colon FibrOut™ (Rat Colon Fibroblast Growth Inhibitors). Mix thoroughly and warm the Complete Colon Media in a 37^{o}C water bath for 10 min prior use.

2.4.2 Treatment of Culture Dishes

To facilitate primary colon cell attachment to the tissue culture plates, the plates should be pre-coated with collagen I (available from Corning, NY) and MUST be pre-treated with the provided Coating Solution (Basal Growth Medium containing 10 μg/ml BSA). Cover the entire surface area of tissue culture plates with the Coating Solution and incubate for 5 min. Aspirate the Coating Solution and allow the dishes to air-dry in a ventilated cell culture hood for 5-10 min prior to use.

2.4.3 Standard primary culture conditions

Seed epithelial tubules and cell clumps isolated from colon tissues (from 2.3.4) into prepared T-25 flasks containing 4 ml of complete media and incubate a 37° C, 5%-CO_2 incubator. The Complete Colon Culture Medium should be changed twice a week. The tubules and cells will attach to the matrix on the plates and the colon epithelial cells will begin to migrate out within 1-2 days. Most of the tubules and cell clumps of epithelium will attach within 7 days, however, larger organoids can take up to 6 weeks to attach. It is important to note that the cells remain viable during this time. (**Important:** Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination).

2.4.2 Alternative Primary Culture conditions

Primary colon epithelium requires a substratum to efficiently attach during tissue culture propagation. While this can be achieved using pre-coated Collagen I plates, the growth of primary colon epithelium is significantly better when a layer of 3T3 feeder cells are used. As the initial colon epithelial colonies expand to several hundred cells per colony, the cells become less dependent on the 3T3 feeder cells, and can be cultured without the feeder cells. All media and solutions supplied in this kit can be used whether cells are grown on collagen coated plates or 3T3 feeder cells.

2.4.5 Subculture and Propagation

Most colon primary cells cannot be passaged by routine procedures using trypsin/EDTA. Colon epithelial cells can be de-attached using a mild dissociation enzyme, dispase as this treatment leads to more successful passaging. A further advantage of using dispase to passage cells is that dispase can only detach epithelial cell but not fibroblast. Thus this property decreases fibroblast contamination of the colon epithelial cells with each successive passage.

8. Add 0.5% Dispase (Sigma, w/v) to the cell monolayer. Use just enough dispase to cover the cells (~2.5 ml/25-cm^2 flask), and incubate for 40-60 min for primary colon cultures and 20-40 min for primary colon cell lines.
9. Once the epithelial layers begin to detach (they do so as sheets rather than single cells), pipette to encourage the detachment and dis-aggregation into smaller clumps.
10. Wash and replate the cells under standard culture conditions. It may take several days for clumps to attach, so take special care when changing the media and feeding the cells.

III Fibroblast Contamination

Several techniques have been published that help prevent fibroblast contamination of primary colon cell cultures. These include: (1) Physically removing isolated fibroblast colonies by scraping off the fibroblast colonies with a sterile cell scraper. This method requires extensive washing to ensure that all the lifted fibroblast that remain are washed away and not allowed to reattach and repopulate the primary colon cultures. (2) Differential trypsinization can be attempted with the carcinomas but may not work well with normal primary colon cells. (3) Using dispase to lift off colon epithelial cells preferentially but not exclusively during routine passaging. In addition, cells can be seeded on mock plates for 4-6 hrs so that the fibroblasts attach, and floating colon cells can be transferred to collagen coated plates under standard culture conditions.

This technique takes advantage of the fact that fibroblasts in general attach much more quickly to plastic than do clumps of epithelial cells. (4) Reducing the concentration of serum to about 2.5-5% to eliminate heavy concentrations of fibroblastic cells. The above methods can be used in combination knowing that normal primary fibroblasts have a finite growth span *in vitro* and if these measures are used persistently the fibroblasts will eventually be forced into senesce.
Important Note: The Rat Colon PrimaCell™ kit includes a fibroblast elimination system Colon FibrOut™, an effective mixture of Rat Colon Fibroblast Growth Inhibitors. It contains a mixture of anti-Thy-1 monoclonal antibody, toxin ricin and formulated serum substitutes. The principle behind the using this system is that Thy-1 antigen is present only on colorectal fibroblasts, but not colorectal epithelial cells. Using this approach, therefore, fibroblasts are eliminated without added toxicity to epithelial cells.

IV References:

1. Bartsch I, Zschaler I, Haseloff M, Steinberg P.: *Establishment of a long-term culture system for rat colon epithelial cells.* In Vitro Cell Dev Biol Anim. 2004 Sep-Oct;40(8-9):278-84.
2. Dzierzewicz Z, Orchel A, Parfiniewicz B, Weglarz L, Stojko J, Swierczek-Zieba G, Wilczok T. *The delay of anoikis due to the inhibition of protein tyrosine dephosphorylation enables the maintenance of normal rat colonocyte primary culture.* Folia Histochem Cytobiol. 2003;41(4):223-8.
3. Dzierzewicz Z, Orchel A, Swierczek-Zieba G, Weglarz L, Stojko J, Wilczok T. *Sodium orthovanadate inhibits apoptosis of rat colonocytes in primary culture.* Acta Pol Pharm. 2004 Dec;61 Suppl:86-7.
4. Dzierzewicz Z, Orchel A, Swierczek-Zieba G, Latocha M, Cwalina B, Wilczok T. *Normal colonocytes in primary culture--an experimental model for molecular pharmacology and biology of large intestine.* Acta Pol Pharm. 2000 Nov;57 Suppl:23-5.
5. Yeh KY, Chopra DP. *Epithelial cell cultures from the colon of the suckling rat.* In Vitro. 1980 Nov;16(11):976-86.
6. Kaeffer B, Benard C, Lahaye M, Blottiere HM, Cherbut C. *Biological properties of ulvan, a new source of green seaweed sulfated polysaccharides, on cultured normal and cancerous colonic epithelial cells.* Planta Med. 1999 Aug;65(6):527-31.

Rat Colon PrimaCell™: Colorectal Epithelium

Rat Colon Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82028	**Rat Colon PrimaCell™ system**	**kit**	**$ 499**
4-21081	Rat Colon Tissue Dissociation System, Colon OptiTDS™ (for 500 ml medium)	1 ml	$ 134
9-41008	Rat Colorectal Epithelium PrimaCell™ Basal Culture Medium	500 ml	$73
9-31081	Rat Colorectal Epithelium PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
7-61081	Rat Colon Fibroblast Growth Inhibitors, Colon FibrOut™	ea	$ 146
9-98008	Rat Colon Tissue Preparation Buffer Set	ea	$75

Rat Primary Colorectal Epithelium Characterization

6-21011	**Rat Colorectal Epithelium Primarker™ Kit**	**kit**	**$220**
6-21012	Rat Colorectal Epithelium Primarker™ antibody set	set	$180
6-21013	Rat Colorectal Epithelium Primarker™ buffer system	set	$90

Rat Endothelium PrimaCell™: Vascular Endothelial Cells
(Cat No. 2-82029)

I. General Description:

The Rat Endothelium PrimaCell kit (Cat No. 2-92111) allows the isolation and tissue culture growth of normal rat vascular endothelial cells from the rat vascular endothelial tissues. This system provides the optimal conditions for endothelial cell culture and yields 5-7 times more cells than most available protocols published in the literature. In addition, this system ensures high cell viability and a pure endothelial cell population that is obtained using CHI's proprietary fibroblast inhibitory system, FibrOut™.

Endothelial cells exist in a single cell layer lining the inner surface of all blood vessels. Historically, the vessels most commonly used to obtain cultured endothelial cells are derived from bovine and rat aorta, bovine adrenal capillaries, rat and rat brain capillaries, human umbilical veins, and human dermal and adipose capillaries. Although all endothelia share some common properties, significant differences exist between the endothelial cells of large and small blood vessels.

The Rat Vascular Endothelial PrimaCell™ kit can be used to isolate most types of endothelial cells from rats ranging from E16 through 3 weeks. E20-22 or 2-3 weeks are only recommended for convenience and obtaining a maximum yield. The preparation of aorta, capillaries or blood vessels for cell culture is usually started within 1-2 h of removal from the animal. If this is impossible, cut the vessels into fine pieces of about 10-15 mm and store overnight at 4° C in washing medium (see below). Endothelial samples containing pathological organism (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Rat Vascular Endothelial PrimaCell™ System

- **Vascular Endothelial Tissue Dissociation System, Vascular Endothelial OptiTDS™, (**2 aliquots) --- *A proprietary mixture of Collagenase, Collagenase I, Collagenase IV, Elastase, Soybean Trysin Inhibitor*
- **Vascular Endothelial Tissue OptiTDS™ Reconstitution Buffer, (**2 x 1 ml)
- **Vascular Endothelial Tissue OptiTDS™ Digestion Buffer, (**2 x 9 ml)
- **Vascular Endothelial Tissue Washing Medium**, (1 x 100 ml) --- *Basal Vascular Endothelial PrimaCell™ Culture Medium with 5% FBS, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Rat Vascular Endothelial Fibroblast Growth Inhibitors, Vascular Endothelial FibrOut™, (**5 x 200 μl) --- *A proprietary mixture of cis-OH-proline, collagenase, D-valine, and special serum substitutes*.
- **Rat Vascular Endothelial PrimaCell™ Basal Culture Medium,** (5 x100 ml) --- *Modified formulation based on medium 199 and DMEM medium*.
- **Rat Vascular Endothelial PrimaCell™ Medium Supplements with Serum,** (5 x 10 ml) --- *A mixture of EGF, VEGF and Heparin, and CHI's proprietary rat serum extracts*.
- **Vascular Endothelial PrimaCell™ I Culture Dish Coating Solution,** (5 x 10 ml) --

- 1.5% gelatin solution, 0.5% bovine serum albumin in PBS.

1.2 Required Materials NOT provided

- 70% sterile ethanol
- Tissue culture dishes
- Pasteur pipettes and 10-ml pipettes
- Falcon Conicals: 15 and 50 ml
- Two clamps or hemostats, 25 mm
- Sharp scissors, 50 mm

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, fresh gelatin-coated plate or culture dishes are recommended (see below for treatment of culture dishes).

2.2 Treatment of Culture Dishes

1. Tissue culture ware used to seed endothelial cells has to be pretreated using Vascular Endothelial PrimaCell™ I Culture Dish Coating Solution.
2. Incubate the plates or dishes with 2-3 ml (enough to cover the medium reaching areas) Vascular Endothelial PrimaCell™ I Culture Dish Coating Solution overnight at room temperature in the tissue culture hood.
3. Remove the Vascular Endothelial PrimaCell™ I Culture Dish Coating Solution (without washing the surface), immediately add complete Rat Vascular Endothelial PrimaCell™ Medium (see 2.4.1 for Medium Preparation) and incubate the medium until the cells are ready for platting.

(Note: *Pre-coated gelatin plates or dishes must be used within 72 after preparation.*)

2.3 Vascular Endothelial Tissue Preparation

4. Rats at ages of E20-22 rats or 2-3 weeks are recommended for convenient of procedures and yielding maximum amount of viable target cells. Rats are sacrificed by CO_2 narcosis.
5. Aseptically isolated blood vessels, preferably in 10-15 mm sections, approximately 5 mm in diameter. If asepsis cannot be guaranteed, clamp both ends of the blood vessel.
6. Incubate blood vessels for up to 10 min in Vascular Endothelial Tissue Washing Medium to prevent infection. (This procedure will not affect endothelial cells viability.)
7. Incubate tissues in 10 ml 70% sterile ethanol for 30 sec.
8. Rinse tissue twice in Vascular Endothelial Tissue Washing Medium for 5 min each and kept on ice.

2.3 Tissue Dissociation

2.3.1 Rat Vascular Endothelial OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Rat Vascular Endothelial Tissue Dissociation System, OptiTDS™, is suited for optimal dissociation of normal adult Vascular Endothelial tissues to yield maximum number of single endothelial cells.

2.3.2 Enzyme Compositions

- Dispase: from *Bacillus polymyxa*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase II: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*

2.3.3 System Components

- Vascular Endothelial Tissue Dissociation System, OptiTDS™, 2 vials.
- Vascular Endothelial OptiTDS™ Reconstitution Buffer, (2 x 1 ml).
- Vascular Endothelial OptiTDS™ Digestion Buffer, (2 x 9 ml).

2.3.4 Procedures For Tissue Preparation and Dissociation

9. Add 1.0 ml of Vascular Endothelial Tissue Dissociation System, OptiTDS™ to one vial of Vascular Endothelial OptiTDS™ Digestion Buffer (9.0 ml). Warm the diluted Vascular Endothelial Tissue OptiTDS™ digestion solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 10 ml diluted Vascular Endothelial OptiTDS™ working solutions.
10. Ligate one end of a 10-cm section of blood vessel 2-10 mm in diameter to a 5-ml plastic syringe.
11. Run the Vascular Endothelial Tissue OptiTDS™ digestion solution through the blood vessel until it appears at the bottom end, clamp that end with a hemostat, and incubate the vessel containing the fresh enzyme working solution at room temperature for 30 min with rocking.
12. Cut the vessel above the clamp with sharp scissors, and collect the Vascular Endothelial Tissue OptiTDS™ digestion solution in a 10-cm Petri dish.
13. Rinse the lumen of the vessel with 10 ml of Vascular Endothelial Tissue Washing Medium, and add this to the Vascular Endothelial Tissue OptiTDS™ digestion solution collected from step 4.
14. Repeat procedure 3-5 until finish all blood vessels and collecting all enzymes digestion solution containing cells.
15. Collect cells by centrifugation at 350 g, washing cells with 10 ml PBS or complete culture medium twice. At the end of washing process, collect cells and dilute cell in 0.5-1.0 ml Complete Rat Vascular Endothelial PrimaCell™ I Culture Medium (see 2.4.1 for Medium Preparation).
16. Count viable cells.
17. Seed cells at 37℃ in Complete Rat Vascular Endothelial PrimaCell™ I Culture Medium at desired densities (see 2.4.1 for Medium Preparation).

Alternative Method: The vessels can be cut along the length to reveal the inner layer of

endothelial cells. The vessels can be attached to a Styrofoam board with the interior of the vessel facing up. Then the entire board revealing the exposed vessel lumen can be inverted into the Vascular Endothelial Tissue OptiTDS™ digestion solution to dissociate the endothelial cells.

2.3.5 Storage:

Diluting tissue dissociation systems should occur before use and can only be stored for 2-4 days at 4 ^{o}C. For long-term use, it should be aliquoted and stored at -20 ^{o}C. Avoid repeated freeze-thaw cycles.

2.4 Culture of Vascular Endothelial Cells

2.4.1 Medium Preparation.

Thaw out the Vascular Endothelial PrimaCell™ Medium Supplements with Serum on ice. To every 100 ml Rat Vascular Endothelial PrimaCell™ Basal Culture Medium, add 10 ml of Vascular Endothelial PrimaCell™ Medium Supplements with Serum; and one vial of Rat Vascular Endothelial Fibroblast Growth Inhibitors, Vascular Endothelial FibrOut™, mix thoroughly and warm the Complete Vascular Endothelial Media in a 37^{o} C water bath for 10 min prior use. (**Important:** Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination).

2.4.2 Primary Culture Protocols.

18. Resuspend the final pellet collected from last step described in 2.3.4 in Complete Vascular Endothelial Media, and seed the cells into fresh prepared gelatin-coated dishes or flasks, with approximately 5 x 10^5 cells derived from one 10-15 cm section of blood vessel, 5 mm in diameter, per 72-cm flask or 10-cm-diateter dish.
19. Subculture by conventional trypsinization method.

2.4.3 Subculture and Propagation

20. Gently rinse the culture dish twice with 1X PBS.
21. Add 3 ml of 0.25% trypsin/0.1% (2.5 mM) EDTA, and incubate at 37℃. Examine the dish under phase microscopy every 5 min to detect cell detachment.
22. When most cells have detached, add 10 ml Complete Vascular Endothelial Media to inactive the trypsin activity.
23. Pipette the contents of the dish to ensure complete endothelial cell detachment.
24. Aspirate and centrifuge the cells for 5 min at 350 g.
25. Aspirate the supernatant, resuspend the cells in a complete growth medium, and re-plate at 2-4 × 10^5 cells per 100-mm dish.
26. Feed the culture twice a week with Complete Vascular Endothelial Media.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the

same tissue sample; the best results are reported when cells from preconfluent primary cultures are used.

27. Detach cells as for the subculture, and centrifuge at 350 g for 10 min.
28. Resuspend cells in complete culture medium with serum, and count.
29. Dispense aliquots of 2 x 10^6 cells/ml in Complete Vascular Endothelial Media with additional 15% FCS and 10% glycerol into cryopreservation tubes.
30. Equilibrate at 4℃ for 1-2 h.
31. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1 ℃ per min.
32. To recover cells:

(i) Thaw cryotubes quickly in a 37℃ water bath.
(ii) Dilute cells tenfold with medium.
(iii) Centrifuge cells and resuspend them at an appropriate concentration in the desired culture medium, and seed culture vessel.

Rat Vascular Endothelial cells can be grown in all media for 5-7 weeks and can be subcultured only 4-5 times.

IV Fibroblast Contamination

Rat Vascular Endothelial PrimaCell™ system includes a fibroblast elimination system, the Rat Vascular Endothelial Fibroblast Growth Inhibitors, Vascular Endothelial FibrOut™. It contains a mixture of cis-OH-proline, collagenase, D-valine, and formulated serum substitutes. This system can effectively eliminate Vascular Endothelial fibroblast contamination while has not affect on the behavior of endothelial cells. (**Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*).

V Confirmation of Vascular Endothelial Cells

Vascular endothelial cells are typically identified by the production of factor VIII, angiotensin-converting, the uptake of acetylated low-density lipoprotein, the presence of Weibel-Palade bodies, and the expression of endothelial-specific cell surface antigens.

VI References:

1. Zetter BR. The endothelial cells of large and small blood vessels. Diabetes. 1981;30(Suppl 2):24-8.
2. Kern PA, Knedler A, Eckel RH. *Isolation and culture of microvascular Vascular Endothelial from human adipose tissue. J Clin Invest. 1983 Jun;71(6):1822-9.*
3. Davison PM, Bensch K, Karasek MA. *Isolation and long-term serial cultivation of endothelial cells from the microvessels of the adult human dermis. In Vitro. 1983 Dec;19(12):937-45.*
4. Jaffe EA, Nachman RL, Becker CG, Minick CR. *Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest. 1973 Nov;52(11):2745-56*
5. Folkman J, Haudenschild CC, Zetter BR. *Long-term culture of capillary endothelial cells. Proc Natl Acad Sci U S A. 1979 Oct;76(10):5217-21.*
6. Bowman PD, Betz AL, Ar D, Wolinsky JS, Penney JB, Shivers RR, Goldstein GW.

Primary culture of capillary Vascular Endothelial from rat brain. In Vitro. 1981 Apr;17(4):353-62.

7. Booyse FM, Sedlak BJ, Rafelson ME Jr. *Culture of arterial endothelial cells: characterization and growth of bovine aortic cells.* Thromb Diath Haemorrh. 1975 Dec 15;34(3):825-39.
8. Kobayashi M, Inoue K, Warabi E, Minami T, Kodama T. *A simple method of isolating rat aortic endothelial cells.* J Atheroscler Thromb. 2005;12(3):138-42.

Rat Endothelium PrimaCell™: Vascular Endothelial Cells

Rat Endothelium Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82029	**Rat Endothelium PrimaCell™ system**	**kit**	**$499**
4-21111	Rat Vascular Endothelial Tissue Dissociation System, Vascular Endothelial OptiTDS™ (for 500 ml medium)	1 ml	$ 134
9-41011	Rat Vascular Endothelial PrimaCell™ Basal Culture Medium	500 ml	$ 85
9-31111	Rat Vascular Endothelial PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
7-61111	Rat Vascular Endothelial Fibroblast Growth Inhibitors, Vascular Endothelial FibrOut™	ea	$ 152
9-98011	Rat Endothelium Tissue Preparation Buffer Set	ea	$75

Rat Primary Vascular Endothelium Characterization

6-21111	**Rat Vascular Endothelium Primarker™ Kit**	**kit**	**$220**
6-21112	Rat Vascular Endothelium Primarker™ antibody set	set	$180
6-21113	Rat Vascular Endothelium Primarker™ buffer system	set	$90

Rat Eye PrimaCell™: Corneal Epithelial Cells
(Cat No. 2-82030)

I. General Description:

This protocol is developed for attachment and growth of normal rat corneal epithelial cells from adult Rat Eye with Rat Eye PrimaCell™ system (Cat No. 2-82030). This system provides an optimal condition of tissue dissociation system, Rat Eye OptiTDS™ that yields 4-7 times of single cells more than most of the tissue dissociation protocols published in the literature. In addition, this system ensures a high viability of the target cells with improved gradient contained in the culture medium. With CHI's proprietary fibroblast inhibitory system, FibrOut™, cells are growing with contamination of minimized amount of the non-epithelial cells.

Rat Eye PrimaCell™ system applies to all type tissue samples from rat at all age though adult tissue samples are recommended for yielding maximum amount of viable target cells. However, tissue samples contain pathological organism (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Rat Eye PrimaCell™ System

- **Rat Eye Tissue Dissociation System, Eye OptiTDS™,** (2 x 1 ml) --- *A mixture of Collagenase II, Collagenase III, Collagenase IV, Elastase, Hyaluronidase I, Trypsin, Papain.*
- **Rat Eye OptiTDS™ Digestion Buffer**, (2 x 9 ml).
- **Rat Eye Tissue Washing Medium**, (1 x 100 ml) --- *Corneal Epithelial Cells PrimaCell™ Basal Culture Medium with 5% FBS, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Rat Eye Fibroblast Growth Inhibitors, Eye FibrOut™** (5 x 200 μl) --- *A mixture of cis-OH-proline, collagenase, D-valine, and formulated serum substitutes*.
- **Rat Corneal Epithelial Cells PrimaCell™ Basal Culture Medium**, (5 x100 ml) --- *Modified formulation based on Weymouth medium*.
- **Rat Corneal Epithelial Cells PrimaCell™ Medium Supplements**, (5 x 1.0 ml) --- A mixture of *Rat EGF, Insulin, Hydrocortisone, bovine pituitary extract, penicillin, and streptomycin*.
- **Rat Eye PrimaCell™ Serum**, (5 x 10 ml) --- *Highly purified special-treated Fetal-bovine serum.*

1.2 Required Materials but not provided

- PBS containing 5% sterilized BSA
- 70% sterile ethanol
- Trypsin-EDTA: Trypsin, 0.05%, EDTA, 0.5 mM
- Rat-tail collagen, type I-coated 6-well plate, (Becton Dickinson)
- Fibronectin-collagen-coated (FNC) culture dishes, 60 mm and 100 mm (Becton Dickinson)
- Scalpels No. 1621 (Becton Dickinson)

- Pipettes (10 and 25 ml)
- Gloves sterilized with autoclave (rat tissue can be contaminated with biologically hazardous agents)
- Controlled atmosphere chamber
- Phase-contrast inverted microscope

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, culture plate (Corning, NY) must be coated with the provided coating solution.

2.2 Surgical specimens

- Carefully remove eye tissue from donor in a way that is minimized damages to the whole corneal area.
- Incubate the eye tissue sequentially in 70% sterile ethanol for 1 min; in Eye Tissue Washing Medium for 10 min. (This procedure will not affect cell viability.)
- Carefully separate the corneas from connective tissues.
- Place corneas epithelial side up on a sterile surface (e.g., a regular cell culture dish), and cut them into 12 triangular shaped wedges, using a single cut of the scalpel and avoiding any sawing motion.

2.3 Tissue Preparation and Dissociation

2.3.1 Rat Eye OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Rat Eye Tissue Dissociation System, OptiTDS™, is suited for optimal dissociation of normal adult cornea samples to yield maximum number of single primary cells of eye tissues.

2.3.2 Enzyme Compositions

- Collagenase II: from *Clostridium Histolyticum*
- Collagenase III: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*
- Elastase: from *Porcine Pancreas*
- Hyaluronidase I: from *Bovine Testes*
- Trypsin: from *Bovine Pancreas*
- Papain: from *Bovine Testes*

2.3.3 System Components

- Rat Eye Tissue Dissociation System, OptiTDS™, (2 x 1 ml)
- Rat Eye OptiTDS™ Digestion Buffer, (2 x 9 ml).

2.3.4 Procedures For Tissue Preparation and Dissociation

1 Prepare Rat Eye OptiTDS™ Digestion solution: Add 1.0 ml of Rat Eye Tissue Dissociation System, OptiTDS™ to one vial of Rat Eye OptiTDS™ Digestion Buffer (9.0 ml).
2 Prepare complete tissue dissociation solution: Mix equal volume of Rat Eye OptiTDS™ Digestion solution and Rat Corneal Epithelial Cells PrimaCell™ Basal Culture Medium (without serum, the kit supplied with extra 50 ml basal culture medium).
3 Warm the complete tissue dissociation solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 10 ml complete tissue dissociation solution.
4 Turn each corneal segment epithelial side down, and place four segments in each well of a six-well tray (precoated with rat-tail collagen, type I, Biocoat, Becton Dickinson).
5 Press each segment down gently with forceps to ensure good contact between the tissue and the tissue culture surface. Allow the tissue to dry for 20 min.
6 Place one drop of complete tissue dissociation solution from step 3 carefully upon each segment. The solution should be applied to fully cover but not to spread over the cornea segments.
7 Incubate the culture for 30 min at 37℃ in 5% CO_2.

2.3.5 Storage:

Reconstituting tissue dissociation solution should occur before use and can only be stored for 2-4 days at 4 ^{o}C. For long-term use, it should be aliquoted and stored at -20 ^{o}C. Avoid repeated freeze-thaw cycles.

2.4 Culture of Primary Rat Keratinocytes

2.4.1 Medium Preparation.

Thaw out the Rat Corneal Epithelial Cells PrimaCell™ Medium Supplements and one vial of Rat Eye Fibroblast Growth Inhibitors, Rat Eye FibrOut™ on ice. To every 100 ml Rat Corneal Epithelial Cells PrimaCell™ Basal Culture Medium, add one vial of Rat Corneal Epithelial Cells PrimaCell™ Medium Supplements; and one vial of Rat Eye Fibroblast Growth Inhibitors, Rat Eye FibrOut™, mix thoroughly and warm the complete culture medium at a 37^{o}C water bath for 10 min prior use. (**Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*).

2.4.2 Primary Culture Conditions.

8 At the end of 30 min incubation, carefully remove the complete tissue dissociation solution remained on each segment by pipetting.
9 Add 1.5 ml of complete culture medium to each well and continue culturing for overnight at 37℃ in 5% CO_2.
10 Next day, exam cells under microscope. When cells are observed to emigrate only from the limbal region of the cornea. Asparate medium and washing cell

once with 1X PBS.

11 Carefully remove the tissue segment with forceps and add 3 ml of complete culture medium. After removal of the donor tissue, adherent cells remained in the plate continuing to proliferate, and within 2 weeks from the time of establishment of the culture, confluent monolayers form, displaying the typical cobblestone morphology associated with epithelia. The yield is approximately 7.5-8 $\times 10^6$ cells/cornea.

2.4.3 Subculture and Propagation

12 Following the initial outgrowth period, feed the cultures twice per week.

13 At 70-80% confluence, rinse the cells in Dulbecco's phosphate-buffered saline (PBS), and release with trypsin/EDTA (0.05% trypsin, 0.53 mM EDTA) for 4 min at 37℃.

14 Stop the reaction with 10% FBS in PBS.

15 Wash the cells (centrifugation followed by resuspension in complete culture medium), count them, and plate at 1×10^4 cells/cm^2 onto tissue culture surfaces coated with FNC.

16 Incubate the culture at 37℃ in 95% air and 5% CO_2.

17 Exchange the culture medium with fresh complete culture medium 1 d after trypsinization and reseeding. Immediately after passage, cells appear more spindles shaped, are refractile, and are highly migratory. Within 6-8 d, control cultures become 70-80% confluent, continue to display cobblestone morphology, and, if allowed to become postconfluent, retain the ability to stratify in discrete areas.

18 Although corneal epithelial cultures can be subcultured up to five times (approximately 9-10 population doublings), most of the proliferation occurs between the first and third passages. Approximate yields are 2.5-3.0 $\times 10^6$ cells/cornea. Senescence always ensues by P5.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample; the best results are reported when cells from preconfluent primary cultures are used.

19. Detach cells as for the subculture, and centrifuge at 100 g for 10 min.
20. Resuspend cells in complete culture medium with serum, and count.
21. Dispense aliquots of 2 x 10^6 cells/ml in complete growth medium with additional 10% FBS and 10% glycerol into cryopreservation tubes.
22. Equilibrate at 4℃ for 1-2 h.
23. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
24. To recover cells:

(i) Thaw cryotubes quickly in a 37℃ water bath.
(ii) Dilute cells tenfold with medium.
(iii) Centrifuge cells and resuspend them at an appropriate concentration in the desired culture medium, and seed culture vessel.

Rat corneal epithelial cells can be grown in the complete culture media for 4-6 weeks and can be subcultured only 5-7 times.

IV Fibroblast Contamination

There are several techniques have been published in the literature to deal with fibroblast contamination during cornea primary cell culture. These include: (1) Physically remove a well-isolated fibroblast colony by scraping it with a sterile blunt instrument (e.g., a cell scraper). Care has to be taken to wash the culture up to six times to remove any fibroblasts that have detached in order to prevent them from reseeding and reattaching to the flask. (2) Differential trypsinization can be attempted with the carcinomas. (3) Dispase preferentially (but not exclusively) removes the epithelium during passaging and leaves behind most of the fibroblastic cells attached to the culture vessel. During subculture, cells that have been removed with dispase can be preincubated in plastic petri dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the epithelium. This technique takes advantage of the fact that fibroblasts in general attach much more quickly to plastic than do clumps of epithelial cells, so that a partial purification step is possible. (4) Reduce the concentration of serum to about 2.5-5% if there are heavy concentrations of fibroblastic cells. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and that using any or all of the preceding techniques will eventually push the cells through so many divisions that any fibroblasts will senesce.

Rat Eye PrimaCell™ system includes a fibroblast elimination system, the Rat Eye Fibroblast Growth Inhibitors, Rat Eye FibrOut™. It contains a mixture of of cis-OH-proline, collagenases, D-valine, and formulated serum substitutes. This system can effectively eliminate Eye fibroblast contamination while has not affect on the behavior of cornea epithelial cells.

V References:

1. Lyu J, Joo CK. *Expression of Wnt and MMP in epithelial cells during corneal wound healing.* Cornea. 2006 Dec;25(10 Suppl 1):S24-8.
2. Adachi W, Ulanovsky H, Li Y, Norman B, Davis J, Piatigorsky J. *Serial analysis of gene expression (SAGE) in the rat limbal and central corneal epithelium.* Invest Ophthalmol Vis Sci. 2006 Sep;47(9):3801-10.
3. Boesch JS, Lee C, Lindahl RG. *Constitutive expression of class 3 aldehyde dehydrogenase in cultured rat corneal epithelium.* J Biol Chem. 1996 Mar 1;271(9):5150-7.
4. Choudhury A, Pakalnis VA, Bowers WE. *Function and cell surface phenotype of dendritic cells from rat cornea.* Invest Ophthalmol Vis Sci. 1995 Dec;36(13):2602-13.
5. Hjortdal JO, Haaskjold E, Sorensen KE, Bjerknes R. *Cell kinetics of normal and healing rat corneal epithelium during organ culture.* Acta Ophthalmol (Copenh). 1993 Feb;71(1):44-50.
6. Chung EH, Bukusoglu G, Zieske JD. *Localization of corneal epithelial stem cells in the developing rat.* Invest Ophthalmol Vis Sci. 1992 Jun;33(7):2199-206.

Rat Eye PrimaCell™: Corneal Epithelial Cells

Rat Eye Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82030	**Rat Eye PrimaCell™ system**	**kit**	**$499**
4-21091	Rat Eye Tissue Dissociation System, Eye OptiTDS™ (for 500 ml medium)	1 ml	$134
9-41009	Rat Corneal Epithelial Cells PrimaCell™ Basal Culture Medium	500 ml	$79
9-31091	Rat Corneal Epithelial Cells PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
7-61091	Rat Eye Fibroblast Growth Inhibitors, Eye FibrOut™	ea	$152
9-98009	Rat Eye Tissue Preparation Buffer Set	ea	$75

Rat Primary Corneal Epithelial Cells Characterization

6-21211	**Rat Corneal Epithelial Cell Primarker™ Kit**	**kit**	**$220**
6-21212	Rat Corneal Epithelial Cell Primarker™ antibody set	set	$180
6-21213	Rat Corneal Epithelial Cell Primarker™ buffer system	set	$90

Rat Fat PrimaCell™: Adipose Cells
(Cat No. 2-82031)

I. General Description

Fat cells, found in adipose tissue, also called adipose cells (adipocytes), are terminally differentiated specialized cells whose primary physiological role hss classically been described as an energy reservoir for the body. The cytoplasm usually being compressed into a thin envelope, with the nucleus at one point in the periphery. The chief chemical constituents of this fat are the neutral glycerol esters of stearic, oleic, and palmitic acids. The size of adipose cells can vary considerably from fat depot to fat depot. Abnormalities in adipose tissue can contribute directly to the pathogenesis of common dieases such as diabetes, hypertension and obesity. Adipocytes are crucial target for pharmacotherapy. While these cells are extremely useful in the laboratory, they are notoriously difficult to isolate and culture. Th protocol described in this kit can be scaled up or down as needed and has been used to provide insulin-responsive cells suitable for DNA transfer by electroporation. The Rat Fat PrimaCell™ kit (Cat No. 2- 82031) is designed for the successful isolation and culture of adipocytes from rat adipose tissue allowing reproducible and dependable results.

1.1 Components of the Rat Fat PrimaCell™ System

- **Rat Fat Tissue Dissociation System, Fat OptiTDS™** (2 × ml) --- *A mixture of collagenase, collagenase I, and trypsin and Rat Fat OptiTDS™ Reconstitution Buffer.*
- **Rat *Fat OptiTDS™ Digestion Buffer,*** (2 × 9 ml)
- **Rat Fat Fibroblast Growth Inhibitors, Fat FibrOut™**---Fat FibrOut™ (5 x 200 µl) --- *A mixture of cis-OH-proline, collagenase and gentamycin.*
- **Rat Adipose Cells PrimaCell™ Basal Culture Medium,** (5 x 100 ml) --- A *Modified DMEM*.
- **Rat Adipose Cells PrimaCell™ Medium Supplements with Serum,** (5 x 1 ml): *Glucose, Glutamine, ®-N6-(1-methyl-2-phenylethyl) adenosine, antibiotics (penicillin, 100 U/ml), streptomycin. Gentamycin, BSA and HEPES.*
- **Rat Adipose Washing Medium,** (1 x 100 ml): *Basal Kerbs-Ringer medium with 10mM NaHCO3, 30mM HEPES, 200nM adenosine and 5% BSA.*

1.2 Required materials but NOT included:

- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or rat fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, fresh gelatin-coated plate or culture dishes are recommended (see below for treatment of culture dishes).

2.2 Principle

Separation of the adipocytes from the adipose tissue is accomplished by enzymatic digestion using the Fat Tissue Dissociation System supplied in this kit. The Fat Tissue Dissociation System contains a mixture of collagenases and trypsin at the optimal concentrations to gently detach the fragile adipose layer from the adipose tissue. The isolated adipose is then further disrupted to release individual adipocytes by enzymatic and mechanical agitation. The mixture is then filtered through Cell Strainers and seeded on specially coated tissue culture plates. The Adipocytes are propagated in serum-free, low-calcium media or growth arrested feeder cells and the corresponding media. Sub-populations of adipocytes can then be isolated based on their selective attachment to specific basement matrix substrates.

2.3 Rat Adipose Tissue Preparation

Rat Adipose: Samples of subcutaneous adipose tissues can be used. Each rat adipose yield large number of cells (4-7 x 10^6/adipose), with a 25-35% plating efficiency.

6. Anesthetize rats in a plastic box with a gas mixture of 70% CO_2, and 30% O_2.
7. Decapitate rats using a guillotine and exsanguinate.
8. Soak the bodies briefly in 70% ethanol.
9. Remove the epididymal fat pads while maintaining the highest level of sterility possible.
   (e) Cut through the skin on the lowest abdomen with one pair of scissors to expose the peritoneum.
   (f) Using a second pair of scissors, open the peritoneum and pull up the testes with a pair of forceps.
   (g) Trim fat pads from epididymides, taking care to leave the blood vessels behind.
   (h) Transport tissue to5-10 ml 70% ethanol and incubate for 1 min, followed by inoculating with 5-10 ml fresh **Rat Adipose Washing Medium** for 5 min.
10. Add 4 g of fat pads (approximately equivalent to 8 fat pads) to a 30-ml low-density polypropylene vial containing **Rat Adipose Washing Medium**. Mince fat pads into pieces approximately 2 mm in diameter with scissors.

2.4 Adipose Separation and Tissue Dissociation

2.4.1 Rat Fat OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Rat Fat Tissue Dissociation System is suited for optimal dissociation of normal rat adipose tissues to yield maximum number of single adipocytes.

2.4.2 Enzyme Compositions

- Trypsin: from *Bovine Pancreas*
- Collagenase: from *Clostridium Histolyticum*
- Collagenase I: from *Clostridium Histolyticum*

2.4.3 System Components

- Rat Fat Tissue Dissociation System, Fat OptiTDS™, (2 × ml).
- Rat Fat OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Dissociation

10. Prepare fresh enzyme working solution: Add 1ml of the reconstituted tissue dissociation solution to one vial of Rat Cervix OptiTDS™ Digestion Buffer (9 ml). Warm the diluted Rat Fat OptiTDS™ working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-4 g tissue samples per 5 ml Rat Fat OptiTDS™ working solutions.
11. Discard the **Rat Fat Washing Medium,** and add 5 ml of Rat Fat Dissociation System working solution to the vial containing the minced fat pads, and incubate the pieces in a shaking water bath at 37˚C for approximately 1 h, until the cell mixture takes on a creamy consistency.
12. After Rat Fat Dissociation System digestion, add 10 ml of **Rat Adipose Washing Medium** at 37˚C to the vial.
13. Mix cells in the vial by swirling, and gently pass the cells through a 250-µm nylon mesh filter into a 50-ml conical tube.

2.6 Adipocytes Isolation

Note: Please read section 2.6 for specific information on Adipocytes culture and platting before preceding this section.

10. Wash the cells by adding 30 ml of **Rat Adipose Washing Medium** buffer at 37˚C to the tube and centrifuging briefly at 200 g in a tabletop centrifuge. Remove infranatant with a pipette. Note that adipose cells will be floating on top of the aqueous buffer.
11. Repeat the washing of cells by adding 40 ml of **Rat Adipose Washing Medium**, centrifuging, and removing infranatant two additional times.
12. Wash cells twice with 40 ml of **Rat Adipose Cells PrimaCell™ Basal Culture Medium** at 37˚C.
13. After the final wash, resuspend the cells from the surface of the medium in **Rat Complete Adipose PrimaCell™ Culture Medium/FibrOut** at a cytocrit of approximately 40%.

2.6 Primary Adipocytes Culture

2.6.1 Medium Preparation.

Thaw out **Rat Adipose Cells PrimaCell™ Basal Culture Medium**, **Rat Adipose Cells PrimaCell™ Medium Su**pplements with Se**rum** on ice.

Rat Complete Adipose Cells PrimaCell™ Culture Medium: To every 100 ml **Rat Adipose Cells PrimaCell™ Basal Culture Medium**, add 1 ml **Rat Adipose Cells PrimaCell™ Medium Supplements with Serum**, mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use.

Rat Complete Adipose Cells PrimaCell™ Culture Medium/FibrOut: To every 100 ml **Rat Adipose Cells PrimaCell™ Basal Culture Medium**, add 1 ml **Rat Adipose Cells PrimaCell™ Medium Supplements with Serum** and 1 ml **Rat Fat Fibroblast Growth Inhibitors, Fat FibrOut™**, mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

2.6.2 Primary Cell Culture

(Important: *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*)

17. Transfer 2 ml of the 40% cytocrit **Rat Complete Adipose PrimaCell™ Culture Medium/FibrOut** suspension, using 200-µl wide-bore pipette tips, into one 60-mm tissue culture dish (Becton Dickinson).
18. Place the cells in a humid incubator at 37˚C with 5% CO_2 for 1.5 h.
19. Add 5 ml of **Rat Complete Adipose PrimaCell™ Culture Medium/FibrOut** to each dish. Change the **Rat Complete Adipose PrimaCell™ Culture Medium/FibrOut** every 2-3 days;

Δ ***Safety Note.*** The rest of the biopsy and all tubes, pipettes, plates, etc., used in the procedure should be treated with hypochlorite before disposal.

2.7 Subculture

Propagating adipocytes in culture can be somewhat challenging, especially when adipocytes are cultured alone on plastic, however the following methods have worked consistently in many laboratories.

17. The primary cultures grew exponentially until confluency was attained.
18. Remove the medium from the cell layer, and wash twice with **Rat Adipose Tissue Washing Medium.**
19. To each culture dish, add enough prewarmed 0.01% EDTA/0.1% trypsin. to cover the cell sheet. Leave the cultures at 37℃ until the adipocytes have detached; check for detachment with a microscope. Do not leave the cells in trypsin for more than 20 min.
20. Remove the cell suspension from the plate and transfer it to a sterile centrifuge tube.
21. Rinse the growth surface with **Rat Complete Adipose PrimaCell™ Culture Medium** and add to the suspension. Mix and dispense the suspension with a 10-ml pipette.
22. Spin the cells at 1,000 rpm for 5 min, remove the supernatant, add 10 ml of **Rat Complete Adipose PrimaCell™ Culture Medium**, and resuspend the cells vigorously with a 10-ml pipette to achieve a single-cell suspension.
23. Count the cells and plate them onto culture dishes at 10^5 cells/cm^2.
24. Cells may be replated and grown as just described or frozen for later recovery.

III Cryopreservation

25. Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Adipocytes cultures derived from preconfluent layers.
    (a) Trypsinize cells as above, and centrifuge at 100 g for 10 min.

(b) Resuspend cells in **Rat Complete Adipose PrimaCell™ Culture Medium** and count cells.

(c) Prepare aliquots of 2 x 10^6 cells/ml in **Rat Complete Adipose PrimaCell™ Culture Medium** and 10% glycerol into cryovials.

(d) Equilibrate at 4℃ for 1-2 h.

(e) Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.

(f) To recover cells:

(i) Thaw cryovials quickly in a 37℃ water bath.

(ii) Dilute cells tenfold with **Rat Complete Adipose PrimaCell™ Culture Medium**.

(iii) Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in **Rat Complete Adipose PrimaCell™ Culture Medium**.

IV Characterization

Morphologically, pre-adipocytes are distinguishable from other primary fibroblasts. There are no biochemical arkers unique to pre-adipocytes in routine use. Morphological changes in pre-adipocytes are apparent when cells start to differentiate. Cells "round up" and lose their fibroblastic morphology, and retain only sparse and tenuous cell-cell contact. As differentiation progresses, intra-cytoplasmic lipid droplets form. It is easy to detect under phase-contrast microscopy, and stain with Oil red-O or Nile red, a simple means of assessing intracytoplasmic lipid accumulation, the classical morphological marker of differentiation. As differentiation continues, more droplets form such that the cytoplasm increases in size and becomes packed with small lipid. Cells tend to differentiate in clumps, with those cells that are refractory to differentiation reverting to a more fibroblastic morphology. The viability of the cells can be checked by glucose uptake assay. Adipocyte markers such as aP2 and adipsin are used in biochemical assays. Lipoprotein lipase (LPL) and pOb24 are widely used as an early marker, and Glycerol 3-phosphate dehydrogenase (G3PDH), along with adipsin and leptin is expressed much later in differentiation. Glucose transporter GLUT4, mediates insulin-stimulated glucose uptake in adipocytes by rapidly moving from intracellular storage sites to the plasma membrane, is a common used adipocyte specific marker. Gene expression assays, such as Northern bloting, real time PCR, etc., are used to detect early or late markers of differentiation.. Retinol-binding protein (RBP) expression occurred mainly and nearly exclusively in mature rat adipocytes. RBP expression in adipose tissue is dependent on the state of adipocyte differentiation.

IV References

1. Freshney, R.I., *Culture of Animal Cells: A Manual of Basic Technique, 4th Edition* 4ed. 2001: Cold Spring Harbor Laboratory Press. 600.
2. Salans, L.B., S.W. Cushman, and R.E. Weismann, *Studies of human adipose tissue. Adipose cell size and number in nonobese and obese patients.* J Clin Invest, 1973. **52**(4): p. 929-41.
3. Dani, C., et al., *Expression and regulation of pOb24 and lipoprotein lipase genes during adipose conversion.* J Cell Biochem, 1990. **43**(2): p. 103-10.
4. Tong, Q., et al., *Function of GATA transcription factors in preadipocyte-adipocyte transition.* Science, 2000. **290**(5489): p. 134-8.
5. Abel, E.D., et al., *Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver.* Nature, 2001. **409**(6821): p. 729-33.
6. Park, S.Y., et al., *Calorie restriction improves whole-body glucose disposal and insulin resistance in association with the increased adipocyte-specific GLUT4 expression in Otsuka*

Long-Evans Tokushima fatty rats. Arch Biochem Biophys, 2005. **436**(2): p. 276-84.
7. Makover, A., et al., *Localization of retinol-binding protein messenger RNA in the rat kidney and in perinephric fat tissue.* J Lipid Res, 1989. **30**(2): p. 171-80.
8. Tsutsumi, C., et al., *Retinoids and retinoid-binding protein expression in rat adipocytes.* J Biol Chem, 1992. **267**(3): p. 1805-10.
9. Zovich, D.C., et al., *Differentiation-dependent expression of retinoid-binding proteins in BFC-1 beta adipocytes.* J Biol Chem, 1992. **267**(20): p. 13884-9.
10. Bjorntorp, P., et al., *Differentiation and function of rat adipocyte precursor cells in primary culture.* J Lipid Res, 1980. **21**(6): p. 714-23.

Rat Fat PrimaCell™: Adipose Cells

Rat Fat Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82031	**Rat Fat PrimaCell™ system**	**kit**	**$499**
4-21101	Rat Fat Tissue Dissociation System, Fat OptiTDS™ (for 500 ml medium)	1 ml	$128
9-41010	Rat Adipose Cells PrimaCell™ Basal Culture Medium	500 ml	$73
9-31101	Rat Adipose Cells PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
7-61101	Rat Fat Fibroblast Growth Inhibitors, Fat FibrOut™	ea	$134
9-98010	Rat Fat Tissue Preparation Buffer Set	ea	$75

Rat Fat Primary Cell Characterization

6-21311	**Rat Adipose Cell Primarker™ Kit**	**kit**	**$220**
6-21312	Rat Adipose Cell Primarker™ antibody set	set	$180
6-21313	Rat Adipose Cell Primarker™ buffer system	set	$90

Rat Glomerular PrimaCell™: Glomerular Endothelial cells
(Cat No. 2-85004)

I. General Description

Glomerular endothelial cells (GEC) are specialized cells with important roles in physiological filtration and glomerular disease. While these cells are extremely useful in the laboratory and clinic research, they are notoriously difficult to isolate and culture. They rapidly divide, become fibroblastic, and lose their biochemical characteristics The Rat GEC PrimaCell™ kit (Cat No. 2-85004) is designed for the successful isolation and culture of GECs from rat glomerular tissue allowing reproducible and dependable results.

1.1 Components of the Rat Glomerular PrimaCell™ System

- **Rat Glomerular Tissue Dissociation System, Glomerular OptiTDS™** (2 × 1 ml) --- A mixture of collagenase, Trypsin, Dexoyribonuclease and Rat Glomerular OptiTDS™ Reconstitution Buffer.
- **Rat Glomerular OptiTDS™ Digestion Buffer**, (2 × 9 ml)
- **Rat Glomerular Fibroblast Growth Inhibitors, Glomerular FibrOut™---**Glomerular FibrOutTM (5 x 200 µl) --- A mixture of D-valine, collagenase and gentamycin.
- **Rat GEC PrimaCell™ Basal Culture Medium**, (5 × 100 ml) --- A Modified Ham F12.
- **Rat GEC PrimaCell™ Medium Supplements**, (5 × 1 ml): Rat EGF, Hydrocortisone, Cholera toxin penicillin (100 U/ml), streptomycin and Weymouth medium.
- **Rat GEC PrimaCell™ Serum**, (50 ml): A modified fetal bovine serum.
- **Rat Glomerular Tissue Washing Medium**, (1 × 100 ml): A modified DMED medium with 10 g/ml amphotericin, 10 g/ml gentamycin, and 10% FCS.

1.2 Required materials but NOT included:

- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or rat fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, fresh gelatin-coated plate or culture dishes are recommended (see below for treatment of culture dishes).

2.2 Principle

Separation of the GEC from the glomerular tissue is accomplished by enzymatic digestion using the Glomerular Tissue Dissociation System supplied in this kit. The Glomerular Tissue Dissociation System contains a mixture of collagenase, and Trypsin at the optimal concentrations to gently detach the fragile glomerular layer from the glomerular tissue. The isolated glomerular tissue is then further disrupted to release individual GEC by enzymatic and mechanical agitation. The mixture is then filtered through Cell Strainers and seeded on flasks or specially coated tissue culture plates. The GECs are propagated in growth arrested feeder cells and the corresponding media. Sub-populations of GECs can then be isolated based on their selective attachment to specific basement matrix substrates.

2.3 Rat Glomerular Tissue Preparation

We isolated GEC from rats, which were maintained under specific pathogen-free conditions,

1. Kidneys were obtained from freshly sacrificed rats perfused through the heart with cold **Rat Glomerular Washing Medium** and glomeruli were prepared by a serial sieving method.
2. Glomerular specimens were incubated in 5-10 ml 70% ethanol for 1 min, followed by inoculating with 5-10 ml fresh Rat Glomerular Washing Medium for 5 min.
3. Minced renal cortex tissue was serially passed through 150, 106 and 75 μm mesh stainless steel screens and glomeruli were collected using 53 μm mesh.
4. The glomeruli, which contain small debris of renal tubule, were suspended in **Rat Glomerular Washing Medium**, washed twice by brief centrifugation (800 x g, 1 min)

2.4 GEC Separation and Tissue Dissociation

2.4.1 Rat Glomerular OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the donor used, enzymes, culture mediums and growth supplements. The Rat Glomerular Tissue Dissociation System is suited for optimal dissociation of normal rat glomerular tissues to yield maximum number of single GEC.

2.4.2 Enzyme Compositions

- Collagenase: from Clostridium Histolyticum
- Trypsin

2.4.3 System Components

- Rat Glomerular Tissue Dissociation System, Glomerular OptiTDS™, (2 × 1 ml).
- Rat Glomerular OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

5. Prepare fresh enzyme working solution: Add 1ml of the reconstituted tissue dissociation solution to one vial of Rat Glomerular OptiTDS™ Digestion Buffer (9 ml). Warm the diluted Rat Glomerular OptiTDS™ working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 5 ml Rat Glomerular

OptiTDS™ working solutions.

6. Discard the Rat Glomerular Washing Medium, and float glomeruli on 5 – 10 ml of 1 mg/ml collagenase Dissociation System working solution (prewarmed to 37℃) and transfer the tissue to a sterile glass universal containing a small plastic-coated magnetic stirrer bar. Place the universal on a magnetic stirrer and stir slowly for 30-60 min at 37 ℃. (or incubate in tube at 37 with occasional vortexing)
7. Allow the suspension to stand at room temperature for 2-3 min (or briefly centrifuge Undigested glomeruli were pelleted by).

2.6 GECs Isolation

Note: Please read section 2.6 for specific information on Cervical Epithelium culture and platting before preceding this section.

8. Combine the supernatant containing single cell suspension of GEC, and filter mixture of cells through a 200-mm nylon mesh.
9. Centrifuge at 200 g for 2 min, and the resulting pellet was resuspended in Rat Glomerular PrimaCell™ Basal Culture Medium.
10. Wash GECs twice with Rat Glomerular PrimaCell™ Basal Culture Medium and centrifuge at 200 g for 2 min.

2.6 Primary GECs Culture

2.6.1 Medium Preparation.

Thaw out Rat Glomerular PrimaCell™ Basal Culture Medium, Rat GEC PrimaCell™ Medium Supplements, and Rat Glomerular PrimaCell™ Serum on ice.

Rat Complete Glomerular PrimaCell™ Culture Medium: To every 100 ml Rat Glomerular PrimaCell™ Basal Culture Medium, add 10 ml Rat Glomerular PrimaCell™ Medium Supplements and 1 ml Rat Glomerular PrimaCell™ Serum, mix thoroughly and warm the complete medium at a 37oC water bath for 10 min prior use.

Rat Complete Glomerular PrimaCell™ Culture Medium/FibrOut: To every 100 ml Rat Glomerular PrimaCell™ Basal Culture Medium, add 10 ml Rat Glomerular PrimaCell™ Medium Supplements, 1 ml Rat Glomerular PrimaCell™ Serum, and 1 ml Rat Glomerular Fibroblast Growth Inhibitors, Glomerular FibrOutTM,mix thoroughly and warm the complete medium at a 37oC water bath for 10 min prior use.

2.6.2 Primary Cell Culture

(Important: Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination)

11. Pellets containing single cell suspension of GEC was suspended in Rat Complete Glomerular PrimaCell™ Culture Medium/FibrOut and plated on fibronectin-coated dishes.
12. Small colonies of GEC were observed within 1 week after plating. To remove contaminating mesangial and epithelial cells, brief trypsinization was performed until a culture with purity over 90% of endothelial cells was achieved. The cells were maintained in collagen-coated

culture dish.

Δ Safety Note. The rest of the biopsy and all tubes, pipettes, plates, etc., used in the procedure should be treated with hypochlorite before disposal.

2.7 Subculture

Propagating GECs in culture can be somewhat challenging, especially when GECs are cultured alone on plastic, however the following methods have worked consistently in many laboratories.

13. Remove culture medium, and wash cells with Rat Glomerular PrimaCell™ Basal Culture Medium.
14. Add a small volume of EDTA gently to the cells and remove it immediately.
15. Add sufficient trypsin solution (0.25%) to form a thin layer over the cells.
16. When cells detach, add 5 to 10 ml of Rat Complete Thyroid PrimaCell™ Culture Medium, pass the culture very gently in and out of a pipette, and then centrifuge the cells for 10 min at 350 g.
17. Count an aliquot and seed the cells at the chosen concentration.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Skeletal muscle cells cultures derived from preconfluent layers.

18. Trypsinize cells as above, and centrifuge at 100 g for 10 min.
19. Resuspend cells in Rat Complete Glomerular PrimaCell™ Culture Medium and count cells.
20. Prepare aliquots of 2 × 106 cells/ml in Rat Complete Glomerular PrimaCell™ Culture Medium and 10% glycerol into cryovials.
21. Equilibrate at 4℃ for 1-2 h.
22. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
23. To recover cells:
    (iv) Thaw cryovials quickly in a 37℃ water bath.
    (v) Dilute cells tenfold with appropriate Rat Complete Glomerular PrimaCell™ Culture Medium.
    (vi) Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in Rat Complete Glomerular PrimaCell™ Culture Medium.

IV Characterization

The GECs can be characterized as endothelial cells by homogeneous monolayer of phase contrast image and positive staining with anti-CD31 in the junctional area and negative with anti-desmin. The negative staining with anti-desmin indicates that there was little contamination of mesangial cells in the primary culture. In addition, RT－PCR analysis is used to confirm CD31 expression in the GEC.

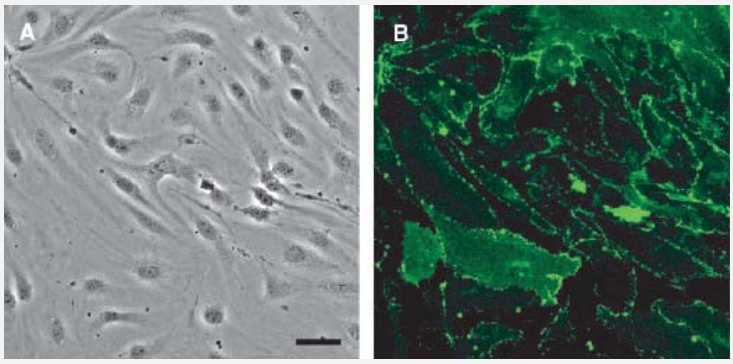


Figure. Phase contrast image of isolated GEC (A) and immunofluorescence image for CD31 (B)[1]

IV Reference

1. Nagao, T., et al., *Up-regulation of adhesion molecule expression in glomerular endothelial cells by anti-myeloperoxidase antibody.* Nephrol Dial Transplant, 2007. **22**(1): p. 77-87.
2. Adler, S., et al., *Complement membrane attack complex stimulates production of reactive oxygen metabolites by cultured rat mesangial cells.* J Clin Invest, 1986. **77**(3): p. 762-7.
3. Hugo, C., et al., *The plasma membrane-actin linking protein, ezrin, is a glomerular epithelial cell marker in glomerulogenesis, in the adult kidney and in glomerular injury.* Kidney Int, 1998. **54**(6): p. 1934-44.

Rat Glomerular PrimaCell™: Glomerular Endothelial cells

Rat Glomerular Primary Cell Culture

Cat No.	Description	Qt.	Price
2-85004	**Rat Glomerular PrimaCell™ system**	**kit**	**$499**
4-27031	Rat Glomerular Tissue Disassociation System, Glomerular OptiTDS™ (for 500 ml medium)	1 ml	$128
9-49003	Rat Glomerular Endothelial Cells PrimaCell™ Basal Culture Medium	500 ml	$ 61
9-38031	Rat Glomerular Endothelial Cells PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$ 140
7-68031	Rat Glomerular Fibroblast Growth Inhibitors, Glomerular FibrOut™	ea	$146
9-97403	Rat Glomerular Tissue Preparation Buffer Set	ea	$75

Rat Primary Glomerular Endothelial Cells Characterization

6-22211	**Rat Glomerular Endothelial Cell Primarker™ Kit**	**kit**	**$220**
6-22212	Rat Glomerular Endothelial Cell Primarker™ antibody set	set	$180
6-22213	Rat Glomerular Endothelial Cell Primarker™ buffer system	set	$90

Rat Heart PrimaCell™ II: Cardiomyocyte
(Cat No. 2-84804)

I. General Description

The muscle cells of heart muscle tissue are called cardiomyocytes. Primary culture of cardiomyocytes has been widely used as a valuable tool for pharmacological and toxicological studies. However, the fact that heart is a solid organ and cardiomyocytes do not proliferate after birth makes the primary myocardial culture a tedious job. The two isolation steps are essential for a successful culture. One is the enzyme digestion step for dissociating cells from heart tissue. The other is the purification step for eliminating non-muscle cells. The latter is critical for ensuring a constant proportion of myocytes.

The Rat Heart PrimaCell™ kit (Cat No. 2-2-84804) is designed for the successful isolation and culture of Cardiomyocytes from rat heart tissue allowing reproducible and dependable results.

1.1 Components of the Rat Heart PrimaCell™ System

- **Rat Heart Tissue Dissociation System, Heart OptiTDS™** (2 × 1 ml) --- *A mixture of collagenase, and Rat Heart OptiTDS™ Reconstitution Buffer.*
- **Rat *Heart OptiTDS™ Digestion Buffer*,** (2 × 9 ml)
- **Rat Heart Fibroblast Growth Inhibitors, Heart FibrOut™**---Heart FibrOut™ (5 x 200 µl) --- *A mixture of D-valine, collagenase and gentamycin.*
- **Rat Cardiomyocyte PrimaCell™ Basal Culture Medium,** (5 × 100 ml) --- *A Modified Ham F12*.
- **Rat Cardiomyocyte PrimaCell™ Medium Supplements,** (5 × 1 ml): *Rat EGF, Hydrocortisone, Cholera toxin penicillin (100 U/ml), streptomycin and Weymouth medium.*
- **Rat Cardiomyocyte PrimaCell™ Serum,** (50 ml): A modified f*etal bovine serum.*
- **Rat Heart Tissue Washing Medium,** (1 × 100 ml): *A modified DMED medium with 10µg/ml amphotericin,* 10*µg/ml gentamycin, and 10% FCS.*

1.2 Required materials but NOT included:

- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or rat fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination.

2.2 Principle

Separation of the Cardiomyocyte from the heart is accomplished by enzymatic digestion using the Heart Tissue Dissociation System supplied in this kit. The Heart Tissue Dissociation System contains a mixture of collagenase and other materials at the optimal concentrations to gently detach the fragile heart layer from the heart tissue. The isolated heart tissue is then further disrupted to release individual Cardiomyocyte by enzymatic and mechanical agitation. The mixture is then filtered through Cell Strainers and seeded on flasks.

2.3 Rat Heart Tissue Preparation

1. Rat pups (Sprague-Dawley or Wistar rats) at the age of postnatal day 1–3 were sacrificed by ethyl ether
2. The animals were decontaminated with 75% ethanol, and transferred to a Luminer flow hood.
3. Surgically remove the beating heart from animals immediately, and keep it in cold **Rat Heart Tissue Washing Medium**
4. Ventricles were excised and transferred to fresh ice-cold **Rat Heart Tissue Washing Medium** and were minced with fine scissors into 1–3 mm3 pieces after washing blood away from the heart lumen. Red blood cells were removed by instant centrifugation for two times.

2.4 Heart Separation and Tissue Dissociation

2.4.1 Rat Heart OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the donor used, enzymes, culture mediums and growth supplements. The Rat Heart Tissue Dissociation System is suited for optimal dissociation of normal rat heart tissues to yield maximum number of single Cardiomyocyte.

2.4.2 Enzyme Compositions

- Collagenase: from *Clostridium Histolyticum*
- Trypsin

2.4.3 System Components

- Rat Heart Tissue Dissociation System, Heart OptiTDS™, (2 × 1 ml).
- Rat Heart OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

5. Prepare fresh enzyme working solutions: to each vial of **Rat Heart Tissue Dissociation System, Bone OptiTDS™**, add 1.0 ml of the **Rat Heart OptiTDS™ Reconstitution Buffer**, Mix well.
6. Add 1.0 ml of the fresh enzyme working solution to one vial of **Rat Heart OptiTDS™ Digestion Buffer** (9 ml). Warm the diluted enzyme working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 5-7 mg tissue samples per 10 ml diluted enzyme working solutions.

7. Transfer the minced tissue to a small sterile bottle with a magnetic stirrer, and add 4 ml of digestion solution. The flask was then settled on ice for 20 min, and shaken every 3 min for better mixing.
8. Stir the solution containing bone fragments at 37℃ for 30 min.
9. Gently pipeting mixture to dispersed cells from the tissue. The cell suspension was settled on ice for 2-3 min
10. Transfer the supernatant to a centrifuge tube and and centrifuge it for 2 min at 580 g at room temperature.
11. Remove the supernatant digestion solution buffer which can be reused, and resuspend the cells in 5 ml of **Rat Heart PrimaCell™ Culture Medium/FibrOut**.
12. In the interim, add an additional 5 ml of digestion solution to the remaining pieces of tissue, and repeat the digestion for 30 min. The released cells are harvested as described in step 12, and, if necessary, the digestion step is repeated several more times.

2.6 Cardiomyocytes Isolation

Note: Please read section 2.6 for specific information on Cervical Epithelium culture and platting before preceding this section.

13. Combine the supension containing single cells, and strain the cell mixture through a sterile cell strainer (70-100μm) into a 50-ml centrifuge tube to remove debris. Cell strainers fit perfectly in 50 ml conical and are very convenient for this procedure. If cell strainers are not available, nylon gauze can be used after rinsed twice in PBS and placed at the opening of a 50 ml conical.
14. Centrifuge the strained mixture at 1000 rmp (800 g) for 5 min at 4°C.
15. Remove the supernatant; add 10 ml of **Rat Heart PrimaCell™ Culture Medium/FibrOut** to the pellet, resuspend the cells vigorously to give a single-cell suspension.

2.6 Primary Cardiomyocytes Culture

2.6.1 Medium Preparation.

Thaw out **Rat Cardiomyocyte PrimaCell™ Basal Culture Medium**, **Rat Cardiomyocyte PrimaCell™ Medium Supplements**, and **Rat CardiomyocytePrimaCell™ Serum** on ice.

Rat Complete Cardiomyocyte PrimaCell™ Culture Medium: To every 100 ml **Rat CardiomyocytePrimaCell™ Basal Culture Medium**, add 10 ml **Rat Cardiomyocyte PrimaCell™ Medium Supplements** and 1 ml **Rat Cardiomyocyte PrimaCell™ Serum** mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

Rat Complete Cardiomyocyte PrimaCell™ Culture Medium/FibrOut: To every 100 ml **Rat Cardiomyocyte PrimaCell™ Basal Culture Medium**, add 10 ml **Rat Cardiomyocyte PrimaCell™ Medium Supplements,** 1 ml **Rat Cardiomyocyte PrimaCell™ Serum**, and 1 ml **Rat Heart Fibroblast Growth Inhibitors, Heart FibrOut™**,mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

2.6.2 Primary Cell Culture

(Important: *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*)

16. The cells were plated in a 40 ml tissue culture flask and incubated at 37 °C in a humidified atmosphere (5% CO2, 95% air).
17. Since non-myocardiocytes attach to the substrata more readily than myocardiocytes, cells were incubated for 1.5 h to allow the attachment of non-myocardiocytes.
18. The majority of myocardiocytes remained in culture medium. The suspended cells were collected and plated at a density of 2 • 105 ml_1 into a new tissue culture flask.
19. Generally, cells isolated from 2 to 3 hearts can be seeded in one 40 ml culture flask.The culture medium was replaced with fresh media every 2-3 days.

Δ ***Safety Note.*** The rest of the biopsy and all tubes, pipettes, plates, etc., used in the procedure should be treated with hypochlorite before disposal.

2.7 Subculture

Propagating Cardiomyocytes in culture can be somewhat challenging, especially when Cardiomyocytes are cultured alone on plastic, however the following methods have worked consistently in many laboratories.

31. Remove culture medium, and wash cells with **Rat Cardiomyocyte PrimaCell™ Basal Culture Medium.**
32. Add a small volume of EDTA gently to the cells and remove it immediately.
33. Add sufficient trypsin solution (0.25%) to form a thin layer over the cells.
34. When cells detach, add 5 to 10 ml of **Rat Complete Cardiomyocyte PrimaCell™ Culture Medium**, pass the culture very gently in and out of a pipette, and then centrifuge the cells for 10 min at 350 g.
35. Count an aliquot and seed the cells at the chosen concentration.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Skeletal muscle cells cultures derived from preconfluent layers.

36. Trypsinize cells as above, and centrifuge at 200 g for 10 min.
37. Resuspend cells in **Rat Complete Cardiomyocyte PrimaCell™ Culture Medium** and count cells.
38. Prepare aliquots of 2×10^6 cells/ml in **Rat Complete Cardiomyocyte PrimaCell™ Culture Medium** and 10% glycerol into cryovials.
39. Equilibrate at 4℃ for 1-2 h.
40. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
41. To recover cells:
    (i) Thaw cryovials quickly in a 37℃ water bath.
    (ii) Dilute cells tenfold with appropriate **Rat Complete Cardiomyocyte PrimaCell™ Culture Medium**.
    (iii) Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in **Rat Complete Cardiomyocyte PrimaCell™ Culture Medium.**

IV Characterization

A-sarcomeric actin is considered as a specific protein in cardiomyocytes, anti-a-sarcomeric actin is applied as the primary antibody to identify cardiomyocytes. Besides, Cardiac troponin I (cTnI), Caveolin-3 is cardiomyocyte specific markers, eHAND, A cardiomyocyte-specific transcription factor, is used to identify cardiomyocyte.

V Reference

1. Fu J., G.J., Pi R. and Liu P., *An optimized protocol for culture of cardiomyocyte from neonatal rat.* Cytotechnology, 2005. **49**: p. 109-116.
2. Kodama, H., et al., *Cardiomyogenic differentiation in cardiac myxoma expressing lineage-specific transcription factors.* Am J Pathol, 2002. **161**(2): p. 381-9.
3. Jiajia Fu, J.G., Rongbiao Pi and Peiqing Liu, *An optimized protocol for culture of cardiomyocyte from neonatal rat.* Cytotechnology, 2005. **49**: p. 109-116.

Mouse Heart PrimaCell™ II: Cardiomyocyte

Mouse Heart Primary Cell II Culture

Cat No.	Description	Qt.	Price
2-84204	**Mouse Heart PrimaCell™ II system**	**kit**	**$499**
4-26231	Mouse Heart Tissue Disassociation System, Heart OptiTDS™ (for 500 ml medium)	1 ml	$128
9-48203	Mouse Cardiomyocyte PrimaCell™ Basal Culture Medium	500 ml	$ 61
9-37231	Mouse Cardiomyocyte PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$ 140
7-67231	Mouse Heart Fibroblast Growth Inhibitors, Heart FibrOut™	ea	$146
9-98203	Mouse Heart Tissue Preparation Buffer Set	ea	$75

Mouse Primary Cardiomyocytes Characterization

6-12311	**Mouse Cardiomyocyte Primarker™ Kit**	**kit**	**$220**
6-12312	Mouse Cardiomyocyte Primarker™ antibody set	set	$180
6-12313	Mouse Cardiomyocyte Primarker™ buffer system	set	$90

Rat Intestine PrimaCell™: Intestinal Epithelial Cells
(Cat No. 2-93203)

I. General Description

Small Intestine Epithelial Cells (IECs) have been difficult to maintain in culture, remaining viable for only hours to several days. Although long term cultures of human and rat small IEC have been established, IEC derived from rats have depended on immortalization by simian virus 40 transfection to remain viable. Successful cultivation of small IEC is dependent on a number of factors such as method of digestion and dissociation; the fine treated and applied serum, and treatment of the culture dishes.

This protocol is developed for attachment and growth of normal Rat Intestine epithelial cells from newborn or adult Rat Intestine with Rat Intestine PrimaCell™ system (Cat No. 2-93203). This system provides an optimal condition of tissue dissociation system, Intestine OptiTDS™ that yields 4-6 times of single cells more than most of the tissue dissociation protocols published in the literature. In addition, this system ensures a high viability of the target cells with improved gradient contained in the culture medium. With CHI's proprietary fibroblast inhibitory system, FibrOut™, cells are growing with contamination of minimized amount of the non-epithelial cells.

The Intestine PrimaCell™ I system is suited for long-term culturing for the monolayer small intestinal epithelium of rat. This kit applies to all types of normal adult rat biopsies samples. Biopsies samples contain pathological organism (virus, parasites, etc.) or tumor may not suitable for this system.

1.3 Components of Rat Kidney PrimaCell™ System

- **Rat Intestine Tissue Dissociation System, Intestine OptiTDS™** ((2 x 1 ml)) --- *A proprietary mixture of Collagenase XIa, Dispase I, Soybean trypsin inhibitor, and BSA.*
- **Rat Intestine OptiTDS™ Digestion Buffer,** (2 x 9 ml)
- **Rat Intestine Fibroblast Growth Inhibitors**, **Intestine FibrOut™** (5 x 200 µl) --- *A mixture of D-valine, collagenase, toxin ricin, and formulated serum.*
- **Rat Intestine Epithelium Basic Culture Medium** (5 x 100 ml) --- *Modified formulation based on DMEM and Ham's F-12*.
- **Rat Intestine Epithelium Culture Medium Supplements with Serum** (5 x 1 ml) --- *A mixture of Insulin, Dexamethasone, Selenium, Transferrin, Triiodothyronine, EGF, HEPES, Glutamine and D-glucose.*
- **Intestinal Epithelium PrimaCell™ Serum** (5 x 10 ml): Charcoal-stripped and highly purified FBS.
- **Buffer Systems for Rat Intestine Preparation** (2 x 100 ml)

1.2 Required Materials NOT provided

- Water bath.
- Pasteur pipettes
- Collagen-I coated culture dishes

- 35-mm tissue culture Petri dishes
- Scalpels, scissors, and forceps
- Pasteur pipettes and 10-ml pipettes
- Test tubes, 12 and 50 ml

II. Procedures

2.1 Preparation of Tissue Culture Plates

All materials used in this experiment must be sterile to prevent contamination. To enhance cell attachment to tissue culture dishes, collagen I pre-coated plates (Corning, NY) MUST be further pre-treated with the Coating Solution included in this kit for 5 min. Aspirate the Coating Solution and allow the dishes to air-dry in a ventilated cell culture hood for 5-10 min before use.

2.2 Surgical specimens

Rat Small Intestines: Adult or newborn (prefer 14-16 days) rat yields a large number of cells (5-10 x 10^7), with a 30-40% plating efficiency. Rats are sacrificed by CO_2 narcosis or a method that is approved by user's institution. Once the intestines is isolated, tissue specimens should be placed immediately in 5-10 ml Intestinal Tissue Washing Medium, transported on ice to the laboratory and worked up within 1 hr of isolation. Intestine tissue specimens are opened longitudinally. With autoclaved scalpels, scissors, and forceps, carefully remove muscle and fat from tissue specimens. Place intestine in a 50 ml conical tube (Falcon) containing 5 ml fresh Intestinal Tissue Washing Medium and incubate while shaking at room temperature for 10 min. For large tissue specimens, use a 50 ml conical tube (Falcon) and a larger volume of Intestinal Tissue Washing Medium to ensure thorough washing. Aspirate the washing medium and repeat the washing procedures using fresh washing medium two more times. After the initial washes, incubate tissue in 70% ethanol for 1 min at room temperature, followed by incubating in 5-10 ml fresh Intestinal Tissue Washing Medium for 5 min. Collect tissue by gentle centrifugation and proceed to dissociation.

2.3 Tissue Preparation and Dissociation

2.3.1 Rat Intestine OptiTDS™

The isolation of primary cells is confounded by several important factors that can greatly affect yield and cell viability. The Intestine Tissue Dissociation System, Intestine OptiTDS™, is developed to produce the optimal conditions that allow for the dissociation of intestinal epithelial cells from normal adult rat tissue samples. This system uses a defined proprietary ratio of specific enzymes to yield the maximum number of single primary cells that remain viable in tissue culture.

2.3.2 Enzyme Compositions

- Collagenase XIa: from *Clostridium Histolyticum*
- Dispase I: from *Bacillus polymyxa*
- Soybean trypsin inhibitor: from *Glycine Max*

2.3.3 System Components

- Intestinal Tissue Dissociation System, OptiTDS™: (2 x 1 ml)
- Intestine OptiTDS™ Digestion Buffer: (2 x 9 ml)

2.3.4 Procedures For Tissue Preparation and Dissociation

8. Prepare Rat Intestine OptiTDS™ working solution: Add 1.0 ml of Rat Intestine Tissue Dissociation System, Intestine OptiTDS™ to 9.0 ml of Rat Intestine OptiTDS™ Digestion Buffer and mix well (Do not Vortex) with hand. It is important that the Rat Intestine OptiTDS™ working solution is freshly prepared and is provided in aliquots for your convenience.
9. Warm the Rat Intestine OptiTDS™ working solution at 37 ℃ for 10 min, just prior to use. For optimal results, we recommend a ratio of 4-5 g intestinal tissue per 10 ml of enzyme solution.
10. Mince the pre-opened and washed intestinal tissue into pieces measuring approximately 2-5 cm in length with scalpel and forceps or scissors.
11. Incubate the minced tissue with the Rat Intestine OptiTDS™ working solution with gentle stirring for 30 min at 37 ℃. Up to 5 g of minced tissue can be incubated with 10 ml of the diluted enzyme solution.
12. Gently pipette sample with a 10 ml pipette, filling the barrel of the pipette at a rate of 2-3 ml per second. Repeat this for 5-6 times.
13. Filter the cell mixture through a cell strainer (100μM). Centrifuge the strained mixture at 100 g. Carefully remove the supernatant and resuspend the cell pellet with1.0 ml of Complete Intestine Culture Medium (See 2.4.1 for preparation).
14. Count and seed the cells in 3 or 4 T-25 collagen I-coated flasks containing 4 ml of Complete Intestine Culture Medium (**Important:** Collagen coated plates must be pre-treated with the provided Coating Solution, see section 2.1). This procedure will give you approximately 2.5-5 x 10^5 Cells/per 5 cm intestinal tissues in length.

2.3.5 Storage of Tissue Dissociation System:

Intestinal Tissue Dissociation System, Intestine OptiTDS™ should only be reconstituted when needed for cell preparation and can be stored for 2-4 days at 4 ^{o}C. For long-term use, it should be aliquoted and stored at -20 ^{o}C. Avoid repeated freeze-thaw cycles.

2.4 Culture of primary rat intestine cells

2.4.1 Medium Preparation

Thaw the Rat Intestinal Epithelium PrimaCell™ Medium Supplements and Rat Intestinal Epithelium PrimaCell™ Serum on ice. To prepare Complete Intestine Culture Media, add one vial of Rat Intestinal Epithelium PrimaCell™ Medium Supplements into 100 ml Rat Intestinal Epithelium PrimaCell™ Basal Culture Medium. Add 10 ml Intestinal Epithelium PrimaCell™ Serum and one vial of Intestine Fibroblast Growth Inhibitors, Intestine FibrOut™. Mix thoroughly and warm the Complete Intestine Media in a 37^{o}C water bath for 10 min prior use. (**Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination).*

2.6.1 Treatment of Culture Dishes

To facilitate primary intestine cell attachment to the tissue culture plates, the plates should be pre-coated with collagen I (available from Corning, NY) and MUST be pre-treated with the provided Coating Solution (Basal Growth Medium containing 10 µg/ml BSA). Cover the entire surface area of tissue culture plates with the Coating Solution and incubate for 5 min. Aspirate the Coating Solution and allow the dishes to air-dry in a ventilated cell culture hood for 5-10 min prior to use.

2.6.1 Standard primary culture conditions

Seed epithelial tubules and cell clumps isolated from intestine tissues (from 2.3.4) into prepared T-25 flasks containing 4 ml of complete media and incubate a 37° C, 5%-CO_2 incubator. The Complete Intestine Culture Medium should be changed twice a week. The tubules and cells will attach to the matrix on the plates and the intestine epithelial cells will begin to migrate out within 1-2 days. Most of the tubules and cell clumps of epithelium will attach within 7 days, however, larger organoids can take up to 6 weeks to attach. It is important to note that the cells remain viable during this time.

2.6.1 Alternative Primary Culture conditions

Primary intestine epithelium requires a substratum to efficiently attach during tissue culture propagation. While this can be achieved using pre-coated Collagen I plates, the growth of primary intestine epithelium is significantly better when a layer of 3T3 feeder cells are used. As the initial intestine epithelial colonies expand to several hundred cells per colony, the cells become less dependent on the 3T3 feeder cells, and can be cultured without the feeder cells. All media and solutions supplied in this kit can be used whether cells are grown on collagen coated plates or 3T3 feeder cells.

2.4.5 Subculture and Propagation

Most intestine primary cells cannot be passaged by routine procedures using trypsin/EDTA. Intestine epithelial cells can be de-attached using a mild dissociation enzyme, dispase as this treatment leads to more successful passaging. A further advantage of using dispase to passage cells is that dispase can only detach epithelial cell but not fibroblast. Thus this property decreases fibroblast contamination of the intestine epithelial cells with each successive passage.

11. Add 0.5% Dispase (Sigma, w/v) to the cell monolayer. Use just enough dispase to cover the cells (~2.5 ml/25-cm^2 flask), and incubate for 40-60 min for primary intestine cultures and 20-40 min for primary intestine cell lines.
12. Once the epithelial layers begin to detach (they do so as sheets rather than single cells), pipette to encourage the detachment and dis-aggregation into smaller clumps.
13. Wash and replate the cells under standard culture conditions. It may take several days for clumps to attach, so take special care when changing the media and feeding the cells.

III Fibroblast Contamination

Several techniques have been published that help prevent fibroblast contamination of primary

intestine cell cultures. These include: (1) Physically removing isolated fibroblast colonies by scraping off the fibroblast colonies with a sterile cell scraper. This method requires extensive washing to ensure that all the lifted fibroblast that remain are washed away and not allowed to reattach and repopulate the primary intestine cultures. (2) Differential trypsinization can be attempted with the carcinomas but may not work well with normal primary intestine cells. (3) Using dispase to lift off intestine epithelial cells preferentially but not exclusively during routine passaging. In addition, cells can be seeded on mock plates for 4-6 hrs so that the fibroblasts attach, and floating intestine cells can be transferred to collagen coated plates under standard culture conditions. This technique takes advantage of the fact that fibroblasts in general attach much more quickly to plastic than do clumps of epithelial cells.
(4) Reducing the concentration of serum to about 2.5-5% to eliminate heavy concentrations of fibroblastic cells. The above methods can be used in combination knowing that normal primary fibroblasts have a finite growth span *in vitro* and if these measures are used persistently the fibroblasts will eventually be forced into senesce. (**Important Note:** The Rat Intestine PrimaCell™ kit includes a fibroblast elimination system Intestine FibrOut™, an effective mixture of Rat Intestine Fibroblast Growth Inhibitors. It contains a mixture of D-valine, collagenase, toxin ricin, and formulated serum. The principle behind the using this system is that low concentration of D-valine is toxic to fibroblast but not epithelial-type cells. Using this approach, therefore, fibroblasts are eliminated without added toxicity to epithelial cells.)

V References:

14. Golaz JL, Vonlaufen N, Hemphill A, Burgener IA. Establishment and characterization of a primary canine duodenal epithelial cell culture.
15. Spottl T, Hausmann M, Gunckel M, Herfarth H, Herlyn M, Schoelmerich J, Rogler G. A new organotypic model to study cell interactions in the intestinal mucosa. Eur J Gastroenterol Hepatol. 2006 Aug;18(8):901-9.
16. Dzierzewicz Z, Orchel A, Swierczek-Zieba G, Latocha M, Cwalina B, Wilczok T. Normal colonocytes in primary culture--an experimental model for molecular pharmacology and biology of large intestine. Acta Pol Pharm. 2000 Nov;57 Suppl:23-5.
17. Follmann W, Weber S, Birkner S. Primary cell cultures of bovine colon epithelium: isolation and cell culture of colonocytes. Toxicol In Vitro. 2000 Oct;14(5):435-45.
18. Slorach EM, Campbell FC, Dorin JR. A rat model of intestinal stem cell function and regeneration. J Cell Sci. 1999 Sep;112 Pt 18:3029-38.
19. Perreault N, Beaulieu JF. Primary cultures of fully differentiated and pure human intestinal epithelial cells. Exp Cell Res. 1998 Nov 25;245(1):34-42.

Rat Intestine PrimaCell™: Intestinal Epithelial Cells

Rat Intestine Primary Cell Culture

Cat No.	Description	Qt.	Price
2-89709	**Rat Intestine PrimaCell™ system**	**kit**	**$499**
4-29781	Rat Intestine Tissue Dissociation System, Intestine OptiTDS™ (for 500 ml medium)	1 ml	$128
9-49708	Rat Intestinal Epithelium PrimaCell™ Basal Culture Medium	500 ml	$61
9-39781	Rat Intestinal Epithelium PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
7-69781	Rat Intestine Fibroblast Growth Inhibitors, Intestine FibrOut™	ea	$146
9-99708	Rat Intestinal Tissue Preparation Buffer Set	ea	$75

Rat Primary Colorectal Epithelium Cell Characterization

6-19711	**Rat Intestinal Epithelium Primarker™ Kit**	**kit**	**$220**
6-19712	Rat Intestinal Epithelium Primarker™ antibody set	set	$180
6-19713	Rat Intestinal Epithelium Primarker™ buffer system	set	$90

Rat Kidney PrimaCell™ I: Kidney Epithelium
(Cat No. 2-82032)

I. General Description

The Rat Kidney PrimaCell™ kit (Cat No. 2-82032) allows the isolation and growth of rat kidney epithelial cells from newborn or adult rats with typical yields of 4-7 times more cells than most protocols published in the literature. In addition, the specially formulated media in the Rat Kidney PrimaCell™ kit ensures high cell viability and the proprietary Kidney Fibroblast Growth Inhibitors, Kidney FibrOut™ minimizes fibroblast contamination of epithelial cultures.

The preparation of tissues is usually started within 1-2 hrs of removal from rats. If this is impossible, tissue can be cut into 1 mm cubes with sterile scalpels and stored overnight at 4°C in the provided washing medium. The Rat Kidney PrimaCell™ system can be used to isolate cells from rat kidneys aged 2-5 months. Kidney samples containing pathological organisms (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Rat Kidney PrimaCell™ System

- **Kidney Tissue Dissociation System**, **Kidney OptiTDS™,** (2 x 1 ml) --- A proprietary mixture of collagenase, collagenase I, collagenase II, collagenase IV, Soybean Trypsin Inhibitor, Dexoyribonuclease I, Trypsin, and Protease.
- **Kidney OptiTDS™ Digestion Buffer,** (2 x 9 ml)
- **Kidney Tissue Washing Medium, (**1 x 100 ml) --- *Basal Kidney PrimaCell™ Culture Medium with 5% FBS, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Kidney Fibroblast Growth Inhibitors, Kidney FibrOut™,** (5 x 200 μl) --- *A proprietary mixture of cis-OH-proline, collagenase, D-valine, and formulated serum substitutes*.
- **Rat Kidney Epithelium PrimaCell™ Basal Culture Medium, (**5 x100 ml) --- *Modified formulation based on medium DMEM and F-12*.
- **Rat Kidney PrimaCell™ Medium Serum, (**1 x 50 ml) --- *Highly purified and special-treated Fetal-bovine serum.*
- **Rat Kidney Epithelium PrimaCell™ Medium Supplements with Serum**, (5 x 10 ml) --- A mixture of insulin, sodium selenite, transferring, 3,3'5-triiodothyronine, highly purified bovine serum albumin, and glutamine, dexamethasone, antibiotics (penicillin, streptomycin), and charcoal-stripped Fetal Bovine Serum.

1.2 Required Materials NOT provided

- Falcon Conicals, 50 ml
- Nylon gauze cell strainer (70-100 μm)
- Plastic tissue culture dishes, collagenase -I coated, 100 mm (Corning, NY)
- Scalpels, curved forceps
- 70% ethanol

- 0.05% EDTA (pH 7.4), sterile
- 0.25% trypsin/0.1% (2.5 mM) EDTA, sterile
- PBSA (PBS containing 10% BSA), sterile

II. Procedures

2.1 Procedure Overview and Materials Preparation

Tissue fragments are excised from the outer cortex of the kidney, minced, washed, and incubated with agitation in Kidney OptiTDS™ solution. The Kidney Dissociation System provides the most optimal conditions to isolate kidney epithelial cells. The kidney epithelium is further dispersed by additional incubations and mechanical disruption. The cells are filtered and platted onto specially treated tissue culture plates. All materials and equipment used in these experiments should be sterilized and rinsed with PBSA prior use.

2.2 Surgical specimens

1. Kidneys from two rats (males and/or females between 2-5 months) are sacrificed using an approved method. The rats are doused with 70% ethanol to minimize contamination. Kidneys are removed using scissors and forceps, immersed in 70% ethanol for 1 min, and immediately placed in a 100-mm tissue culture dish containing 10 ml of Kidney Tissue Washing Medium (each kidney is placed in a separate dish). After all the kidneys are removed, transfers into fresh 100-mm dishes containing 10 ml of Kidney Tissue Washing Medium. Whole kidneys are minced into 1 mm cubes using sterile razor blades. The minced tissues are transferred into sterile 15 ml conical tubes containing Kidney Tissue Washing Medium. After allowing the minced tissue to settle, aspirate off the Kidney Tissue Washing Medium, and wash once more with Kidney Tissue Washing Medium.
2. Incubate kidney pieces sequentially in 10 ml 70% ethanol for 1 min, in 10 ml PBSA for 2 min, and in 20 ml Kidney Tissue Washing Medium for 10 min. These steps reduce the risk of infection and do not interfere with cell viability.
3. Keep tissues on ice until ready to proceed with tissue dissociation.

2.3 Tissue Preparation and Dissociation

2.3.1 Rat Kidney OptiTDS™

Several important factors can affect the yield and viability of primary cells, including the tissue type, species, age of the animals, enzymes, culture media and growth supplements. The Rat Kidney Tissue Dissociation System, OptiTDS™, is optimized for the efficient dissociation of kidney epithelial cells from normal adult and newborn rat kidneys yielding the maximum number of single primary cells.

2.3.2 Enzyme Compositions

- Collagenase: from *Clostridium Histolyticum*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase II: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*
- Soybean Trysin Inhibitor: from *Glycine Max*

- Dexoyribonuclease I: from *Bovine Pancreas*
- Trypsin: from *Bovine Pancreas*
- Protease: from *Staph Aureus*

2.3.3 System Components

- **Kidney Tissue Dissociation System, OptiTDS™,** (2 x 1 ml).
- **Kidney OptiTDS™ Digestion Buffer,** (2 x 9 ml).

2.3.4 Procedures For Tissue Preparation and Dissociation

1 Prepare fresh enzyme working solutions: Add 1.0 ml of Kidney Tissue Dissociation System, OptiTDS™ to 9 ml of Kidney OptiTDS™ Digestion Buffer. Warm this diluted enzyme solution at 37° C for 10 min prior to use. For optimal results we recommend using 10 ml diluted enzyme solution per 2-3 g of tissue.
2 Mince pre-washed tissue into pieces measuring approximately 1 mm with scalpels or sterile razors.
3 Incubate minced tissue with the fresh Kidney Dissociation enzyme working solution (the diluted enzyme solutions prepared in step 1) by one of the following steps:

   a. Rapid dissociation: Incubate kidney tissue in Kidney Dissociation enzyme working solution with rocking for 2-3 hrs at 37° C. This works particularly well with thicker/older kidneys.
   b. Slow dissociation: Incubate kidney tissue in ice-cold Kidney Dissociation enzyme working solution with rocking at 4° C for 15-24 hrs.

4 Prepare Nylon cell strainers by placing inside a 50 ml conical tube. Alternatively, rinse sterile woven cloth 2X in sterile PBSA and place into sterile funnel on top of sterile beaker and use to strain the cell mixture in order to remove debris. Save the Kidney Dissociation enzyme working solution.
5 Tirturate the cell mixture with a sterile pipette several times to facilitate a single cell mixture. Collect the cells by passing the mixture through the Nylon cell strainers into a 50 ml conical.
6 The collected cells are centrifuged at 800 g for 5 min. The resulting pellet contains the primary kidney epithelial cells. This pellet can be resuspended in 5 ml of Complete Kidney Epithelium Medium (see medium preparation below 2.4.1). Save the supernatant for repeating tissue dissociation process in step 4.
7 To the remaining pieces of tissue, repeat the tissue dissociation process with the saved Kidney Dissociation working solutions from step 4 to yield additional cells. This process can repeat up to 3 times if it is necessary without changing the enzyme working solution. In general, kidneys from each 5-6 month old rat should produce six to eight 60-mm dishes of primary culture cells.
8 Count viable cells and plate cells at a density of 1×10^5 cells per 100-mm collagenase -I coated culture dish.

Note: Kidneys can be pooled for this preparation; however, it is very important that the concentration of the dissociation solutions be kept constant at 10 ml /2-3 g kidney tissues.

2.3.5 Storage:

The tissue dissociation systems should be reconstituted just before use and can only be stored for 2-4 days at 4° C. For long-term use, the reconstituted tissue dissociation system solutions should be aliquoted and stored at -20° C. Avoid repeated freeze-thaw cycles.

2.4 Culture of Primary Rat Kidney Epithelium

2.4.1 Medium Preparation

Thaw out Rat Kidney Epithelium PrimaCell™ Basal Culture Medium, 1 ml Rat Kidney Epithelium PrimaCell™ Medium Supplements, and 1 ml Rat Kidney Fibroblast Growth Inhibitors, Kidney FibrOut™ on ice. To every 100 ml Rat Kidney Epithelium PrimaCell™ Basal Culture Medium, add 1 ml of Rat Kidney PrimaCell™ Medium Supplements, 1 ml of Rat Kidney Fibroblast Growth Inhibitors, Kidney FibrOut™, mix thoroughly and warm the Complete Kidney Media at a 37° C water bath for 10 min prior use. (**Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*).

2.4.2 Primary Cell Culture

Primary cultures are incubated at 37 °C, 5% CO^2 incubator for 24 hrs to allow cells to adhere. After 24 hrs the cells should be washed twice with sterile PBS to remove non-adherent cells (i.e. non epithelial cells) and tissue fragments. Return cells to 37° C incubator in Complete Kidney Media described above for another 2–3 days, or until they reach 60–80% confluency. At this time, cells from the same organ are trypsinized, combined, and split into the number of 60-mm tissue-culture dishes required for a single experiment (usually,17–24 dishes). Cells usually require an additional 3-4 days of growth before the appropriate density is reached for experimentation. Please note that individual kidneys can be kept separate or pooled if the concentrations of reagents are scaled up as described per kidney.

2.4.3 Subculture and Propagation

9. Gently rinse the culture dish twice with 0.02% (0.7 mM) EDTA.
10. Add 3 ml of 0.25% trypsin/0.1% (2.5 mM) EDTA, and incubate at 37° C. Examine the dish under a microscopy every 5 min to detect cell detachment.
11. When most cells have detached, add 10 ml Complete Kidney Media to inactive the trypsin.
12. Pipette the contents of the dish to ensure all the cells have detached.
13. Collect the cells and centrifuge at 350 g for 5 min.
14. Aspirate the supernatant; resuspend the cell pellet in a Complete Kidney Media, and plate at a density of 1×10^5 cells per cm^2.
15. Feed the cultures twice a week with Complete Kidney Media.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample; the best results are obtained from preconfluent primary cultures.
16. Trypsinize cells using method used above, and centrifuge cells at 100 g for 10 min.
17. Resuspend the cell pellet in Complete Kidney Media with serum, and count cells.

18. Dispense aliquots of 2 x 10^6 cells/ml in Complete Kidney Medium supplemented with an additional 10% FBS and 10% glycerol into cryopreservation vials.
19. Equilibrate at 4° C for 1-2 hrs.
20. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of one degree per min.

To recover cells:

(i) Thaw cryovials quickly in a 37° C water bath.
(ii) Dilute cells tenfold with medium.
(iii) Centrifuge cells; resuspend them at an appropriate density in Complete Kidney Media and plate.

Rat cells can be grown for several weeks and can be subcultured only 4-6 passages, in Complete Kidney Media.

IV Fibroblast Contamination

Several techniques have been published in the literature to address fibroblast contamination of primary kidney primary cell cultures. These include (1) Physically removing well-isolated fibroblast colonies by scraping and following with several careful washes to remove any fibroblasts that have detached (2) Differential trypsinization of carcinomas (3) Using dispase to preferentially (but not exclusively) to remove epithelial cells during passaging. During subculture, cells that have been removed with dispase can be pre-incubated in plastic culture dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the epithelium. This technique takes advantage of the fact that fibroblasts in general attach much more quickly to plastic than do clumps of epithelial cells, so that a partial purification step is possible. (4) Reduce the concentration of serum between 2.5-5% to discourage fibroblast growth. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and that using any or all of the preceding techniques will eventually push fibroblasts through enough divisions to allow the fibroblasts to senesce.

The Rat Kidney PrimaCell™ I system includes a unique fibroblast elimination system, the Rat Kidney Fibroblast Growth Inhibitors, Kidney FibrOut™. It contains a proprietary mixture of cis-OH-proline, collagenase, D-valine, and formulated serum substitutes that most effectively eliminate kidney fibroblast contaminations and does not interfere with the propagation and biology of kidney epithelial cells.

V References:

1. Keynan S, Asipu A, Hooper NM, Turner AJ, Blair GE. *Stable and temperature-sensitive transformation of baby rat kidney cells by SV40 suppresses expression of membrane dipeptidase.* Oncogene. 1997 Sep 4;15(10):1241-5.
2. Lynch MJ, Trainer DL. *Immortalization of primary baby rat kidney cells by retroviral mediated gene transfer of adenovirus E1A genes.* Cancer Res. 1989 Oct 1;49(19):5429-34.
3. Elliget KA, Trump BF. *Primary cultures of normal rat kidney proximal tubule epithelial cells for studies of renal cell injury.* In Vitro Cell Dev Biol. 1991 Sep;27A(9):739-48.
4. Ritter D, Needleman P, Greenwald JE. *Synthesis and secretion of an atriopeptin-like protein in rat kidney cell culture.* J Clin Invest. 1991 Jan;87(1):208-12.
5. Chen ML, King RS, Armbrecht HJ. *Sodium-dependent phosphate transport in primary cultures of renal tubule cells from young and adult rats.* J Cell Physiol. 1990

Jun;143(3):488-93.
6. Larsson SH, Yun S, Kolare S, Aperia A. *Post-natal changes in growth of rat proximal tubule cells: a study of cells in short primary culture.* Acta Physiol Scand. 1990 Feb;138(2):243-4.

Rat Kidney PrimaCell™ Kidney Epithelium

Rat Kidney Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82032	**Rat Kidney PrimaCell™ system**	**kit**	**$550**
4-21121	Rat Kidney Tissue Dissociation System, Kidney OptiTDS™ (for 500 ml medium)	1 ml	$ 128
9-41012	Rat Kidney Epithelium PrimaCell™ Basal Culture Medium	500 ml	$73
9-31121	Rat Kidney Epithelium PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$ 140
7-61121	Rat Kidney Fibroblast Growth Inhibitors, Kidney FibrOut™	ea	$ 146
9-91012	Rat Kidney Tissue I Preparation Buffer Set	ea	$75

Rat Primary Kidney Epithelium Characterization

6-21411	**Rat Kidney Epithelium Primarker™ Kit**	**kit**	**$220**
6-21412	Rat Kidney Epithelium Primarker™ antibody set	set	$180
6-21413	Rat Kidney Epithelium Primarker™ buffer system	set	$90

Rat Kidney PrimaCell™ II: Proximal Tubular Cells
(Cat No. 2-85204)

I. General Description

Renal cell culture remains an essential tool to investigate kidney cell function, transport processes and a variety of cytotoxic or ischemic effects. Conventional primary cultures of rat proximal tubular cells (PTC) are time-consuming, have low yields of starting material, and expose the cells to oxidative or mechanical aggression that influence cell differentiation.

While these cells are extremely useful in the laboratory they are notoriously difficult to isolate and culture. They rapidly divide, become fibroblastic, and lose their biochemical characteristics. Our protocol is a combination of careful dissection of the kidney, enzymatic digestion and sieving. The Rat Kidney Cortice PrimaCell™ kit (Cat No. 2-85204) is designed for the successful isolation and culture of PTCs from rat kidney cortice tissue allowing reproducible and dependable results.

1.1 Components of the Rat Kidney cortice PrimaCell™ System

- **Rat Kidney Cortice Tissue Dissociation System, Kidney cortice OptiTDS™** (2 × 1 ml) --- *A mixture of collagenase, and Rat Kidney cortice OptiTDS™ Reconstitution Buffer.*
- **Rat *Kidney cortice OptiTDS™ Digestion Buffer,*** (2 × 9 ml)
- **Rat Kidney cortice Fibroblast Growth Inhibitors, Kidney cortice FibrOut™---**Kidney cortice FibrOut™ (5 x 200 µl) --- *A mixture of D-valine, collagenase and gentamycin.*
- **Rat PTC PrimaCell™ Basal Culture Medium,** (5 × 100 ml) --- *A Modified Ham F12*.
- **Rat PTC PrimaCell™ Medium Supplements,** (5 × 1 ml): *Rat EGF, Hydrocortisone, Cholera toxin penicillin (100 U/ml), streptomycin and Weymouth medium.*
- **Rat PTC PrimaCell™ Serum,** (50 ml): A modified f*etal bovine serum.*
- **Rat Kidney Cortice Tissue Washing Medium,** (1 × 100 ml): *A modified DMED medium with 10µg/ml amphotericin,* 10*µg/ml gentamycin, and 10% FCS.*

1.2 Required materials but NOT included:

- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or rat fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination.

2.2 Principle

Separation of the PTC from the Kidney Cortice is accomplished by enzymatic digestion using the Kidney cortice Tissue Dissociation System supplied in this kit. The Kidney Cortice Tissue Dissociation System contains a mixture of collagenase and other materials at the optimal concentrations to gently detach the fragile kidney cortice layer from the kidney cortice tissue. The isolated kidney cortice tissue is then further disrupted to release individual PTC by enzymatic and mechanical agitation. The mixture is then filtered through Cell Strainers and seeded on flasks.

2.3 Rat Kidney Cortice Tissue Preparation

1. Rats were killed at 3-4 weeks of age.
2. Surgically remove the renal cortices from animals immediately, and keep it in cold **Rat Kidney Cortice Tissue Washing Medium**
3. Renal cortices were dissected visually in fresh ice-cold **Rat Kidney Cortice Tissue Washing Medium** and sliced into pieces of approximately 1mm wide.

2.4 Kidney cortice Separation and Tissue Dissociation

2.4.1 Rat Kidney Cortice OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the donor used, enzymes, culture mediums and growth supplements. The Rat Kidney Cortice Tissue Dissociation System is suited for optimal dissociation of normal rat kidney cortice tissues to yield maximum number of single PTC.

2.4.2 Enzyme Compositions

- Collagenase: from *Clostridium Histolyticum*
- Trypsin: from *Clostridium Histolyticum*

2.4.3 System Components

- Rat Kidney cortice Tissue Dissociation System, Kidney cortice OptiTDS™, (2 × 1 ml).
- Rat Kidney cortice OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

4. Prepare fresh enzyme working solutions: to each vial of **Rat Kidney Cortice Tissue Dissociation System, Bone OptiTDS™**, add 1.0 ml of the **Rat Kidney Cortice OptiTDS™ Reconstitution Buffer**, Mix well.
5. Add 1.0 ml of the fresh enzyme working solution to one vial of **Rat Kidney Cortice OptiTDS™ Digestion Buffer** (9 ml). Warm the diluted enzyme working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 mg tissue samples per 5 ml diluted enzyme working solutions.
6. Transfer the minced tissue to a small sterile bottle with a magnetic stirrer, and add 5 ml of digestion solution.
7. Stir the solution containing bone fragments at 37℃ for 30 min.

8. The cell suspension was settled on ice for 2-3 min

2.6 PTCs Isolation

Note: Please read section 2.6 for specific information on Cervical Epithelium culture and platting before preceding this section.

9. The supernatant containing single cells, and strain the cell mixture through two sterile cell strainers (250μm and 80μm) to remove debris and substantial contamination of other nephron segments or glomeruli. The longer proximal tubule fragments remained in the 80μm sieve.
10. Wash cells in 80μm sieve with **Rat Kidney Cortice Tissue Washing Medium.**
11. Resuspend cells by flushing the sieve in the reverse direction with **Rat Kidney Cortice Tissue Washing Medium**.
12. Centrifuge the PTCs at 200 g for 5 min at 4°C and resuspend cells with **Rat Kidney Cortice Tissue Washing Medium**.

2.6 Primary PTCs Culture

2.6.1 Medium Preparation.

Thaw out **Rat PTC PrimaCell™ Basal Culture Medium**, **Rat PTC PrimaCell™ Medium Supplements**, and **Rat PTCPrimaCell™ Serum** on ice.

Rat Complete PTC PrimaCell™ Culture Medium: To every 100 ml **Rat PTCPrimaCell™ Basal Culture Medium**, add 10 ml **Rat PTC PrimaCell™ Medium Supplements** and 1 ml **Rat PTC PrimaCell™ Serum** mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use.

Rat Complete PTC PrimaCell™ Culture Medium/FibrOut: To every 100 ml **Rat PTC PrimaCell™ Basal Culture Medium**, add 10 ml **Rat PTC PrimaCell™ Medium Supplements,** 1 ml **Rat PTC PrimaCell™ Serum**, and 1 ml **Rat Kidney Cortice Fibroblast Growth Inhibitors, Kidney Cortice FibrOut™**,mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use. (**Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*)

2.6.2 Primary Cell Culture

13. The PTCs present in **Rat Kidney cortice Tissue Washing Medium** were centrifuged for 5min at 200 x g, and then resuspended into **Rat PTC PrimaCell™ Culture Medium/FibrOut**.
14. The cells were plated in a collagen-coated permeable PTFE-filter supports and incubated at 37 °C in a humidified atmosphere (5% CO2, 95% air).
15. The culture medium was replaced with fresh media every 2-3 days. After 7 days, cell cultures were organized as a confluent monolayer.

Δ ***Safety Note.*** The rest of the biopsy and all tubes, pipettes, plates, etc., used in the procedure should be treated with hypochlorite before disposal.

2.7 Subculture

Propagating PTCs in culture can be somewhat challenging, especially when PTCs are cultured

alone on plastic, however the following methods have worked consistently in many laboratories.

27. Remove culture medium, and wash cells with **Rat Thyroid PrimaCell™ Basal Culture Medium.**
28. Add a small volume of EDTA gently to the cells and remove it immediately.
29. Add sufficient trypsin solution (0.25%) to form a thin layer over the cells.
30. When cells detach, add 5 to 10 ml of **Rat Complete Thyroid PrimaCell™ Culture Medium**, pass the culture very gently in and out of a pipette, and then centrifuge the cells for 10 min at 350 g.
31. Count an aliquot and seed the cells at the chosen concentration.

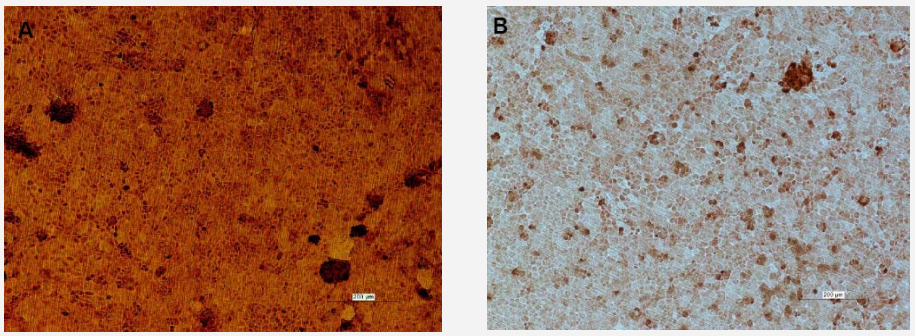
III Cryopreservation

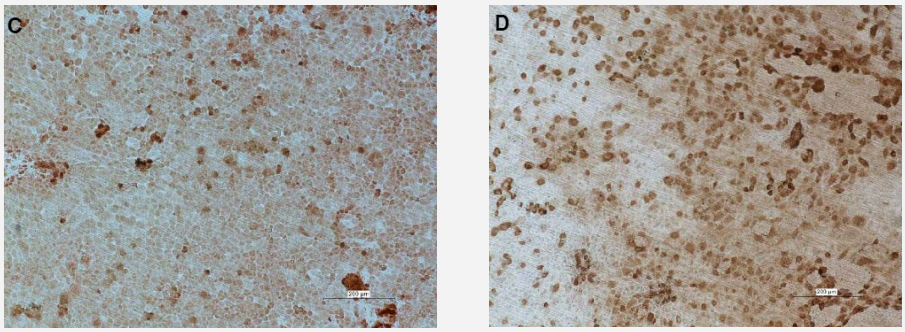
Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Skeletal muscle cells cultures derived from preconfluent layers.

32. Trypsinize cells as above, and centrifuge at 200 g for 10 min.
33. Resuspend cells in **Rat Complete PTC PrimaCell™ Culture Medium** and count cells.
34. Prepare aliquots of 2×10^6 cells/ml in **Rat Complete PTC PrimaCell™ Culture Medium** and 10% glycerol into cryovials.
35. Equilibrate at 4℃ for 1-2 h.
36. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
37. To recover cells:
    a. Thaw cryovials quickly in a 37℃ water bath.
    b. Dilute cells tenfold with appropriate **Rat Complete PTC PrimaCell™ Culture Medium**.
    c. Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in **Rat Complete PTC PrimaCell™ Culture Medium.**

IV Characterization

At low magnification, single cells grew as a homogeneous monolayer of cells with cobblestone-like appearance. The cells were structurally polarized: the apical pole, facing the free surface, differed from the basal pole, toward the underlying support layer. The presence of specific markers, like transporter proteins or brush border enzymes, was established to characterize these primary cultures. The PTC shows a clear immunoreactivity against AQP1, while no positive signal for early distal tubule expressing AQP2 or NCC was obtained. The multiligand receptor, megalin, is located at the brush border of PT cells and participates in protein reabsorption from the primitive urine. The chloride channel, ClC-5, and the vacuolar proton pump, V-ATPase, are both localized in endosomes ensuring correct endosomal acidification and protein trafficking in PT cells. All of these components of the endocytic apparatus were detected in primary PT cells





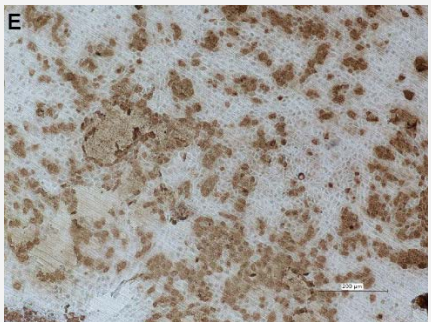


Figure. Immunostaining of 7-days old primary PTC with primary antibodies raised against AQP1 (A), ClC-5 (B), megalin (C), SGLT1 (D) or SGLT2 (E).[1]

V Reference

1. Terryn, S., et al., *A primary culture of rat proximal tubular cells, established on collagen-coated membranes.* Am J Physiol Renal Physiol, 2007.
2. Pollock, C.A. and M.J. Field, *Compensatory renal hypertrophy: tubular cell growth and transport studied in primary culture.* Nephron, 1993. **64**(4): p. 615-20.
3. Fine, L.G. and L.M. Sakhrani, *Proximal tubular cells in primary culture.* Miner Electrolyte Metab, 1986. **12**(1): p. 51-7.
4. Qi, W., et al., *Isolation, propagation and characterization of primary tubule cell culture from human kidney.* Nephrology (Carlton), 2007. **12**(2): p. 155-9.

Rat Kidney PrimaCell™ II: Proximal Tubular Cells

Rat Kidney Primary Cell II Culture

Cat No.	Description	Qt.	Price
2-85204	**Rat Kidney PrimaCell™ II system**	**kit**	**$499**
4-27231	Rat Kidney Tissue Disassociation System II, Kidney OptiTDS™ (for 500 ml medium)	1 ml	$128
9-49203	Rat Kidney Proximal Tubular Cells PrimaCell™ Basal Culture Medium	500 ml	$ 61
9-38231	Rat Kidney Proximal Tubular Cells PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$ 140
7-68231	Rat Kidney Fibroblast Growth Inhibitors II, Kidney FibrOut™	ea	$146
9-99203	Rat Kidney Tissue II Preparation Buffer Set	ea	$75

Rat Primary Kidney Proximal Tubular Cells Characterization

6-22411	**Rat Proximal Tubular Cell Primarker™ Kit**	**kit**	**$220**
6-22412	Rat Proximal Tubular Cell Primarker™ antibody set	set	$180
6-22413	Rat Proximal Tubular Cell Primarker™ buffer system	set	$90

Rat Liver PrimaCell™: Hepatocytes
(Cat No. 2-82033)

I. General Description:

The basic two-step perfusion procedure can be used to isolate hepatocytes from the livers of rats, rabbits, guinea pigs, or woodchucks, by proportionally adapting the volume and flow rate of the solutions to the size of the liver. The general technique used for livers involves perfusion of the whole liver or a biopsy sample (15-30 ml/min, depending on the size of the tissue sample). Complete isolation of hepatocytes into a single-cell suspension can be obtained by an additional incubation at 37℃ with collagenase under gentle stirring for 10- 20 min (especially for human liver). Fish hepatocytes can be isolated by cannulation of the intestinal vein and incision of the heart to avoid excessive pressure. Perfusion is performed at room temperature at a flow rate of 12-15 ml per min.

This protocol has been developed for the attachment and growth of normal rat liver hepatocytes from adult rat liver tissue using the Rat Liver PrimaCell™ system (Cat No. 2-82033). This system provides the optimal tissue dissociation system, Liver OptiTDS™ that yields 4-7 times the single cells than most tissue dissociation protocols published in the literature. CHI's proprietary fibroblast inhibitory system, Liver FibrOut™, allows for minimal to no contamination of the hepatocyte cultures by non-epithelial cells types. In addition, the media and supplements provided have been supplied to ensure a robust culture of hepatocytes for experimentation.

The preparation of tissue specimens for cell culture should be started within 1-2 h of organ removal or sacrifice of the animal. If this is impossible, store the tissue overnight at 4℃ in washing medium (see below) as this has also given satisfactory results.

Rat Liver PrimaCell™ system applies to normal adult rat liver samples. Livers containing pathological organisms (virus, parasites, etc.) or tumor may not be suitable for this system.

1.1 Components of Rat Liver PrimaCell™ System

- **Rat Liver Tissue Dissociation System, Liver OptiTDS™,** (2 x 1 ml) *A proprietary mixture of collagenase, collagenase I, collagenase II, collagenase IV, Hyaluronidase I, andDispase*
- **Rat Liver OptiTDS™ Digestion Buffer**, (4 x 100 ml)
- **Rat Liver Tissue Washing Medium**, (2 x 100 ml) *Basal Liver PrimaCell™ Culture Medium with NaCl, KCl, Na2HPO4.12H2O, HEPES, and 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*
- **Rat Liver Fibroblast Growth Inhibitors, Liver FibrOut™** (5 x 200 µl) *A mixture of D-valine, collagenase, and formulated serum substitutes*
- **Rat Liver PrimaCell™ Basal Culture Medium**, (5 x100 ml) *Modified formulation based on Ham's F12 and Weymouth medium*
- **Rat Liver PrimaCell™ Medium Supplements with Serum**, (5 x 1.0 ml) A mixture of *Basal culture medium containing bovine albumin (grade V), bovine Insulin, penicillin, streptomycin, EGF, dexamethasone, pyruvate and formulated serum substitutes*

1.2 Required Materials not provided

- Tygon tube (ID, 3.0 mm; OD, 5.0mm)
- Disposable scalp vein infusion needles, 20G
- Sewing thread for cannulation
- Graduated bottles and Petri dishes
- Surgical instruments (Sharp, straight, and curved scissors and clips)
- 2 × 1-ml disposable syringes
- Collagen I-coated plate (Corning, NY)
- Chronometer
- Peristaltic pump (10 to 200 rpm)
- Water bath

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, collagen I-coated plate (Corning, NY) MUST be pre-treated with the Rat Liver PrimaCell™ Basal Culture Medium and incubated for 5 min. After 5 min incubation aspirate the Rat Liver PrimaCell™ Basal Culture Medium and allow the dishes to air-dry in a ventilated cell culture hood for 5-10 min.

2.2 Surgical specimens

Perfusion of liver tissue must be performed within 2 hours of the rat being anesthetized to ensure a smooth and efficient perfusion. Immediately after the rat is anesthetized, open the abdomen, places a loosely tied ligature around the portal vein (approximately 5 mm from the liver), insert the cannula up to the liver, and ligate. Briefly dip the liver tissue in 70% ethanol for 1 min, followed by a rinse with 10 ml PBS and 10 ml Liver Tissue Washing Medium. Initiate the perfusion with 100 ml Liver Tissue Washing Medium, followed by 100 ml of Liver OptiTDS™ Digestion Buffer, and finally by 300 ml 100 ml of Liver OptiTDS™ Digestion working solution (see below for details).

2.3 Tissue Preparation and Dissociation

2.3.1 Rat Liver OptiTDS™

Several important factors can affect yield and viability of primary cell culture, including tissue type, origin of species, tissue age, enzymes, culture medium and growth supplements. The Liver Tissue Dissociation System, OptiTDS™, is suited for optimal dissociation of whole liver or a biopsy sample of a normal adult rat liver to yield the maximum number of single primary hepatocytes.

2.3.2 Enzyme Compositions

- Collagenase: from *Clostridium Histolyticum*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase II: from *Clostridium Histolyticum*

- Collagenase IV: from *Clostridium Histolyticum*
- Hyaluronidase I: from *Bovine Testes*
- Dispase: from *Bacillus polymyxa*

2.3.3 System Components

- Liver Tissue Dissociation System, OptiTDS™, (2 x 1.0 ml)
- Liver OptiTDS™ Digestion Buffer, (4 x 100 ml)

2.3.4 Procedures For Tissue Preparation and Dissociation

1. Warm the 200 ml Liver Tissue Washing Medium and 400 ml Liver OptiTDS™ Digestion Buffer in a water bath (usually approximately 38-39℃ to achieve 37℃ in the liver) for 10 min. Oxygenation is not necessary. Combine 300 ml Liver OptiTDS™ Digestion Buffer and 1 ml Liver Tissue Dissociation System, OptiTDS™, mix well and allow to incubate in the water bath until use. The remaining 100 ml Liver OptiTDS™ Digestion Buffer will be used for flushing the portal vein described in step 5 below.
2. Set the pump flow rate at 30 ml/min.
3. Anesthetize the rat (180-200 g) by intraperitoneal injection of Nembutal (100 µl/100 g), and inject heparin into the femoral vein (1,000 IU).
4. Open the abdomen, tie a loose ligature around the portal vein (approximately 5 mm from the liver), insert the cannula up to the liver and ligate.
5. Rapidly make an incision in the sub-hepatic vessels to avoid excess pressure, and start the perfusion with 100 ml Liver Tissue Washing Medium followed by 100 ml of Liver OptiTDS™ Digestion Buffer (without adding tissue dissociation enzymes) at a flow rate of 30 ml/min; verify that the liver whitens within a few seconds.
6. Perfuse 300 ml of the Liver Tissue Dissociation System, OptiTDS™ (working solution prepared in step 1) at a flow rate of 15 ml/min for 30 min. The liver should swell.
7. Remove the liver and wash with 100 ml Liver Tissue Washing Medium; after disrupting the Glisson capsule, disperse the cells in 100 ml of complete Rat Hepatocytes PrimaCell™ Medium (see preparation below in 2.4.1).

2.3.5 Storage:

Tissue dissociation systems should be reconstituted just before use and should only be stored for 2-4 days at 4 ^{o}C. For long-term use, solutions should be aliquoted and stored at -20 ^{o}C. Avoid repeated freeze-thaw cycles.

2.4 Culture of primary Rat Liver cells

2.4.1 Medium Preparation

Thaw out the Hepatocytes PrimaCell™ Medium Supplements with Serum and Liver Fibroblast Growth Inhibitors, Liver FibrOut™ on ice. To every 100 ml Rat Hepatocytes PrimaCell™ Basal Culture Medium add 1 ml of Rat Hepatocytes PrimaCell™ Medium Supplements with Serum; and 1 ml of Liver Fibroblast Growth Inhibitors, Liver FibrOut™, mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use. (**Important:** *Long term use of FibrOut™ in the culture medium may result in loss of targeted primary cells, therefore, we recommend using FibrOut™ in 3-5 cycles of changing culture medium or to until an acceptable level of fibroblasts is attained*).

2.4.2 Treatment of Culture Dishes

To enhance cell attachment to the culture dishes, collagen I-coated plate (Corning, NY) MUST be pre-treated with the complete Rat Hepatocytes PrimaCell™ Culture Medium (enough to cover the whole cell-growth area) and incubated for 5 min. After a 5 min incubation, aspirate the medium and allow the dishes to air-dry in a ventilated cell culture hood for 5-10 min.

2.4.3 Standard primary culture conditions

8. Filter the suspension through two-layers of gauze or 60-80-μm nylon mesh and allow the viable cells to sediment for 20 min (usually at room temperature). Discard the supernatant (60 ml) containing debris and dead cells.
9. Wash the cells twice with 1 x PBS and once with complete Rat Hepatocytes PrimaCell™ Medium by slow centrifugations (50 g for 40 s) to remove tissue dissociation enzymes, damaged cells, and non-parenchymal cells.
10. Collect the hepatocytes in 10 ml complete Rat Hepatocytes PrimaCell™ Medium.
11. Count the cells and seed at 5-7 x 10^5 cells/100-mm dishes. Isolated Hepatocytes can grow for 4-6 days and undergo 1-3 round of cell division on collagen I-coated culture dish without obvious differentiation. In our experiments freshly isolated rat hepatocytes were seeded at a density of 2×10^5 to 3×10^5 cells/ml in medium on collagen-coated plates. Confluence after plating was 80–90%, with hepatocyte viability of greater than 90% as assessed by Trypan blue exclusion. After one round of plating, hepatocytes were maintained in the complete culture medium without the addition of Liver FibrOut™. Medium was changed daily.

2.4.4 Alternative Primary Culture conditions

The attachment of hepatocytes during primary culture and subculture is more reproducible and efficient when cells are inoculated onto collagen-coated flasks. When short-term (4-6 hours) growth of cell is acceptable for the experiment, plain culture dish without biomatrix can be used.

2.4.5 Subculture and Propagation

Most primary cultures cannot be passaged presently using routine trypsin / EDTA procedures. Disaggregation to single cells of the cultured liver cells with 0.1% trypsin in 0.25 mM (0.1%) EDTA will result in extremely poor or no growth. To avoid these consequences Dispase is used. An advantage with this procedure is that dispase can only efficiently detach epithelial cell but not fibroblast, thereby, increasing the purity of the target cell population.

12. Add 0.5% Dispase (Sigma, w/v) to the cell monolayer, just enough to cover the cells (~2.5 ml/25-cm^2 flask), and leave the solution to stand for 40-60 min for primary cultures and 20-40 min for cell lines.
13. Once the epithelial layers begin to detach (they do so as sheets rather than single cells), pipette to help detachment and dis-aggregation into smaller clumps.
14. Wash and replate the cells under standard culture conditions. It may take several days for clumps to attach, so replace the medium carefully when feeding.

III Fibroblast Contamination

There are several techniques have been published in the literature to deal with fibroblast

contamination during primary cell culture. These include: (1) Physically remove a well-isolated fibroblast Livery by scraping it with a sterile blunt instrument (e.g., a cell scraper). Care has to be taken to wash the culture up to six times to remove any fibroblasts that have detached in order to prevent them from reseeding and reattaching to the flask. (2) Differential trypsinization can be attempted with the carcinomas. (3) Dispase preferentially (but not exclusively) removes the epithelium during passaging and leaves behind most of the fibroblastic cells attached to the culture vessel. During subculture, cells that have been removed with dispase can be preincubated in plastic Petri dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the epithelium. Clumps of epithelial cells still floating can be transferred to new flasks under standard culture conditions. This technique takes advantage of the fact that fibroblasts in general attach more quickly to plastic than do clumps of epithelial cells, so that a partial purification step is possible. (4) Reduce the concentration of serum to about 2.5-5%, if there are heavy concentrations of fibroblasts. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and using any or all of the techniques mentioned above will eventually push the cells through so many divisions that the fibroblasts will eventually senesce.

Rat Liver PrimaCell™ includes a fibroblast elimination system, the Rat Liver Fibroblast Growth Inhibitors, Liver FibrOut™. It contains a mixture of D-valine, collagenase, and formulated serum substitutes. The FibrOut™ kills contaminating fibroblasts, but shows no signs of toxicity toward the target cells, whether derived from an adenoma, a carcinoma or normal liver tissues.

IV References:

1. Seglen PO. *Protein degradation in isolated rat hepatocytes is inhibited by ammonia.* Biochem Biophys Res Commun. 1975 Sep 2;66(1):44-52.
2. Gaboriau F, Laupen-Chassay C, Pasdeloup N, Pierre JL, Brissot P, Lescoat G. *Modulation of cell proliferation and polyamine metabolism in rat liver cell cultures by the iron chelator O-trensox.* Biometals. 2006 Dec;19(6):623-32. Epub 2006 Aug 31.
3. Kamiya T, Kwon AH, Kanemaki T, Matsui Y, Uetsuji S, Okumura T, Kamiyama Y. *A simplified model of hypoxic injury in primary cultured rat hepatocytes.* In Vitro Cell Dev Biol Anim. 1998 Feb;34(2):131-7.
4. Tokiwa T, Kano J, Meng XY, Kodama M. *Primary culture of adult rat hepatocytes on porous expanded polytetrafluoroethylene.* Transplant Proc. 1997 Jun;29(4):2120-2.
5. Zurlo J, Arterburn LM. *Characterization of a primary hepatocyte culture system for toxicological studies.* In Vitro Cell Dev Biol Anim. 1996 Apr;32(4):211-20.
6. Kocarek TA, Schuetz EG, Guzelian PS. *Biphasic regulation of cytochrome P450 2B1/2 mRNA expression by dexamethasone in primary cultures of adult rat hepatocytes maintained on matrigel.* Biochem Pharmacol. 1994 Nov 1;48(9):1815-22.

Rat Liver PrimaCell™: Hepatocytes

Rat Liver Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82033	**Rat Liver PrimaCell™ system**	**kit**	**$499**
4-21131	Rat Liver Dissociation System, Muscle OptiTDS™ (for 500 ml medium)	1 ml	$128
9-41013	Rat Hepatocytes PrimaCell™ Basal Culture Medium	500 ml	$73
9-31131	Rat Hepatocytes PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
7-61131	Rat Liver Fibroblast Growth Inhibitors, Liver FibrOut™	ea	$146
9-91013	Rat Liver Tissue Preparation Buffer Set	ea	$75

Rat Primary Hepatocytes Epithelium Characterization

6-21511	**Rat Hepatocyte Primarker™ Kit**	**kit**	**$220**
6-21512	Rat Hepatocyte Primarker™ antibody set	set	$180
6-21513	Rat Hepatocyte Primarker™ buffer system	set	$90

Rat Lung PrimaCell™: Alveolar Epithelial Cell II
(Cat No. 2-84704)

I. General Description

Alveolar epithelial type II (AEC II) is small, cuboidal cells that constitute ~60% of the pulmonary alveolar epithelium. These cells are crucial for repair of the injured alveolus by differentiating into alveolar epithelial type I cells. AEC II cells are a promising source of cells that could be used therapeutically to treat distal lung diseases. While these cells are extremely useful in the laboratory they are notoriously difficult to isolate and culture.

The Rat Alveolar PrimaCell™ kit (Cat No. 2-84704) is designed for the successful isolation and culture of AEC IIs from rat tissue allowing reproducible and dependable results.

1.1 Components of the Rat Alveolar PrimaCell™ System

- **Rat Alveolar Tissue Dissociation System, alveolar OptiTDS™** (2 × 1 ml) --- *A mixture of collagenase and Rat Alveolar OptiTDS™ Reconstitution Buffer.*
- **Rat Alveolar *OptiTDS™ Digestion Buffer*,** (2 × 9 ml)
- **Rat Alveolar Fibroblast Growth Inhibitors, alveolar FibrOut™---**Alveolar FibrOut™ (5 x 200 µl) --- *A mixture of D-valine, collagenase and gentamycin.*
- **Rat AEC II PrimaCell™ Basal Culture Medium,** (5 × 100 ml) --- *A Modified Ham F12*.
- **Rat AEC II PrimaCell™ Medium Supplements,** (5 × 1 ml): *Rat EGF, Hydrocortisone, Cholera toxin penicillin (100 U/ml), streptomycin and Weymouth medium.*
- **Rat AEC II PrimaCell™ Serum,** (50 ml): A modified f*etal bovine serum.*
- **Rat Alveolar Tissue Washing Medium,** (1 × 100 ml): *A modified DMED medium with 10µg/ml amphotericin,* 10*µg/ml gentamycin, and 10% FCS.*

1.2 Required materials but NOT included:

- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or rat fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination.

2.2 Principle

Separation of the AEC II from the pancreases is accomplished by enzymatic digestion using the Alveolar Tissue Dissociation System supplied in this kit. The Alveolar Tissue Dissociation System contains a mixture of collagenase and other reagents at the optimal concentrations to gently isolate alveolars.

2.3 Rat Alveolar Tissue Preparation

1. The rats (200-300 g) were anesthetized with pentobarbital sodium (65 mg/kg body wt).
2. The trachea was clamped to prevent aspiration of blood into the lungs, and the heart and lungs were removed en bloc.
3. Blood was removed from the lungs by **Rat alveolar tissue washing medium** through the pulmonary artery and veins at room temperature until the lungs were blanched.
4. The heart, adipose tissue, blood vessels, and loose connective tissue were then removed.
5. Free alveolar macrophages were removed by lavaging the lung 10 times with ice-cold **Rat alveolar washing medium**

2.4 Alveolar Separation and Tissue Dissociation

2.4.1 Rat Alveolar OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the donor used, enzymes, culture mediums and growth supplements. The Rat Alveolar Tissue Dissociation System is suited for optimal dissociation of normal rat alveolar tissues to yield maximum number of single AEC II.

2.4.2 Enzyme Compositions

- Collagenase: from *Clostridium Histolyticum*
- Trypsin: from *Clostridium Histolyticum*

2.4.3 System Components

- Rat Alveolar Tissue Dissociation System, Alveolar OptiTDS™, (2 × 1 ml).
- Rat Alveolar OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

6. Prepare fresh enzyme working solution: Add 1ml of the reconstituted tissue dissociation solution to one vial of Rat Alveolar OptiTDS™ Digestion Buffer (9 ml). Warm the diluted Rat Alveolar OptiTDS™ working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 5 ml Rat Alveolar OptiTDS™ working solutions.
7. Lungs were lavaged once with 5-10 ml of **Rat alveolar Tissue Dissociation Solution** incubated at 37°C for 30 min to free lung cells from the pulmonary epithelium.
8. Following enzymatic digestion, large airways and connective tissue were removed and the lungs finely minced with a tissue chopper adjusted for a slice thickness of 0.5 mm.

Digestion was arrested by suspending minced lung tissue in 20 ml of **Rat Complete AEC II PrimaCell™ Culture Medium/FibrOut™**.

9. This suspension was incubated at 37°C for 10 min with agitation to free lung cells from the tissue mass. The resultant suspension was strained through nylon mesh of successively smaller pore size (150, 330, and 440 mesh; New York Silk Stencil, New York, NY).

2.6 AEC IIs Isolation

Note: Please read section 2.6 for specific information on Cervical Epithelium culture and platting before preceding this section.

10. Isolated cells in this whole-lung digest were then concentrated by centrifugation at 1,000 g for 5 min at 2°C and resuspended in **Rat Complete AEC II PrimaCell™ Culture Medium FibrOut™**.
11. The cell pellet was resuspended in **Rat Complete AEC II PrimaCell™ Culture Medium FibrOut™**and panned on an IgG-coated bacteriological plastic dish at 37°C for 1 h to remove macrophages.
12. The unattached cells were centrifuged and suspended in **Rat Complete AEC II PrimaCell™ Culture Medium FibrOut™**.

2.6 Primary AEC IIs Culture

2.6.1 Medium Preparation.

Thaw out **Rat AEC II PrimaCell™ Basal Culture Medium**, **Rat AEC II cell PrimaCell™ Medium Supplements**, and **Rat AEC II PrimaCell™ Serum** on ice.

Rat Complete AEC II PrimaCell™ Culture Medium: To every 100 ml **Rat AEC II PrimaCell™ Basal Culture Medium**, add 10 ml **Rat AEC II PrimaCell™ Medium Supplements** and 1 ml **Rat AEC II PrimaCell™ Serum**, mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

Rat Complete AEC II PrimaCell™ Culture Medium/FibrOut: To every 100 ml **Rat Alveolar PrimaCell™ Basal Culture Medium**, add 10 ml **Rat Alveolar PrimaCell™ Medium Supplements,** 1 ml **Rat Alveolar PrimaCell™ Serum**, and 1 ml **Rat Alveolar Fibroblast Growth Inhibitors, Alveolar FibrOut™**,mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

2.6.2 Primary Cell Culture

(Important: Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination)

13. The type II cells were plated on a 35mm plastic tissue culture dish.
14. Replace medium every 2-3 days.

Δ ***Safety Note.*** The rest of the biopsy and all tubes, pipettes, plates, etc., used in the procedure should be treated with hypochlorite before disposal.

2.7 Subculture

Propagating AEC II in culture can be somewhat challenging, especially when AEC IIs are cultured alone on plastic, however the following methods have worked consistently in many laboratories.

15. Remove culture medium, and wash cells with **Rat Alveolar Washing Medium.**
16. Add a small volume of HBSS containing trypsin (0·05%) and EDTA (0·02%) for 5 min Add an equal volume of culture medium and mechanically dispersed the remaining intact alveolars by mild trituration.
17. The dispersed alveolars were washed and suspended in **Rat Complete Alveolar PrimaCell™ Culture Medium**.
18. Count an aliquot and seed the cells at the chosen concentration.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Skeletal muscle cells cultures derived from preconfluent layers.

19. Trypsinize cells as above, and centrifuge at 100 g for 10 min.
20. Resuspend cells in **Rat Complete Alveolar PrimaCell™ Culture Medium** and count cells.
21. Prepare aliquots of 2×10^6 cells/ml in **Rat Complete Alveolar PrimaCell™ Culture Medium** and 10% glycerol into cryovials.
22. Equilibrate at 4℃ for 1-2 h.
23. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
24. To recover cells:
    a. Thaw cryovials quickly in a 37℃ water bath.
    b. Dilute cells tenfold with appropriate **Rat Complete Alveolar PrimaCell™ Culture Medium**.
    c. Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in **Rat Complete Alveolar PrimaCell™ Culture Medium.**

IV Characterization

AEC II were identified by the modified Papanicolaou or Nile red staining and immunocytochemistry using AEC II-specific antibodies such as anti-RTII70 and anti-LB180 antibodies. Phosphine 3R is a lipophilic fluorescent dye concentrated in lamellar bodies of type II cells and can, therefore, be used to identify type II cells. P2X7 and GABRP (gamma-aminobutyric acid receptor pi subunit), specifically expressed in alveolar epithelial type II cells (AEC II) and AEC I, could be used as potential AEC marker for studying lung epithelial cell biology and monitoring lung injury.

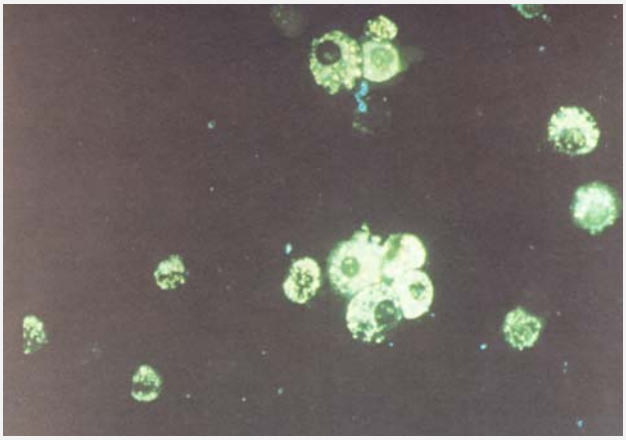


Figure. AECs were equilibrated for 2 min with phosphine 3R dye (0.002%) and observed under a fluorescent microscope[2]

V References

1. Chen, J., et al., *Isolation of highly pure alveolar epithelial type I and type II cells from rat lungs.* Lab Invest, 2004. **84**(6): p. 727-35.
2. Jones, G.S., et al., *Ionic content and regulation of cellular volume in rat alveolar type II cells.* J Appl Physiol, 1982. **53**(1): p. 258-66.
3. Chen, Z., et al., *Identification of two novel markers for alveolar epithelial type I and II cells.* Biochem Biophys Res Commun, 2004. **319**(3): p. 774-80.
4. Dobbs, L.G., R. Gonzalez, and M.C. Williams, *An improved method for isolating type II cells in high yield and purity.* Am Rev Respir Dis, 1986. **134**(1): p. 141-5.
5. Liu, L., et al., *Involvement of annexin II in exocytosis of lamellar bodies from alveolar epithelial type II cells.* Am J Physiol, 1996. **270**(4 Pt 1): p. L668-76.

Rat Lung PrimaCell™: Alveolar Epithelial Cell II

Rat Lung Primary Cell Culture

Cat No.	Description	Qt.	Price
2-84704	**Rat Lung PrimaCell™ system**	**kit**	**$499**
4-26731	Rat Lung Tissue Disassociation System, Lung OptiTDS™ (for 500 ml medium)	1 ml	$128
9-48703	Rat Lung Type II Alveolar Epithelial Cells PrimaCell™ Basal Culture Medium	500 ml	$ 61
9-36731	Rat Lung Type II Alveolar Epithelial Cells PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$ 140
7-67731	Rat Lung Fibroblast Growth Inhibitors II, Lung FibrOut™	ea	$146
9-98703	Rat Lung Tissue Preparation Buffer Set	ea	$75

Rat Primary Alveolar Epithelial Cell II Characterization

6-22511	**Rat Alveolar Epithelial Cell II Primarker™ Kit**	**kit**	**$220**
6-22512	Rat Alveolar Epithelial Cell II Primarker™ antibody set	set	$180
6-22513	Rat Alveolar Epithelial Cell II Primarker™ buffer system	set	$90

Rat Muscle PrimaCell™: Skeletal Muscle Cells
(Cat No. 2-82035)

I. General Description

Of all the different tissues and organs in the body of any mammalian species, skeletal muscles are the most frequent organ. In adult rat skeletal muscle the majority of myonuclei are located in syncytial myotubes that were formed by myoblast fusion during fetal and postnatal development. These myonuclei are terminally postmitotic. However, a small fraction of myonuclei is in mononucleate precursor cells called muscle satellite cells which are located between the basal lamina and sarcolemma of myofibers. In healthy adult rodent muscle, satellite cells are mitotically quiescent and do not detectably express MRFs (MyoD family muscle regulatory factors). When stimulated by damage to the muscle or by explant and culture manipulations, some fractions of satellite cells are activated to reenter the cell cycle and/or to express myogenic regulatory factors. The resulting myoblasts subsequently differentiate and fuse to form new replacement myofibers. During embryogenesis, mononuclear precursor cells, or myoblasts, fuse to form these huge muscle fibers. Later life during regenerative process subsequent to muscle damage, quiescent mononuclear stem cells (satellite cells) lacated between the sarcolemma and basal lamina of the muscle fiber are activated. These cells begin to proliferate and either fuse with each other into novel myotubes, or they fuse with damaged muscle fibers. Both myoblasts and satellite cells can be isolated from the body and grown in tissue culture, and in optimal culture media they will fuse and differentiate into mature, spontaneously-contracting myotubes. Culture derived from normal and diseased rat skeletal muscle cells therefore provides an excellent model to study several aspects of early muscle development under normal and pathological conditions.

The Rat Muscle PrimaCell™ kit (Cat No. 2-82035) is designed for the successful isolation and culture of skeletal muscle cells from rat muscle allowing reproducible and dependable results.

1.1 Components of the Rat Muscle PrimaCell™ System

- **Rat Muscle Tissue Dissociation System , Muscle OptiTDS™** (2 × 1 ml) --- *A mixture of collagenase, collagenase II, collagenase IV, Elastase, Hyaluronidase I, Pronase, trypsin and Muscle OptiTDS™ Reconstitution Buffer*.
- **Muscle Tissue Washing Medium**, (2 × 1 m)
- **Rat Muscle OptiTDS™ Digestion Buffer,** (1 × 100ml) --- *A modified Ham's F12 without NaHCO3, with 20 mM HEPES, 200 U/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Rat Muscle Fibroblast Growth Inhibitors, Muscle FibrOut™ ---**Muscle FibrOut™ (5 × 200 μl) --- *A mixture of D-valine, collagenase, and gentamycin.*
- **Rat Skeletal Muscle Cells PrimaCell™ Basal Culture Medium** (5 × 100 ml) --- *A modified Ham's F12*.
- **Rat Skeletal Muscle Cells PrimaCell™ Medium Supplements with Serum** (10 × 10 ml): *Highly purified and special-treated fetal bovine serum, penicillin, streptomycin.*

1.2 Required materials but NOT included:

- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or rat fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, fresh gelatin-coated plate or culture dishes are recommended (see below for treatment of culture dishes).

2.2 Principle

It is possible to culture myogenic cells from adult skeletal muscle of several species under conditions in which the cells continue to express at least some of their differentiated traits. A cell type called *satellite cells*; the myogenic cells partially mimic the first steps of skeletal muscle differentiation. They proliferate and migrate randomly on the substratum and then align and finally form multinucleated myotubes through a fusion process. Although three to four passages can be performed by means of trypsinization, subculture is no longer possible once differentiation (i.e., fusion) has taken place. For the same reason, proliferation is very difficult to estimate once some cells have started to fuse. Primary cultures from rat healthy muscle biopsies are highly enriched in myogenic cells, as evidenced by at least 80% positively to desmin by immunostaining at day 7 after seeding.

Primary cultures can be grown easily in Rat Complete Skeletal Muscle PrimaCell™ Culture Medium included in this system. Without modifying the culture conditions, these cells proliferate and differentiate by fusing to form multinucleated myotubes, confirming the myogenicity of the cultivated cells.

2.3 Rat Muscle Tissue Preparation

Rat Muscle: Skeletal muscle cells are usually taken from fresh non-fixed and non-frozen muscle specimens such thigh origin of muscle.

1. The muscle specimens for culture were immediately placed in a 100-mm tissue culture dish containing 5-10 ml of **Rat Muscle Tissue Washing Medium**.
2. Trim off nonmuscle tissue from the specimens with a scalpel, and rinse in **Rat Muscle Tissue Washing Medium**.
3. Cut the muscle tissue into fragments parallel to the fibers and wash in **Rat Muscle Tissue Washing Medium** prior to weighing the biopsy.
4. Place the fragments parallel to each other in the lid of a Petri dish, cut the fragments into thinner cylinders and then, finally into 1-mm^3 pieces, without crushing the tissue. The final cutting can be done in a tube with long scissors, again avoiding crushing.

5. Discard the **Rat Muscle Tissue Washing Medium** and incubate tissue specimens with 5-10 ml 70% ethanol for 1 min, followed by inoculating with 5-10 ml fresh **Rat Muscle Tissue Washing Medium** for 5 min.

2.4 Skeletal muscle cell Separation and Tissue Dissociation

2.4.1 Rat Muscle OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Rat Muscle Tissue Dissociation System is suited for optimal dissociation of normal newborn rat muscle tissues to yield maximum number of single skeletal muscle cell.

2.4.2 Enzyme Compositions

- Trypsin: from *Bovine Pancreas*
- Collagenase: from *Clostridium Histolyticum*
- Collagenase II: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*
- Elastase: from *Bovine Pancreas*
- Hyaluronidase I: from *Bovine Pancreas*
- Pronase: from *Clostridium Histolyticum*

2.4.3 System Components

- Rat Muscle Tissue Dissociation System, Muscle OptiTDS™, (2 × 1 ml).
- Rat Muscle OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

6. Prepare fresh enzyme working solution: Add 1.0 ml of **Rat Muscle Tissue Dissociation System, Bone OptiTDS™** to one vial of **Rat Muscle OptiTDS™ Digestion Buffer** (9 ml). Warm the diluted enzyme working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 mg tissue samples per 10 ml diluted enzyme working solutions.
7. Rinse muscle tissue with **Rat Muscle Tissue Washing Medium** and let the pieces settles; discard the supernatant.
8. Dissociation can be achieved in either of the following two methods

   (a) Rapid dissociation: Float muscle on Rat Muscle Tissue Dissociation System working solution for 2-3 hrs at 37 ℃. This works particularly well with full-thickness muscle. Monitor the separation of the muscle carefully when using the rapid dissociation method.

   (b) Slow dissociation: Float the samples on ice-cold Rat Muscle Tissue Dissociation System working solution at 4℃ for 15-24 h. This is particularly convenient for flexible scheduling of skeletal muscle cells preps.

2.6 Primary skeletal muscle cells Culture

2.5.1 Medium Preparation.

Thaw out **Rat Skeletal Muscle PrimaCell™ Basal Culture Medium**, **Rat Skeletal Muscle PrimaCell™ Medium Supplements With Serum** on ice.

Rat Complete Skeletal Muscle PrimaCell™ Culture Medium: To every 100 ml **Rat Skeletal Muscle PrimaCell™ Basal Culture Medium**, add 10 ml **Rat Skeletal Muscle PrimaCell™ Medium Supplements With Serum**, mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

Rat Complete Skeletal Muscle PrimaCell™ Culture Medium/FibrOut: To every 100 ml **Rat Skeletal Muscle PrimaCell™ Basal Culture Medium**, add 10 ml **Rat Skeletal Muscle PrimaCell™ Medium Supplements With Serum**, one vial of **Rat Muscle Fibroblast Growth Inhibitors, Bone FibrOut™**, mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use. (Important: *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*)

2.5.2 Primary Cell Culture

24. Triturate the culture with a pipette after incubation. The medium should become increasingly opaque as more and more cells are released.
25. Let the fragments settle to the bottom by gravity, forming pellet P1 and supernatant S1.
26. Filter S1 through a 100-µm nylon mesh and into a 20-ml centrifuge tube. Shake or pipette the supernatant gently to resuspend the cells.
27. Centrifuge the tube 8-10 min at 350 g. Discard the supernatant by aspiration.
28. Resuspend the pellet very, very gently by means of a rubber-bulb pipette in precisely 10 ml of **Rat Complete Skeletal Muscle PrimaCell™ Culture Medium/FibrOut l™**, and count the cells with a hemocytometer.
29. Dilute the suspension in growth medium to seed culture flasks with about 1.5×10^4 cells/ml. About $1 – 2 \times 10^5$ cells/g are obtained from healthy donor biopsies.
30. Add 15 ml of digestion medium to P1, and incubate the fragments for 30 min in a water bath at 37˚C, with periodic shaking.
31. Pipette the suspension to disaggregate the cells and then filter the suspension through nylon mesh. Rinse the filter with 20 ml of **Rat Complete Skeletal Muscle PrimaCell™ Culture Medium/FibrOut l™**.
32. Centrifuge the suspension for 8 – 10 min at 350 g, count the cells, and seed as before.
33. Transfer the flasks to a 37˚C humidified incubator with 5% CO_2.
34. Maintenance of Cultures: Change the **Rat Complete Skeletal Muscle PrimaCell™ Culture Medium/FibrOut l™** very gently 24 h after seeding and then every 3-4 d. The development of these cultures is mainly towards differentiation. The timing of the three phases for rat muscle cells is about 4-6 d for peak proliferation; then the cells align at about day 8, and around day 10 to 12 an increase in cell fusion and the formation of myotubes are observed. Nevertheless, one must keep in mind that some cells may differentiate earlier and that others will still proliferate when the majority of the culture is undergoing differentiation. Change culture medium to **Rat Complete Skeletal Muscle PrimaCell™ Culture Medium** after 3-5 cycles or an acceptable level of fibroblast cell contamination is observed.

Δ ***Safety Note.*** The rest of the biopsy and all tubes, pipettes, plates, etc., used in the procedure should be treated with hypochlorite before disposal.

2.6 Subculture

35. Add a small volume of EDTA gently to the cells and remove it immediately.
36. Add sufficient trypsin solution (0.25%) to form a thin layer over the cells.
37. When cells detach, add 5 to 10 ml of **Rat Complete Skeletal Muscle PrimaCell™ Culture Medium**, pass the culture very gently in and out of a pipette, and then centrifuge the cells for 10 min at 350 g.
38. Count an aliquot and seed the cells at the chosen concentration.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Skeletal muscle cells cultures derived from preconfluent layers.

24. Trypsinize cells as above, and centrifuge at 100 g for 10 min.
25. Resuspend cells in complete **Rat Complete Skeletal Muscle PrimaCell™ Culture Medium** and count cells.
26. Prepare aliquots of 2×10^6 cells/ml in **Rat Complete Skeletal Muscle PrimaCell™ Culture Medium** and 10% glycerol into cryovials.
27. Equilibrate at 4℃ for 1-2 h.
28. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
29. To recover cells:
    a. Thaw cryovials quickly in a 37℃ water bath.
    b. Dilute cells tenfold with appropriate **Rat Complete Skeletal Muscle PrimaCell™ Culture Medium**.
    c. Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in **Rat Complete Skeletal Muscle PrimaCell™ Culture Medium**.

IV Characterization

The growth curves of rat myogenic cell cultures obtained in **Rat Complete Skeletal Muscle PrimaCell™ Culture Medium** show the three traditional phases: the lag phase, the exponential phase, and the plateau, which corresponds to the onset of fusion. The last, evaluated in terms of the number of nuclei incorporated into myobubes or in terms of a fusion index (the percentage of nuclei incorporated into myotubes relative to the total number of nuclei), commences usually around day 8 after plating and rises dramatically around day 10. According to the sample, this chronology can gain or lose one day. Hence, differentiation, expressed as the number of nuclei per myotube/cm^2, may be observed morphologically. But the differentiation process can also be monitored by the use of biochemical markers (such as the sarcomeric proteins), enzymes involved in differentiation(e.g., creatine phosphokinase and its time-dependent muscle-specific isoform shift), or the appearance of α-actin Expression of the c-met receptor tyrosine kinase can serve as an effective molecular marker for quiescent or activated satellite cells. A family of genes, such as myogenin, MyoD1 and desmin, the best known of which is MyoD, was shown to activate muscle-specific gene expression in myogenic progenitors. Myogenic cell differentiation involves either the activation of a variety of other genes with concurrent changes in cell surface adhesive properties or the recently shown requirement of cell surface plasminogen activator urokinase and its receptor.

V References

1. Cornelison, D.D. and B.J. Wold, *Single-cell analysis of regulatory gene expression in quiescent and activated rat skeletal muscle satellite cells.* Dev Biol, 1997. **191**(2): p. 270-83.
2. Grounds, M.D., et al., *Identification of skeletal muscle precursor cells in vivo by use of MyoD1 and myogenin probes.* Cell Tissue Res, 1992. **267**(1): p. 99-104.
3. Koller, M.R.P., B. O.; Masters, J. R. W., *Human Cell Culture: Primary Mesenchymal Cells* Vol. 5. 2001: Springer.
4. Bischoff, R., *Proliferation of muscle satellite cells on intact myofibers in culture.* Dev Biol, 1986. **115**(1): p. 129-39.
5. Lawson-Smith, M.J. and J.K. McGeachie, *The identification of myogenic cells in skeletal muscle, with emphasis on the use of tritiated thymidine autoradiography and desmin antibodies.* J Anat, 1998. **192 (Pt 2)**: p. 161-71.
6. Metzinger, L., P. Poindron, and A.C. Passaquin, *A rapid preparation of primary cultures of rat skeletal muscle cells.* Cytotechnology, 1993. **13**(1): p. 55-60.
7. Sejersen, T., et al., *Rat skeletal myoblasts and arterial smooth muscle cells express the gene for the A chain but not the gene for the B chain (c-sis) of platelet-derived growth factor (PDGF) and produce a PDGF-like protein.* Proc Natl Acad Sci U S A, 1986. **83**(18): p. 6844-8.
8. Askanas, V., A. Bornemann, and W.K. Engel, *Immunocytochemical localization of desmin at human neuromuscular junctions.* Neurology, 1990. **40**(6): p. 949-53.
9. Buckingham, M., *Making muscle in mammals.* Trends Genet, 1992. **8**(4): p. 144-8.
10. Dodson, M.V., B.A. Mathison, and B.D. Mathison, *Effects of medium and substratum on ovine satellite cell attachment, proliferation and differentiation in vitro.* Cell Differ Dev, 1990. **29**(1): p. 59-66.
11. Pegolo, G., V. Askanas, and W.K. Engel, *Expression of muscle-specific isozymes of phosphorylase and creatine kinase in human muscle fibers cultured aneurally in serum-free, hormonally/chemically enriched medium.* Int J Dev Neurosci, 1990. **8**(3): p. 299-308.
12. Quax, P.H., et al., *Modulation of activities and RNA level of the components of the plasminogen activation system during fusion of human myogenic satellite cells in vitro.* Dev Biol, 1992. **151**(1): p. 166-75.

Rat Muscle PrimaCell™: Skeletal Muscle Cells

Rat Muscle Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82035	**Rat Muscle PrimaCell™ system**	**kit**	**$499**
4-21141	Rat Muscle Tissue Dissociation System, Muscle OptiTDS™ (for 500 ml medium)	1 ml	$128
9-41014	Rat Skeletal Muscle Cells PrimaCell™ Basal Culture Medium	500 ml	$61
9-31141	Rat Skeletal Muscle Cells PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
7-61141	Rat Muscle Fibroblast Growth Inhibitors, Muscle FibrOut™	ea	$158
9-91014	Rat Muscle Tissue Preparation Buffer Set	ea	$75

Rat Primary Skeletal Muscle Cells Characterization

6-21611	**Rat Skeletal Muscle Cell Primarker™ Kit**	**kit**	**$220**
6-21612	Rat Skeletal Muscle Cell Primarker™ antibody set	set	$180
6-21613	Rat Skeletal Muscle Cell Primarker™ buffer system	set	$90

Rat Pancreas PrimaCell™ I: Pancreatic Epithelium

(Cat No. 2-82036)

I. General Description:

This protocol is developed for attachment and growth of normal Rat Pancreatic epithelial cells from 1-3 mm^3 biopsies with Rat Pancreatic PrimaCell™ system (Cat No. 2-82036). This system provides an optimal condition of tissue dissociation system, Pancreatic OptiTDS™ that yields 4-7 times of single cells more than most of the tissue dissociation protocols published in the literature. In addition, this system ensures a high viability of the target cells with improved gradient contained in the culture medium. With CHI's proprietary fibroblast inhibitory system, Pancreatic FibrOut™, cells are growing with contamination of minimized amount of the non-epithelial cells.

The preparation of tissue specimens for cell culture is usually started within 1-2 h of removal from the patient. If this is impossible, fine cutting of the tissue into small pieces (1-2 mm) with scalpels and storage overnight at 4℃ in washing medium (see below) can also prove successful.

Rat Pancreatic PrimaCell™ system applies to all types of normal adult Rat biopsies samples. Biopsies samples contain pathological organism (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Rat Pancreatic PrimaCell™ System

- **Rat Pancreatic Tissue Dissociation System, Pancreatic OptiTDS™,** (2 aliquots) --- *A mixture of collagenase I, collagenase III, collagenase IV, collagenase, and trypsin*.
- **Rat Pancreatic OptiTDS™ Reconstitution Buffer**, (2 x 1 ml).
- **Rat Pancreatic OptiTDS™ Digestion Buffer**, (2 x 10 ml).
- **Rat Pancreatic Tissue Washing Medium**, (5 x 10 ml) --- *Basal Pancreatic PrimaCell™ Culture Medium with 5% FBS, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Rat Pancreatic Fibroblast Growth Inhibitors, Pancreatic FibrOut™** (5 x 200 µl) --- *A mixture of* anti-Thy-1 *monoclonal antibody, toxin ricin, and formulated serum substitutes*.
- **Rat Pancreatic PrimaCell™ Basal Culture Medium**, (5 x100 ml) --- *Modified formulation based on NCTC 168 and Weymouth medium*.
- **Rat Pancreatic PrimaCell™ Medium Supplements**, (5 x 1.0 ml) --- A mixture of *ethanolamine, phosphoethanolamine, hydrocortisone, ascorbic acid, transferring, insulin, epidermal growth factor, pentagastrin, and deoxycholic acid*.
- **Rat Pancreatic PrimaCell™ Serum**, (1 x 50 ml) --- H*eat-inactivated and special-treated Fetal-bovine serum.*
- **Coating Solution**, (5 x 10 ml) --- *Basal growth medium containing 10 μg/ml BSA.*

1.2 Required Materials but not provided

- Hank's balanced salt solution (HBSS)
- Dispase (Sigma)
- Pasteur pipettes

- Collagen I-coated Culture dishes
- Scalpels, scissors, and forceps
- Pasteur pipettes and 10-ml pipettes
- Test tubes, 12 and 50 ml

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, collagen I-coated plate (Corning, NY) MUST be pre-treated with the provided BSA by adding 5 ml 10 μg/ml BSA in growth medium and incubate for 5 min. Aspirate the BSA solution; let the dishes be air-dry in the ventilated cell culture hood for 5-10 min.

2.2 Surgical specimens

Carefully remove the rat pancreas and place specimens in a 10 ml falcon tube contain 5 ml Pancreatic Tissue Washing Medium followed by inoculating for 10 min at the room temperature. For large tissue specimens, 50 ml falcon tube and more wash medium is needed to ensure thoroughly washing. Aspirate the washing medium and repeat the washing procedures with fresh washing medium two more time. Washing tissue specimens sequentially in 70% ethanol for 1 min at the room temperature, in PBS for 5 min, and in fresh Pancreatic Tissue Washing Medium for 5 min. Collecting tissue specimen by centrifugation prior to tissue dissociation procedures (see below).

2.3 Tissue Preparation and Dissociation

2.3.1 Rat Pancreatic OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Pancreatic Tissue Dissociation System, OptiTDS™, is suited for optimal dissociation of normal adult Rat biopsies samples to yield maximum number of single primary cells of Pancreatic tissues.

2.3.2 Enzyme Compositions

- Collagenase I: from *Clostridium Histolyticum*
- Collagenase III: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*
- Collagenase: from *Clostridium Histolyticum*
- Trypsin: from *Bovine Pancreas*

2.3.3 System Components

- Pancreatic Tissue Dissociation System, OptiTDS™, 2 vials.
- Pancreatic OptiTDS™ Reconstitution Buffer, (2 x 1 ml).
- Pancreatic OptiTDS™ Digestion Buffer, (2 x 9 ml).

2.3.4 Procedures For Tissue Preparation and Dissociation

1. Prepare fresh enzyme working solutions: to each vial of Pancreatic Tissue Dissociation System, OptiTDS™, add 1.0 ml of the Pancreatic OptiTDS™ Reconstitution Buffer, Mix well.
2. Add 1.0 ml of the fresh enzyme working solution to one vial of Pancreatic OptiTDS™ Digestion Buffer (9.0 ml). Warm the diluted enzyme working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 4-5 mg tissue samples per 10 ml diluted enzyme working solutions.
3. Mince pre-washed tissue into pieces approximately 0.2-0.5 mm^2 in diameter with two scalpel and forceps.
4. Incubate minced tissues with the diluted enzyme working solutions by incubating minced tissue samples (up to 5 mg) in 10 ml diluted enzyme working solutions with slow magnetic stirring for 30 min at 37 ℃.
5. At the end of tissue dissociation period, gentle triturating tissue with a 10 ml pipette, constitutes filling the empty the barrel at a rate of 2-3 ml per second. Repeat this procedure for 5-6 times.
6. Collecting cells by filtration the mixture through a cell strainer followed by centrifugation at 1 x 100 g. Carefully remove the medium and resuspend the cell pellet with 1.0 ml complete culture medium.
7. Count the cells and seed cells in 3-4 T-25 collagen I-coated flasks (**Important:** pre-treat the flask with the provided BSA containing growth medium, see below) upon the density of viable cells (2.5-5 x 10^5 Cells/Dish).

2.3.5 Storage:

Reconstituted tissue dissociation systems should occur before use and can only be stored for 2-4 days at 4 ^oC. For long-term use, it should be aliquoted and stored at -20 ^oC. Avoid repeated freeze-thaw cycles.

2.4 Culture of primary Rat Pancreatic cells

2.4.1 Medium Preparation.

Thaw out the Rat Pancreatic PrimaCell™ Medium Supplements and Rat Pancreatic PrimaCell™ Serum on ice. To every 100 ml Rat Pancreatic PrimaCell™ Basal Culture Medium, add one vial of Rat Pancreatic PrimaCell™ Medium Supplements; 10 ml Rat Pancreatic PrimaCell™ Serum; and one vial of Rat Pancreatic Fibroblast Growth Inhibitors, Pancreatic FibrOut™, mix thoroughly and warm the complete medium at a 37^oC water bath for 10 min prior use.

2.4.2 Treatment of Culture Dishes.

To enhance cell attachment to the culture dishes, collagen I-coated plate (Corning, NY) MUST be pre-treated with the provided Coating Solution (Basal growth medium containing 10 µg/ml BSA) by adding appropriate volume of the Coating Solution (enough to cover the whole cell-growth area) and incubate for 5 min. Aspirate the BSA solution; let the dishes be air-dry in the ventilated cell culture hood for 5-10 min.

.

2.4.3 Standard primary culture conditions.

Inoculate epithelial tubules and clumps of cells derived from tissue specimens into T-25 flasks

coated with collagen type I with pre-treatment of coating solution at 37℃ in a 5%-CO_2 incubator with 4 ml of complete culture medium. Change the culture medium twice weekly. The tubules and cells start to attach to the substratum, and epithelial cells migrate out within 1-2 d. Most of the tubules and small clumps of epithelium attach within 7 d, but the larger organoids can take up to 6 weeks to attach, although they will remain viable all that time.

2.4.4 Alternative Primary Culture conditions.

The attachment of epithelium during primary culture and subculture is more reproducible and efficient when cells are inoculated onto collagen-coated flasks, and significantly better growth is obtained with 3T3 feeders than without. When the epithelial Breasties expand to several hundred cells per Breasty, they become less dependent on 3T3 feeders, and no further addition of feeders is necessary. All medium and solutions described in the standard culture condition are applicable in this culture method.

2.4.5 Subculture and Propagation

Most colorectal primary cultures cannot at present be passaged by routine trypsin / EDTA procedures. Disaggregation to single cells of the cultured Pancreatic cells with 0.1% trypsin in 0.25 mM (0.1%) EDTA will result in extremely poor or even zero growth, so Dispase is used instead. One of the advantage using dispase is that dispase can only detach epithelial cell but not the fibroblast, thus increase the purity of epithelium.

8. Add 0.5% Dispase (Sigma, w/v) to the cell monolayer, just enough to cover the cells (~2.5 ml/25-cm^2 flask), and leave the solution to stand for 40-60 min for primary cultures and 20-40 min for cell lines.
9. Once the epithelial layers begin to detach (they do so as sheets rather than single cells), pipette to help detachment and disaggregation into smaller clumps.
10. Wash and replate the cells under standard culture conditions. It may take several days for clumps to attach, so replace the medium carefully when feeding.

III Fibroblast Contamination

There are several techniques have been published in the literature to deal with fibroblast contamination during colorectal primary cell culture. These include: (1) Physically remove a well-isolated fibroblast Breasty by scraping it with a sterile blunt instrument (e.g., a cell scraper). Care has to be taken to wash the culture up to six times to remove any fibroblasts that have detached in order to prevent them from reseeding and reattaching to the flask. (2) Differential trypsinization can be attempted with the carcinomas. (3) Dispase preferentially (but not exclusively) removes the epithelium during passaging and leaves behind most of the fibroblastic cells attached to the culture vessel. During subculture, cells that have been removed with dispase can be preincubated in plastic Petri dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the epithelium. Clumps of epithelial cells still floating can be transferred to new flasks under standard culture conditions. This technique takes advantage of the fact that fibroblasts in general attach much more quickly to plastic than do clumps of epithelial cells, so that a partial purification step is possible. (4) Reduce the concentration of serum to about2.5-5% if there are heavy concentrations of fibroblastic cells. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and that using any or all of the preceding techniques will eventually push the cells through so many divisions that any fibroblasts will senesce.

Rat Pancreatic PrimaCell™ includes a fibroblast elimination system, the Rat Pancreatic Fibroblast Growth Inhibitors, Pancreatic FibrOut™. It contains a mixture of anti-Thy-1 monoclonal antibody, toxin ricin and formulated serum substitutes. Thy-1 antigen is present on colorectal fibroblasts, but not colorectal epithelial cells; therefore, the conjugate kills contaminating fibroblasts, but shows no signs of toxicity toward the epithelium, whether derived from an adenoma, a carcinoma or normal Pancreatic tissues.

IV References:

1. Youngman KR, Simon PL, West GA, Cominelli F, Rachmilewitz D, Klein JS, Fiocchi C: Localisation of intestinal interleukin 1 activity and protein and gene expression to lamina propria cells. Gastroenterology 1993, 104:749-758.
2. Gibson PR, van de Pol E, Maxwell LE, Gabriel A, Doe WF: Isolation of Pancreatic crypts that maintain structural and metabolic viability in vitro. Gastroenterology 1989, 96:283-291.
3. Whitehead RH, Brown A, Bhathel PS: A method for the isolation and culture of Rat Pancreatic crypts in collagen gels. In Vitro 1986, 23:436-442.
4. Knoll N, Weise A, Claussen U, Sendt W, Marian B, Glei M, Pool-Zobel BL. 2-Dodecylcyclobutanone, a radiolytic product of palmitic acid, is genotoxic in primary Rat Pancreatic cells and in cells from preneoplastic lesions.
5. Buset M, Winawer S, Friedman E. Defining conditions to promote the attachment of adult Rat Pancreatic epithelial cells.

Rat Pancreas PrimaCell™: Pancreatic Epithelium

Rat Pancreas Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82036	**Rat Pancreas PrimaCell™ system**	**kit**	**$499**
4-21161	Rat Pancreas Tissue Dissociation System, Pancreas OptiTDS™ (for 500 ml medium)	1 ml	$ 134
9-41016	Rat Pancreatic Epithelium PrimaCell™ Basal Culture Medium	500 ml	$ 79
9-31161	Rat Pancreatic Epithelium PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$ 140
7-61161	Rat Pancreas Fibroblast Growth Inhibitors, Pancreas FibrOut™	ea	$ 146
9-91016	Rat Pancreas Tissue I Preparation Buffer Set	ea	$75

Rat Primary Pancreatic Epithelium Characterization

6-21711	**Rat Pancreatic Epithelium Primarker™ Kit**	**kit**	**$220**
6-21712	Rat Pancreatic Epithelium Primarker™ antibody set	set	$180
6-21713	Rat Pancreatic Epithelium Primarker™ buffer system	set	$90

Rat Pancreas PrimaCell™ II: Islet Cells
(Cat No. 2-85104)

I. General Description

The endocrine cells of the pancreas are grouped in the islets of Langerhans. Each islet contains approximately one thousand cells and is 50-500 um in diameter.Hormones produced in the Islets of Langerhans are secreted directly into the blood flow by (at least) islet cells.
While these cells are extremely useful in the laboratory they are notoriously difficult to isolate and culture. The Rat Islet PrimaCell™ kit (Cat No.2-85104) is designed for the successful isolation and culture of islet cells from rat tissue allowing reproducible and dependable results.

1.1 Components of the Rat Islet PrimaCell™ System

- **Rat Islet Tissue Dissociation System, Islet OptiTDS™** (2 × 1 ml) --- *A mixture of collagenase and Rat Islet OptiTDS™ Reconstitution Buffer.*
- **Rat *Islet OptiTDS™ Digestion Buffer,*** (2 × 9 ml)
- **Rat Islet Fibroblast Growth Inhibitors, Islet FibrOut™**---Islet FibrOut™ (5 x 200 µl) --- *A mixture of D-valine, collagenase and gentamycin.*
- **Rat Islet cell PrimaCell™ Basal Culture Medium,** (5 × 100 ml) --- *A Modified Ham F12*.
- **Rat Islet cell PrimaCell™ Medium Supplements,** (5 × 1 ml): *Rat EGF, Hydrocortisone, Cholera toxin penicillin (100 U/ml), streptomycin and Weymouth medium.*
- **Rat Islet PrimaCell™ Serum,** (50 ml): A modified f*etal bovine serum.*
- **Rat Islet Tissue Washing Medium,** (1 × 100 ml): *A modified DMED medium with 10µg/ml amphotericin, 10µg/ml gentamycin, and 10% FCS.*

1.2 Required materials but NOT included:

- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or rat fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination.

2.2 Principle

Separation of the islet cell from the pancreases is accomplished by enzymatic digestion using the Islet Tissue Dissociation System supplied in this kit. The Islet Tissue Dissociation System contains a mixture of collagenase and other reagents at the optimal concentrations to gently isolate islets.

2.3 Rat Islet Tissue Preparation

Rat Islets: Most islets are obtained from pregnant rats at 25 days of gestation (The day of mating was counted as day 0).

1. Pregnant rats were killed by cervical dislocation and fetuses were rapidly removed. All the procedures were performed under sterile conditions.
2. The fetal pancreases were removed aseptically, placed into culture dish with cold **Rat Islet Washing Medium**.
3. Tissues were finely minced, and washed with **Rat Islet Washing Medium**.

2.4 Islet Separation and Tissue Dissociation

2.4.1 Rat Islet OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the donor used, enzymes, culture mediums and growth supplements. The Rat Islet Tissue Dissociation System is suited for optimal dissociation of normal rat islet tissues to yield maximum number of single islet cell.

2.4.2 Enzyme Compositions

- Collagenase: from *Clostridium Histolyticum*
- Trypsin: from *Clostridium Histolyticum*

2.4.3 System Components

- Rat Islet Tissue Dissociation System, Islet OptiTDS™, (2 × 1 ml).
- Rat Islet OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

4. Prepare fresh enzyme working solution: Add 1ml of the reconstituted tissue dissociation solution to one vial of Rat Islet OptiTDS™ Digestion Buffer (9 ml). Warm the diluted Rat Islet OptiTDS™ working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 5 ml Rat Islet OptiTDS™ working solutions.
5. Discard the **Rat Islet Washing Medium,** and float samples on 5 ml of Rat Islet Tissue Dissociation System working solution (prewarmed to 37℃) and transfer the minced tissue to a sterile centrifuge tube (10–12 pancreases each).
6. The tissue was digested in a shaking water bath at 37 C for 10-30 min.

2.5 Islet cells Isolation

Note: Please read section 2.6 for specific information on Cervical Epithelium culture and platting before preceding this section.

7. The resulting digests were washed three times with cold **Rat Islet Washing Medium.**
8. The pellets were resuspended in **Rat Complete Islet PrimaCell™ Culture Medium/FibrOut** and distributed in 50 mm plastic culture dishes.
9. The islets were cultured for 5 days in 5 ml **Rat Complete Islet PrimaCell™ Culture Medium/FibrOut** at 37 ^{o}C in a humidified atmosphere of 5% CO2.

2.6 Primary Islet cells Culture

2.7.1 Medium Preparation.

Thaw out **Rat Islet PrimaCell™ Basal Culture Medium**, **Rat Islet cell PrimaCell™ Medium Supplements**, and **Rat Islet PrimaCell™ Serum** on ice.

Rat Complete Islet PrimaCell™ Culture Medium: To every 100 ml **Rat Islet PrimaCell™ Basal Culture Medium**, add 10 ml **Rat Islet PrimaCell™ Medium Supplements** and 1 ml **Rat Islet PrimaCell™ Serum** mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use.

Rat Complete Islet PrimaCell™ Culture Medium/FibrOut: To every 100 ml **Rat Islet PrimaCell™ Basal Culture Medium**, add 10 ml **Rat Islet PrimaCell™ Medium Supplements,** 1 ml **Rat Islet PrimaCell™ Serum**, and 1 ml **Rat Islet Fibroblast Growth Inhibitors, Islet FibrOut™**,mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use. (**Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*)

2.7.2 Primary Cell Culture

10. The medium was changed every day. At the end of the preculture period, the islets attached to the bottom of the culture dishes were gently blown free using a sterilized Pasteur pipette under a stereomicroscope. The fibroblast layer remaining on the bottom of the culture dishes
11. The detached islets were cultured free-floating in 50 mm Petri dishes in **Rat Complete Islet PrimaCell™ Culture Medium/FibrOut** changed every other day.

Δ ***Safety Note.*** The rest of the biopsy and all tubes, pipettes, plates, etc., used in the procedure should be treated with hypochlorite before disposal.

2.7 Subculture

Propagating Islet cells in culture can be somewhat challenging, especially when Islet cells are cultured alone on plastic, however the following methods have worked consistently in many laboratories.

12. Remove culture medium, and wash cells with **Rat Islet Washing Medium.**
13. Add a small volume of HBSS containing trypsin (0·05%) and EDTA (0·02%) for 5 min Add an equal volume of culture medium and mechanically dispersed the remaining intact islets by mild trituration.

14. The dispersed islets were washed and suspended in **Rat Complete Islet PrimaCell™ Culture Medium**.
15. Count an aliquot and seed the cells at the chosen concentration.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Skeletal muscle cells cultures derived from preconfluent layers.

16. Trypsinize cells as above, and centrifuge at 100 g for 10 min.
17. Resuspend cells in **Rat Complete Islet PrimaCell™ Culture Medium** and count cells.
18. Prepare aliquots of 2×10^6 cells/ml in **Rat Complete Islet PrimaCell™ Culture Medium** and 10% glycerol into cryovials.
19. Equilibrate at 4℃ for 1-2 h.
20. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
21. To recover cells:
    a. Thaw cryovials quickly in a 37℃ water bath.
    b. Dilute cells tenfold with appropriate **Rat Complete Islet PrimaCell™ Culture Medium**.
    c. Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in **Rat Complete Islet PrimaCell™ Culture Medium.**

IV Characterization

Adenylylcyclase can be used as a marker to assess islet cell viability as well as differences in preservation media and may predict islet cell transplant success. Besides, CGRP (Calcitonin gene-related peptide)-containing cells were found primarily in the peripheral portion of the pancreatic islets. Neuropilin-2 is a novel marker expressed in pancreatic islet cells and endocrine pancreatic tumours.

V References

1. Cohen, T., et al., *Neuropilin-2 is a novel marker expressed in pancreatic islet cells and endocrine pancreatic tumours.* J Pathol, 2002. **198**(1): p. 77-82.
2. Navarro-Tableros, V., et al., *Autocrine regulation of single pancreatic beta-cell survival.* Diabetes, 2004. **53**(8): p. 2018-23.
3. Fragner, P., S.L. Lee, and S. Aratan de Leon, *Differential regulation of the TRH gene promoter by triiodothyronine and dexamethasone in pancreatic islets.* J Endocrinol, 2001. **170**(1): p. 91-8.
4. Sjoholm, A., *Differential effects of cytokines on long-term mitogenic and secretory responses of fetal rat pancreatic beta-cells.* Am J Physiol, 1992. **263**(1 Pt 1): p. C114-20.
5. Islam, M.S., A. Sjoholm, and V. Emilsson, *Fetal pancreatic islets express functional leptin receptors and leptin stimulates proliferation of fetal islet cells.* Int J Obes Relat Metab Disord, 2000. **24**(10): p. 1246-53.
6. Lu, M., J. Seufert, and J.F. Habener, *Pancreatic beta-cell-specific repression of insulin gene transcription by CCAAT/enhancer-binding protein beta. Inhibitory interactions with basic helix-loop-helix transcription factor E47.* J Biol Chem, 1997. **272**(45): p. 28349-59.
7. Rosenbaum, T., M.C. Sanchez-Soto, and M. Hiriart, *Nerve growth factor increases insulin secretion and barium current in pancreatic beta-cells.* Diabetes, 2001. **50**(8): p. 1755-62.

8. Cabrera-Valladares, G., et al., *Effect of retinoic acid on glucokinase activity and gene expression and on insulin secretion in primary cultures of pancreatic islets.* Endocrinology, 1999. **140**(7): p. 3091-6.

Rat Pancreas PrimaCell™ II: Islet Cells

Rat Pancreas Primary Cell II Culture

Cat No.	Description	Qt.	Price
2-85104	**Rat Pancreas PrimaCell™ II system**	**kit**	**$499**
4-27131	Rat Pancreas Tissue Dissociation System II, Pancreas OptiTDS™ (for 500 ml medium)	1 ml	$128
9-49103	Rat Pancreatic Islets PrimaCell™ II Basal Culture Medium	500 ml	$61
9-38131	Rat Pancreatic Islets PrimaCell™ II Medium Supplements with Serum (for 500 ml medium)	set	$140
7-68131	Rat Pancreas Fibroblast Growth Inhibitors II, Pancreas FibrOut™	ea	$146
9-99103	Rat Pancreas Tissue II Preparation Buffer Set	ea	$75

Rat Primary Islet Cells Characterization

6-21811	**Rat Islet Cell Primarker™ Kit**	**kit**	**$220**
6-21812	Rat Islet Cell Primarker™ antibody set	set	$180
6-21813	Rat Islet Cell Primarker™ buffer system	set	$90

Rat Prostate PrimaCell™: Prostate Epithelium
(Cat No. 2-82037)

I. General Description:

This protocol is developed for attachment and growth of normal Rat Prostate epithelial (NHBE) cells from newborn or adult Rat Prostate with Rat Prostate PrimaCell™ system (Cat No. 2-82037). This system provides an optimal condition of tissue dissociation system, Prostate OptiTDS™ that yields 4-7 times of single cells more than most of the tissue dissociation protocols published in the literature. In addition, this system ensures a high viability of the target cells with improved gradient contained in the culture medium. With CHI Scientific's proprietary fibroblast inhibitory system, FibrOut™, cells are growing with contamination of minimized amount of the non-epithelial cells. This procedure involves explanting fragments of large Prostate tissue in a serum-free medium (LHC-9) in order to initiate and subsequently propagate fibroblast-free outgrowths of NHBE cells; four subculturings and 30 population doublings are routine.

Rat Prostate PrimaCell™ system applies to all type tissue samples from Rat at all age though younger tissue samples are recommended for yielding maximum amount of viable target cells. However, tissue samples contain pathological organism (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Rat Prostate PrimaCell™ System

- **Prostate Tissue Dissociation System, Prostate OptiTDS™,** (2 aliquots) --- *A mixture of collagenase I, collagenase II, collagenase IV, dispase and trypsin*.
- **Prostate OptiTDS™ Digestion Buffer**, (2 x 9 ml).
- **Coating Solution:** *A Mixure of Rat fibronectin, collagen, and crystallized bovine serum albumin (BSA), in basal culture medium.*
- **Prostate Tissue Washing Medium**, (1 x 100 ml) --- *Basal Prostate PrimaCell™ Culture Medium with 5% FBS, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Rat Prostate Fibroblast Growth Inhibitors, Prostate FibrOut™** (5 x 200 μl) --- *A mixture of cis-OH-proline, collagenase, D-valine, and formulated serum substitutes*.
- **Rat Prostate PrimaCell™ Basal Culture Medium**, (5 x100 ml) --- *Modified formulation based on medium 199 and Weymouth medium*.
- **Rat Prostate PrimaCell™ Medium Supplements**, (5 x 1.0 ml) --- A mixture of *of Rat fibronectin, collagen, and crystallized bovine serum albumin (BSA)*.
- **Rat Prostate PrimaCell™ Serum**, (1 x 50 ml) --- *Highly purified special-treated Fetal-bovine serum.*

1.2 Required Materials but not provided

- Plastic tissue culture dishes (60 and 100 mm)
- Scalpels No. 1621 (Becton Dickinson)
- Surgical scissors
- Half-curved microdissecting forceps

- Pipettes (10 and 25 ml)
- Trypsin (Cooper Biomedical), 0.02%, EGTA (Sigma), 0.5 mM, and polyvinylpyrrolidine (USB), 1% solution
- High-O2 gas mixture (50% O2, 45% N2, 5% CO2)
- Gloves sterilized with autoclave (Rat tissue can be contaminated with biologically hazardous agents)
- Controlled atmosphere chamber
- Rocker platform
- Phase-contrast inverted microscope

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, culture plate (Corning, NY) must be coated with the provided coating solution.

2.2 Surgical specimens

1. Prostate tissue from autopsy of noncancerous rats is recommended for yielding a large number of cells with a 30-40% plating efficiency.
2. Incubate Prostate tissues for up to 10 min in Prostate Tissue Washing Medium to prevent infection followed. (This procedure should not affect cell viability.)
3. Incubate Prostate tissues in 10 ml 70% sterile ethanol for 2 min.
4. Rinse tissue twice in Prostate Tissue Washing Medium for 10 min each and kept on ice.

2.3 Tissue Preparation and Tissue Dissociation

2.3.1 Rat Prostate OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the donor used, enzymes, culture mediums and growth supplements. The Rat Prostate Tissue Dissociation System is suited for optimal dissociation of normal rat islet tissues to yield maximum number of single islet cell.

2.3.2 Enzyme Compositions

- Collagenase: from *Clostridium Histolyticum*
- Trypsin: from *Clostridium Histolyticum*

2.3.3 System Components

- Rat Prostate Tissue Dissociation System, Prostate OptiTDS™, (2 × 1 ml).
- Rat Prostate OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.3.4 Procedures For Tissue Preparation and Dissociation

5. Prepare fresh enzyme working solution: Add 1ml of the reconstituted tissue dissociation solution to one vial of Rat Prostate OptiTDS™ Digestion Buffer (9 ml). Warm the diluted Rat Prostate OptiTDS™ working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 5 ml Rat Prostate OptiTDS™ working solutions.
6. Discard the **Rat Prostate Washing Medium,** and float samples on 5 ml of Rat Prostate Tissue Dissociation System working solution (prewarmed to 37℃) and transfer the minced tissue to a sterile centrifuge tube (10–12 pancreases each).
7. The tissue was digested in a shaking water bath at 37 C for 10-30 min.

2.4 Prostate Epithelial cells Isolation

Note: Please read section 2.5 for specific information on Cervical Epithelium culture and platting before preceding this section.

8. The resulting digests were washed three times with cold **Rat Prostate Washing Medium.**
9. The pellets were resuspended in **Rat Complete Prostate PrimaCell™ Culture Medium/FibrOut** and distributed in 50 mm plastic culture dishes.
10. The Prostates were cultured for 5 days in 5 ml **Rat Complete Prostate PrimaCell™ Culture Medium/FibrOut** at 37 ^{o}C in a humidified atmosphere of 5% CO2.

2.5 Primary Prostate Epithelial Cells Culture

2.6.1 Medium Preparation.

Thaw out **Rat Prostate PrimaCell™ Basal Culture Medium**, **Rat Prostate cell PrimaCell™ Medium Supplements**, and **Rat Prostate PrimaCell™ Serum** on ice.

Rat Complete Prostate PrimaCell™ Culture Medium: To every 100 ml **Rat Prostate PrimaCell™ Basal Culture Medium**, add 10 ml **Rat Prostate PrimaCell™ Medium Supplements** and 1 ml **Rat Prostate PrimaCell™ Serum** mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use.

Rat Complete Prostate PrimaCell™ Culture Medium/FibrOut: To every 100 ml **Rat Prostate PrimaCell™ Basal Culture Medium**, add 10 ml **Rat Prostate PrimaCell™ Medium Supplements,** 1 ml **Rat Prostate PrimaCell™ Serum**, and 1 ml **Rat Prostate Fibroblast Growth Inhibitors, Prostate FibrOut™**,mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use. (**Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*)

2.6.2 Primary Cell Culture

11. The medium was changed every day. At the end of the preculture period, the Prostates attached to the bottom of the culture dishes were gently blown free using a sterilized Pasteur pipette under a stereomicroscope. The fibroblast layer remaining on the bottom of the culture dishes
12. The detached Prostates were cultured free-floating in 50 mm Petri dishes in **Rat Complete Prostate PrimaCell™ Culture Medium/FibrOut** changed every other

day.

Δ ***Safety Note.*** The rest of the biopsy and all tubes, pipettes, plates, etc., used in the procedure should be treated with hypochlorite before disposal.

2.5.3 Subculture and Propagation

13. Gently rinse the culture dish twice with 0.02% (0.7 mM) EDTA.
14. Add 3 ml of 0.25% trypsin/0.1% (2.5 mM) EDTA, and incubate at 37℃. Examine the dish under phase microscopy every 5 min to detect cell detachment.
15. When most cells have detached, add 12 ml complete melanocyte growth medium to inactive the trypsin activity.
16. Pipette the contents of the dish to ensure complete melanocyte melanocyte detachment.
17. Aspirate and centrifuge the cells for 5 min at 350 g.
18. Aspirate the supernatant, resuspend the cells in a complete growth medium, and replate at 2-4 $\times$ 10^4 cells per 100-mm dish.
19. Refeed the culture twice a week with complete melanocyte growth medium.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample; the best results are reported when cells from preconfluent primary cultures are used.

20. Detach cells as for the subculture, and centrifuge at 100 g for 10 min.
21. Resuspend cells in complete culture medium with serum, and count.
22. Dispense aliquots of 2 x 10^6 cells/ml in complete growth medium with additional 10% FBS and 10% glycerol into cryopreservation tubes.
23. Equilibrate at 4℃ for 1-2 h.
24. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
25. To recover cells:
    (i) Thaw cryotubes quickly in a 37℃ water bath.
    (ii) Dilute cells tenfold with medium.
    (iii) Centrifuge cells and resuspend them at an appropriate concentration in the desired culture medium, and seed culture vessel.

Rat cells can be grown in all media for 4-7 weeks and can be subcultured only 4-5 times.

IV Fibroblast Contamination

There are several techniques have been published in the literature to deal with fibroblast contamination during colorectal primary cell culture. These include: (1) Physically remove a well-isolated fibroblast colony by scraping it with a sterile blunt instrument (e.g., a cell scraper). Care has to be taken to wash the culture up to six times to remove any fibroblasts that have detached in order to prevent them from reseeding and reattaching to the flask. (2) Differential trypsinization can be attempted with the carcinomas. (3) Dispase preferentially (but not exclusively) removes the epithelium during passaging and leaves behind most of the fibroblastic cells attached to the culture vessel. During subculture, cells

that have been removed with dispase can be preincubated in plastic petri dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the epithelium. This technique takes advantage of the fact that fibroblasts in general attach much more quickly to plastic than do clumps of epithelial cells, so that a partial purification step is possible. (4) Reduce the concentration of serum to about 2.5-5% if there are heavy concentrations of fibroblastic cells. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and that using any or all of the preceding techniques will eventually push the cells through so many divisions that any fibroblasts will senesce.

Rat Prostate PrimaCell™ I system includes a fibroblast elimination system, the Rat Prostate Fibroblast Growth Inhibitors, Prostate FibrOut™. It contains a mixture of of cis-OH-proline, collagenase, D-valine, and formulated serum substitutes. This system can effectively eliminate Prostate fibroblast contamination while has not affect on the behavior of targeted cells.

VI References:

1. Yim HW, Slebos RJ, Randell SH, Umbach DM, Parsons AM, Rivera MP, Detterbeck FC, Taylor JA. *Smoking is associated with increased telomerase activity in short-term cultures of Rat Prostate epithelial cells.* Cancer Lett. 2006 Mar 3;
2. Doherty GM, Christie SN, Skibinski G, Puddicombe SM, Warke TJ, de Courcey F, Cross AL, Lyons JD, Ennis M, Shields MD, Heaney LG. *Non-bronchoscopic sampling and culture of Prostate epithelial cells in children.* Clin Exp Allergy. 2003 Sep;33(9):1221-5.
3. Mattinger C, Nyugen T, Schafer D, Hormann K. *Evaluation of serum-free culture conditions for primary Rat nasal epithelial cells.* Int J Hyg Environ Health. 2002 Apr;205(3):235-8.
4. de Jong PM, van Sterkenburg MA, Kempenaar JA, Dijkman JH, Ponec M. *Serial culturing of Rat Prostate epithelial cells derived from biopsies.* In Vitro Cell Dev Biol Anim. 1993 May;29A(5):379-87.
5. Robbins RA, Koyama S, Spurzem JR, Rickard KA, Nelson KJ, Gossman GL, Thiele GM, Rennard SI. *Modulation of neutrophil and mononuclear cell adherence to Prostate epithelial cells.* Am J Respir Cell Mol Biol. 1992 Jul;7(1):19-29.
6. Lechner JF, Wang Y, Siddiq F, Fugaro JM, Wali A, Lonardo F, Willey JC, Harris CC, Pass HI. *Rat lung cancer cells and tissues partially recapitulate the homeobox gene expression profile of embryonic lung.* Lung Cancer. 2002 Jul;37(1):41-7.
7. Lechnewr JF, & LeVeck MA. *A serum free method for culturing normal Rat Prostate epithelial cells at clonal density.* J. Tissue Cult. Methods 9: 43-48.

Rat Prostate PrimaCell™: Prostate Epithelium

Rat Prostate Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82037	**Rat Prostate PrimaCell™ system**	**kit**	**$ 499**
4-21171	Rat Prostate Tissue Dissociation System, Prostate OptiTDS™ (for 500 ml medium)	1 ml	$ 128
9-41017	Rat Prostate Epithelium PrimaCell™ Basal Culture Medium	500 ml	$73
9-31171	Rat Prostate Epithelium PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
7-61171	Rat Prostate Fibroblast Growth Inhibitors, Prostate FibrOut™	ea	$ 146
9-91017	Rat Prostate Tissue II Preparation Buffer Set	ea	$75

Rat Primary Prostate Epithelium Characterization

6-21911	**Rat Prostate Epithelium Primarker™ Kit**	**kit**	**$220**
6-21912	Rat Prostate Epithelium Primarker™ antibody set	set	$180
6-21913	Rat Prostate Epithelium Primarker™ buffer system	set	$90

Rat Skin PrimaCell™ I: Melanocytes
(Cat No. 2-82038)

I. General Description:

This protocol is developed for attachment and growth of normal rat skin melanocytes from newborn or adult rat skin with Rat Skin PrimaCell™ I system (Cat No. 2-82038). This system provides an optimal condition of tissue dissociation system, Skin OptiTDS™ that yields 4-7 times of single cells more than most of the tissue dissociation protocols published in the literature. In addition, this system ensures a high viability of the target cells with improved gradient contained in the culture medium. With CHI's proprietary fibroblast inhibitory system, FibrOut™, cells are growing with contamination of minimized amount of the non-epithelial cells. The preparation of tissue specimens for cell culture is usually started within 1-2 h of removal from rats. If this is impossible, fine cutting of the tissue into small pieces (2 x 2 mm) with scalpels and storage overnight at 4℃ in washing medium (see below) can also prove successful.

Rat SkinPrimaCell™ I system applies to all types skin samples from rats at all age though newborn rats are recommended for yielding maximum amount of viable target cells. Skin samples contain pathological organism (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Rat Skin PrimaCell™ I System

- **Skin Tissue Dissociation System, Skin OptiTDS™,** (2 aliquots) --- *A mixture of collagenase I, collagenase II, collagenase IV, dispase and trypsin*.
- **Skin OptiTDS™ Reconstitution Buffer**, (2 x 1 ml).
- **Skin OptiTDS™ Digestion Buffer**, (2 x 10 ml).
- **Skin Tissue Washing Medium**, (5 x 10 ml) --- *Basal Skin PrimaCell™ I Culture Medium with 5% FBS, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Rat Skin Fibroblast Growth Inhibitors, Skin FibrOut™** (5 x 200 μl) --- *A mixture of cis-OH-proline, collagenase, D-valine, and formulated serum substitutes*.
- **Rat Skin PrimaCell™ I Basal Culture Medium**, (5 x100 ml) --- *Modified formulation based on medium 199 and Weymouth medium*.
- **Rat Skin PrimaCell™ I Medium Supplements**, (5 x 1.0 ml) --- A mixture of *basic fibroblast growth factor, cholera toxin, hydrocortisone, Insulin, transferring, insulin, and epidermal growth factor*.
- **Rat Skin PrimaCell™ I Serum**, (1 x 50 ml) --- *Highly purified special-treated Fetal-bovine serum.*

1.2 Required Materials but not provided

- Pasteur pipettes
- Collagen I-coated Culture dishes
- Scalpels, scissors, and forceps
- Pasteur pipettes and 10-ml pipettes
- Test tubes, 12 and 50 ml

- Nylon gauze cell strainer

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, collagen I-coated plate (Corning, NY) is recommended.

2.2 Surgical specimens

- Newborn (prefer 1-2 days) rats epidermis is recommended for yielding a large number of cells (5-10 x 10^6/epidermis), with a 30-40% plating efficiency. Rats are sacrificed by CO_2 narcosis 1-4 days postpartum (prior to the appearance of hair). Using an aseptic technique, limbs and tails are amputated, a longitudinal incision is made from tail to snout, and skin is peeled off the carcass using forceps.
- Incubate skin biopsies for up to 10 min in Skin Tissue Washing Medium to prevent infection. (This procedure should not affect Melanocytes viability.)
- Incubate skin biopsies in 10 ml 70% sterile ethanol for 2 min.
- Rinse tissue twice in Skin Tissue Washing Medium for 10 min each and kept on ice.

2.3 Tissue Preparation and Dissociation

2.3.1 Rat Skin OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Rat Skin Tissue Dissociation System, OptiTDS™, is suited for optimal dissociation of normal adult and newborn skin biopsies samples to yield maximum number of single primary cells of colonic tissues.

2.3.2 Enzyme Compositions

- Trypsin: from *Bovine Pancreas*
- Dispase: from *Bacillus polymyxa*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase II: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*

2.3.3 System Components

- Skin Tissue Dissociation System, OptiTDS™, 2 vials.
- Skin OptiTDS™ Reconstitution Buffer, (2 x 1 ml).
- Skin OptiTDS™ Digestion Buffer, (2 x 9 ml).

2.3.4 Procedures For Tissue Preparation and Dissociation

5. Prepare fresh enzyme working solutions: to each vial of Skin Tissue Dissociation System, OptiTDS™, add 1.0 ml of the Skin OptiTDS™ Reconstitution Buffer, Mix

well.

6. Add 1.0 ml of the fresh enzyme working solution to one vial of Skin OptiTDS™ Digestion Buffer (9.0 ml). Warm the diluted Skin OptiTDS™ working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 10 ml diluted Skin OptiTDS™ working solutions.
7. Mince pre-washed tissue into pieces approximately 0.2-0.5 mm^2 in diameter with two scalpel and forceps.
8. Incubate tissue with Skin Dissociation System by one of the following steps:
   (a) Rapid dissociation: Float skin samples in Skin Dissociation System solution for 2-3 h min at 37 ℃. This works particularly well also with full-thickness skin.
   (b) Slow dissociation: Float the samples on ice-cold Skin Dissociation System at 4℃ for 15-24 h.
9. Monitor the separation of the epidermis carefully. When the first detachment of the epidermis is visible at the cut edges of skin samples, carefully separate epidermis from dermis. Place the epidermis (dermis side down) in 100-mm plastic Petri dishes and irrigate with 10 ml fresh Skin OptiTDS™ solution.
10. Once all pelts have been processed, use scissors to cut epidermis and disrupt beta pleated sheets in the epidermis. Transfer all solution containing skin samples into a sterile beaker or container.
11. Rinse sterile stir bar in PBS and place in cell mixture and stir gently for 20-30 min.
12. Rinse woven cloth 2X in PBS and place into sterile funnel on top of sterile beaker and strain the cell mixture to remove debris (alternatively, a Nylon gauze cell strainer can be used).
13. Passing the cell mixture with sterile pipette several times to facilitate a single cell mixture, pass through the Nylon gauze cell strainer.
14. Collect cells by centrifugation at 100 g, washing cells with 10 ml PBS or washing medium twice. At the end of washing process, collect cells and dilute cell in 0.5-1.0 ml complete culture medium.
15. Count viable cells and plate 1.0 x 10^6/ 100mm dish.
16. Seed cells at 37℃ in Complete Rat Skin PrimaCell™ I Culture Medium at desired densities:

2.3.5 Storage:

Reconstituted tissue dissociation systems should occur before use and can only be stored for 2-4 days at 4 ^oC. For long-term use, it should be aliquoted and stored at -20 ^oC. Avoid repeated freeze-thaw cycles.

2.4 Culture of Primary Rat Melanocytes

2.4.1 Medium Preparation.

Thaw out the Rat Skin PrimaCell™ Medium Supplements and Rat Skin PrimaCell™ I Serum on ice. To every 100 ml Rat Skin PrimaCell™ I Basal Culture Medium, add one vial of Rat Skin PrimaCell™ I Medium Supplements; 10 ml Rat Skin PrimaCell™ I Serum; and one vial of Rat Skin Fibroblast Growth Inhibitors, Skin FibrOut™, mix thoroughly and warm the complete medium at a 37^oC water bath for 10 min prior use.

2.6.1 Primary Culture Conditions.

Inoculate epithelial cells derived from tissue specimens contains both Melanocytes and Melanocytes. Seed cells into T-25 flasks coated with collagen type I in a 5%-CO_2 incubator with 4 ml of complete culture medium. Change the culture medium twice weekly. The cultures will contain primary Melanocytes with scattered melanocytes. Melanocytes proliferation should cease within several days, and colonies should begin to detach during the second week. By the end of the third week, only melanocytes should remain. In most cases, cultures attain near confluence and are ready to passage within 2-4 weeks.

2.4.3 Subculture and Propagation

17. Gently rinse the culture dish twice with 0.02% (0.7 mM) EDTA.
18. Add 3 ml of 0.25% trypsin/0.1% (2.5 mM) EDTA, and incubate at 37℃. Examine the dish under phase microscopy every 5 min to detect cell detachment.
19. When most cells have detached, add 12 ml complete melanocyte growth medium to inactive the trypsin activity.
20. Pipette the contents of the dish to ensure complete melanocyte melanocyte detachment.
21. Aspirate and centrifuge the cells for 5 min at 350 g.
22. Aspirate the supernatant, resuspend the cells in a complete growth medium, and replate at 2-4 × 10^4 cells per 100-mm dish.
23. Refeed the culture twice a week with complete melanocyte growth medium.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample; the best results are reported when cells from preconfluent primary cultures are used.

24. Detach cells as for the subculture, and centrifuge at 100 g for 10 min.
25. Resuspend cells in complete culture medium with serum, and count.
26. Dispense aliquots of 2 x 10^6 cells/ml in complete growth medium with additional 10% FBS and 10% glycerol into cryopreservation tubes.
27. Equilibrate at 4℃ for 1-2 h.
28. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
29. To recover cells:
    (i) Thaw cryotubes quickly in a 37℃ water bath.
    (ii) Dilute cells tenfold with medium.
    (iii) Centrifuge cells and resuspend them at an appropriate concentration in the desired culture medium, and seed culture vessel.

Rat cells can be grown in all media for 4-7 weeks and can be subcultured only 4-5 times.

IV Fibroblast Contamination

There are several techniques have been published in the literature to deal with fibroblast contamination during colorectal primary cell culture. These include: (1) Physically remove a well-isolated fibroblast colony by scraping it with a sterile blunt instrument (e.g., a cell scraper). Care has to be taken to wash the culture up to six times to remove any fibroblasts that have detached in order to prevent them from reseeding and reattaching to the flask. (2) Differential trypsinization can be attempted with the carcinomas. (3) Dispase preferentially (but not exclusively) removes the epithelium during passaging and leaves

behind most of the fibroblastic cells attached to the culture vessel. During subculture, cells that have been removed with dispase can be preincubated in plastic petri dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the epithelium. This technique takes advantage of the fact that fibroblasts in general attach much more quickly to plastic than do clumps of melanocytes, so that a partial purification step is possible. (4) Reduce the concentration of serum to about 2.5-5% if there are heavy concentrations of fibroblastic cells. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and that using any or all of the preceding techniques will eventually push the cells through so many divisions that any fibroblasts will senesce.

Rat Skin PrimaCell™ I system includes a fibroblast elimination system, the Rat Skin Fibroblast Growth Inhibitors, Skin FibrOut™. It contains a mixture of of cis-OH-proline, collagenase, D-valine, and formulated serum substitutes. This system can effectively eliminate skin fibroblast contamination while has not affect on the behavior of melanocytes.

V Confirmation of Melanocytic Identity

Melanocyte cultures may be contaminated initially with melanocytes and at any time by dermal fibroblasts. Both forms of contamination are rare in cultures established and maintained by an experienced technician or investigator, but are common problems for the novice. The cultured cells can be confirmed to be melanocytes with moderate certainty by frequent examination of the culture under phase microscopy, assuming that the examiner is familiar with the respective cell morphologies. More definitive identification is provided by electron microscopic examination, DOPA staining, or immunofluorescent staining with Mel 5 antibody, directed against tyrosinase-related protein-1.

VI References:

1. Fetissov SO, Barcza MA, Meguid MM, Oler A. *Hypophysial and meningeal melanocytes in the Zucker rat.* Pigment Cell Res. 1999 Oct;12(5):323-30.
2. Glimcher ME, Szabo G. *Giant pigment granules in dermal melanocytes of rat scrotal skin.* Experientia. 1978 Aug 15;34(8):1079-80.
3. Glimcher ME, Garcia RI, Szabo G. *Organ culture of mammalian skin and the effects of ultraviolet light and testosterone on melanocyte morphology and function.* J Exp Zool. 1978 May;204(2):229-37.

Mouse Skin PrimaCell™ I: Melanocytes

Mouse Skin Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82018	**Mouse Skin PrimaCell™ I system**	**kit**	**$ 520**
4-22181	Mouse Skin Tissue Dissociation System, Skin OptiTDS™ (for 500 ml medium)	1 ml	$128
9-42018	Mouse Melanocytes PrimaCell™ Basal Culture Medium	500 ml	$ 73
9-32181	Mouse Melanocytes PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$ 140
7-62181	Mouse Skin Fibroblast Growth Inhibitors, Skin FibrOut™	ea	$146
9-92018	Mouse Skin Tissue I Preparation Buffer Set	ea	$75

Mouse Primary Melanocytes Characterization

6-12011	**Mouse Melanocyte Primarker™ Kit**	**kit**	**$220**
6-12012	Mouse Melanocyte Primarker™ antibody set	set	$180
6-12013	Mouse Melanocyte Primarker™ buffer system	set	$90

Rat Skin PrimaCell™ II: Epidermal Keratinocytes
(Cat No. 2-82040)

I. General Description

Keratinocytes have a unique biology and thus are widely used for experiments to study the activity of oncogenes in epithelial neoplasias, and the molecular mechanisms implicated in warts and other skin associated disorders. In addition, several *in vitro* skin models have been developed that accurately mimic the epidermis making it possible to study the skin in a physiologically relevant context. While these cells are extremely useful in the laboratory they are notoriously difficult to isolate and culture. The Rat Skin PrimaCell™ II kit (Cat No. 2-82040) is designed for the successful isolation and culture of epidermal keratinocytes from Rat skin allowing reproducible and dependable results.

1.1 Components of the Rat Skin PrimaCell™ II System

- **Rat Skin Tissue Dissociation System II, Skin OptiTDS™ II** (2 aliquots) --- *A mixture of collagenase I, collagenase III, collagenase IV, collagenase, and trypsin*.
- **Rat Skin OptiTDS™ II Reconstitution Buffer,** (2 x 1 ml)
- **Rat Skin OptiTDS™ II Digestion Buffer,** (4 x 4.5 ml)
- **Rat Skin Fibroblast Growth Inhibitors** ---Skin FibrOut™ (5 x 200 µl) --- *A mixture of toxin rici , collagenase, D-valine, cis-OH-proline, and formulated serum.*
- **Rat Skin Keratinocytes Basic Culture Medium** (5 x 95 ml) --- *Modified formulation based on NCTC 168 and Weymouth medium*.
- **Rat Skin Keratinocytes Culture Medium Supplements with Serum** (5 x 5 ml): *insulin, hydrocortisone, EGF, transferring, highly purified serum substitute, and L-ascorbic acid, $CaCl_2$, Bovine Pituitary Extract*, *antibiotics (penicillin, 100 U/ml), and streptomycin.*
- **Buffer Systems for Rat Skin Preparation** (1 x 100 ml): Basal Culture medium containing Betadine solution.

1.2 Required materials but NOT included:

- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or rat fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To

enhance cell attachment to the culture dishes, fresh gelatin-coated plate or culture dishes are recommended (see below for treatment of culture dishes).

2.2 Principle

Separation of the epidermis from the dermis is accomplished by enzymatic digestion using the Skin Tissue Dissociation System II supplied in this kit. The Skin Tissue Dissociation System II contains a mixture of trypsin, dispase and type I, II, III, and IV collagenases at the optimal concentrations to gently detach the fragile epidermal layer from the dermis. The isolated epidermis is then further disrupted to release individual keratinocytes by enzymatic and mechanical agitation. The mixture is then filtered through Cell Strainers and seeded on specially coated tissue culture plates. The Keratinocytes are propagated in serum-free, low-calcium media or growth arrested feeder cells and the corresponding media. Sub-populations of keratinocyte stem cells can then be isolated based on their selective attachment to specific basement matrix substrates.

2.3 Rat Skin Tissue Preparation

Rat Skin: Skin tissues from adult or newborn after birth can be used, however, newborn or embryonic tissue specimen are preferred since they proliferate well. Each 5-7 cm^2 rat epidermis yield large number of cells (5-10 x 10^6/epidermis), with a 20-40% plating efficiency.

1. Skin Tissue specimen must be kept on ice and in cold DMEM medium containing 10% FBS all the time prior to next step. It is strongly recommended that tissue specimen must not be stored exceed more than 12 hours.
2. Incubate skins in Betadine solution for up to 5 min to prevent infection. (This procedure should not affect keratinocytes cell viability.)
3. Rinse skins twice in cold PBSA for 5 min while keeping on ice.

2.4 Epidermal Separation and Tissue Dissociation

2.4.1 Rat Skin OptiTDS™ II

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Rat Skin Tissue Dissociation System II is suited for optimal dissociation of normal newborn Rat skin tissues to yield maximum number of single keratinocyte cells.

2.4.2 Enzyme Compositions

- Trypsin: from *Bovine Pancreas*
- Dispase: from *Bacillus polymyxa*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase II: from *Clostridium Histolyticum*
- Collagenase III: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*

2.4.3 System Components

- Rat Skin Tissue Dissociation System II, Skin OptiTDS™ II, 2 vials.
- Rat Skin OptiTDS™ II Reconstitution Buffer, (2 x 1 ml).
- Rat Skin OptiTDS™ II Digestion Buffer, (4 x 4.5 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

4. Reconstitute tissue dissociation solution: to each vial of Rat Skin Tissue Dissociation System II, Skin OptiTDS™ II, add 1.0 ml of the Rat Skin OptiTDS™ II Reconstitution Buffer, Mix well.
5. Prepare fresh enzyme working solution: Add 500 µl of the reconstituted tissue dissociation solution to one vial of Rat Skin OptiTDS™ II Digestion Buffer (4.5 ml). Warm the diluted Rat Skin OptiTDS™ II working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 5 ml Rat Skin OptiTDS™ II working solutions.
6. Dissociation can be achieved in either of the following two methods

   (a) Rapid dissociation: Float skins on Rat Skin Tissue Dissociation System II working solution for 2-3 hrs at 37 ℃. This works particularly well with full-thickness skin.
   (b) Slow dissociation: Float the samples on ice-cold Rat Skin Tissue Dissociation System II working solution at 4℃ for 15-24 h. This is particularly convenient for flexible scheduling of Keratinocytes preps.

7. Monitor the separation of the epidermis carefully when using the rapid dissociation method. When the first detachment of the epidermis is visible at the edge of each skin carefully separate the epidermis from dermis. Place the entire skin on a clean and dry tissue culture plate epidermis side up. Starting at the edge of the skin tease out the epidermis from the dermis using two forceps. Once you have the epidermis, gently start pulling it away from the dermis, while using one of the forceps to stabilize the dermis on the plate. Place the epidermis in 10 ml DMEM containing 10% FBS, antibiotics, and 500 µl reconstituted Rat Skin Tissue Dissociation System II (not the working solution).

2.5 Keratinocytes Isolation

Note: Please read section 2.6 for specific information on Keratinocytes culture and platting before preceding this section.

8. Once all epidermis have been separated, use scissors to mince the epidermi in order to disrupt the beta pleated sheets that hold the keratinocytes together. Particular attention should be given to this step to ensure that the epidermi are completely minced to a fine pulp. Transfer all medium containing the epidermal pulp into a sterile beaker or plastic container.
9. Rinse a sterile stir bar in PBSA and then place the stir bar into the pulp mixture. Stir gently for 30 min.
10. Strain the cell mixture through a sterile cell strainer (70-100µm) to remove debris. Cell strainers fit perfectly in 50 ml conical and are very convenient for this procedure. If cell strainers are not available, nylon gauze can be used after rinsed twice in PBS and placed at the opening of a 50 ml conical.
11. Centrifuge the strained mixture at 1500 rmp for 10 min at 4°C.
12. Carefully pour off the supernatant and discard.
13. Add 10 ml of fresh DMEM containing 10% FBS and 100µl Rat Skin FibrOut™ to the Keratinocytes pellet and pipette with a sterile pipette several times to ensure that the

keratinocytes are in a single cell suspension.
14. Count viable cells and plate 1.0 x 10^6/ 100mm dish.
15. Check cells after 2-3 hrs for attachment. Please be sure cells are attached before preceding the following.
16. At which time most cells have attached, carefully aspirate off DMEM, and add 10ml of complete Rat Skin Keratinocytes Culture Medium (see below).

2.6 Primary Keratinocytes Culture

17. Primary keratinocytes can be cultured alone or on a layer of feeder cells depending on the nature of the experiments.

(a) Keratinocyte Culture with feeder layer cells:

- Prepare feeder layers by culturing 3T3 cells or rat fibroblasts for 3 days. When the cells have reached confluence irradiate the 3T3 at 30 Gy or the rat fibroblasts at 70 Gy.
- Prepare Keratinocytes as usual (see procedure above) in complete DMEM containing 10% FBS and seed on dishes that contain the already irradiated feeder layers. Seed Keratinocytes at 2-5 x 10^4 cells/cm^2.
- Change to complete Rat Skin Keratinocytes Culture Medium after 2-3 hrs or visible attachment, however, the time should not exceed 5 hours.

(b) Keratinocyte Culture without feeder layer:

- Prepare complete DMEM containing 10% FBS, add 100µl Rat Skin FibrOut™
- Prepare complete Rat Skin Keratinocytes Culture Medium (95 ml Rat Skin Keratinocytes Basic Culture Medium + 5 ml Rat Skin Keratinocytes Culture Medium Supplements with Serum +100 µl Skin FibrOut™).
- Pre-coat tissue culture dishes with collagen freshly. Dilute collagen solution (Vitrogen 100, Palo Alto, CA) in sterile PBS and completely coat tissue culture dishes. Place in the incubator for at least 1 hr before platting the Keratinocytes. When ready to plate the cells, aspirate off the collagen solution and seed the Keratinocytes. (We have directly used collagen-coated plates from Vitrogen 100 but resulted lower efficiency).
- Place the isolate Keratinocytes from step 13 (1-5 x 10^4 cells/cm^2) and seed in the collagen coated plates with 10 ml complete DMEM for 2-3 hr. After cells have attached aspirate off DMEM and add complete Rat Skin Keratinocytes Culture Medium.

Note: Keratinocytes do well when maintained at high densities on tissue culture dishes

18. Keratinocytes will form stable layers for 1-3 days. To maintain viability in culture rinse cells several times with Rat Skin Keratinocytes Basic Culture Medium to eliminate nonattached dead and differentiated cells. If cells are to be cultured further then it is necessary to change culture medium every 2 days. Differentiation and growth arrest can be achieved by increasing the Ca^{2+} concentration to 1.2 mM by adding $CaCl_2$ to complete Rat Skin Keratinocytes Culture Medium which contains 0.05 mM $CaCl_2$.

2.7 Subculture

19. Propagating keratinocytes in culture can be somewhat challenging, especially when

keratinocytes are cultured alone on plastic, however the following methods have worked consistently in many laboratories.

(a) Keratinocytes grown on a feeder layer:
- (i) Incubate in 0.05-0.1% EDTA for 5-15 min to initiate cell detachment. Cells start to detach when the areas around cells become enlarged.
- (ii) Incubate in 0.1% trypsin and 1.3 mM (0.05%) EDTA at 37 ℃ for 5-10 min, followed by gentle pipetting, to completely detach the cells.

(b) Cultures in complete Rat Skin Keratinocytes Culture Medium:
- (i) EDTA should NOT be used when keratinocytes are cultured without feeder cells.
- (ii) Remove complete Rat Skin Keratinocytes Culture Medium and wash keratinocytes in 0.1% trypsin two times. Place just enough trypsin (0.1 % trypsin without EDTA) to moisten keratinocyte layer (200-500 µl depending on size of the dish). Incubate for 1-5 min at 37°C.
- (iii) Gently pipette keratinocytes and resuspend in complete Rat Skin Keratinocytes Culture Medium (FibrOut™ is not necessarily needed at this step) for counting and replatting on collagen coated tissue culture plates.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Keratinocytes cultures derived from preconfluent layers.

20. Trypsinize cells as above, and centrifuge at 100 g for 10 min.
21. Resuspend cells in complete complete Rat Skin Keratinocytes Culture Medium without FibrOut™ and count cells.
22. Prepare aliquots of 2 x 10^6 cells/ml in complete DMEM medium with additional normal 10% FBS (total 20-25% FBS) and 10% glycerol into cryovials.
23. Equilibrate at 4℃ for 1-2 h.
24. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
25. To recover cells:
    - (i) Thaw cryovials quickly in a 37℃ water bath.
    - (ii) Dilute cells tenfold with appropriate culture media.
    - (iii) Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in Media A or B depending on whether feeder layers are used.

IV Characterization

Keratinocytes can be characterized based on their specific for their epidermal (epithelial) phenotype to exclude contamination by mesenchymal cells. This is best achieved using cytokeratin-specific antibodies for the epithelial cells. Contaminating endothelial cells can be identified by antibodies against CD31 or factor VIII-related antigen. Identifying fibroblasts unequivocally is difficult, because the use of antibodies against vimentin (the mesenchymal cytoskeletal element) is not specific; Keratinocytes *in vitro* may initiate vimentin synthesis at frequencies that depend on culture conditions. As a practical assessment for mesenchymal cell contamination, cells should be plated at clonal densities (1-5 x 10^2 cells/cm^2) on feeder cells, and clone morphology should be identified at low magnification following fixation and haematoxylin and eosin (H&E) staining of 10- to 14-d cultures. A more specific and highly sensitive method to

identify contaminating fibroblasts is the analysis of expression of Keratinocytes growth factor (KGF) by RT-PCR. Since this factor is produced in fibroblasts and not in Keratinocytes, it represents a selective marker. Moreover, KGF expression is enhanced by co-cultured Keratinocytes so that a minority of contaminating fibroblasts will be detected by this assay.

V References:

1. Albarenque SM, Doi K. 2005. T-2 toxin-induced apoptosis in rat keratinocyte primary cultures.. *Exp Mol Pathol. Apr;78(2):144-9.*
2. Albarenque SM, Doi K. 1981. Requirement of hydrocortisone and insulin for extended proliferation and passage of rat keratinocytes. *In Vitro*. Nov;17(11):941-6.
3. Dlugosz, A. A., Glick, A. B., Tennenbaum, T., Weinberg, W. C., and Yuspa, S. H. 1995. Isolation and utlization of epidermal keratinocytes for oncogene research. *Methods in Enzymol.* **254**:3-20.
4. Bayley SA, Stones AJ, Smith CG. 1988. Immortalization of rat keratinocytes by transfection with polyomavirus large T gene. *Exp Cell Res.* 1988 Jul;177(1):232-6.
5. Parenteau NL, Pilato A, Rice RH. 1986. Induction of keratinocyte type-I transglutaminase in epithelial cells of the rat. *Differentiation*. 33(2):130-41.
6. Stark, H.-J., Baur, M., Breitkreutz, D., Mirancea, N. and Fusenig, N. E. 1999. Organotype keratinocyte cocultures in defined medium with regular epidermal morphogenesis and differentiation. *J. Invest. Derm.* **112**:681-691.
7. Oku H, Kumamoto C, Miyagi T, Hiyane T, Nagata J, Chinen I. 1994. Serum-free culture of rat keratinocytes. *In Vitro Cell Dev Biol Anim.* Aug;30A(8):496-503.

Rat Skin PrimaCell™ II: Epidermal Keratinocytes

Rat Skin Primary Cell II Culture

Cat No.	Description	Qt.	Price
2-82040	**Rat Skin PrimaCell™ II system**	**kit**	**$499**
4-29181	Rat Skin Tissue Dissociation System, Skin OptiTDS™ (for 500 ml medium)	1 ml	$128
9-41019	Rat Keratinocytes PrimaCell™ Basal Culture Medium	500 ml	$86
9-31191	Rat Keratinocytes PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$140
7-91181	Rat Skin Fibroblast Growth Inhibitors, Skin FibrOut™	ea	$146
9-91019	Rat Skin Tissue II Preparation Buffer Set	ea	$75

Rat Primary Epidermal Keratinocytes Characterization

6-22711	**Rat Epidermal Keratinocyte Primarker™ Kit**	**kit**	**$220**
6-22712	Rat Epidermal Keratinocyte Primarker™ antibody set	set	$180
6-22713	Rat Epidermal Keratinocyte Primarker™ buffer system	set	$90

Rat Thyroid PrimaCell™: Thyroid Epithelium

(Cat No. 2-84904)

I. General Description

Thyroid epithelial cells are cells in the thyroid gland which produce and secrete thyroxine and triiodothyronine. They are simple cuboidal epithelium and are arranged in spherical follicles surrounding colloid. They have thyrotropin receptors on their surface, which respond to thyroid-stimulating hormone. While these cells are extremely useful in the laboratory they are notoriously difficult to isolate and culture. They rapidly divide, become fibroblastic, and lose their biochemical characteristics The Rat Thyroid PrimaCell™ kit (Cat No. 2-84904) is designed for the successful isolation and culture of thyroid cells from rat thyroid tissue allowing reproducible and dependable results.

1.1 Components of the Rat Thyroid PrimaCell™ System

- **Rat Thyroid Tissue Dissociation System, Thyroid OptiTDS™** (2 × 1 ml) --- *A mixture of collagenase, Trypsin, Dexoyribonuclease and Rat Thyroid OptiTDS™ Reconstitution Buffer.*
- **Rat *Thyroid OptiTDS™ Digestion Buffer*,** (2 × 9 ml)
- **Rat Thyroid Fibroblast Growth Inhibitors, Thyroid FibrOut™---**Thyroid FibrOut™ (5 x 200 µl) --- *A mixture of D-valine, collagenase and gentamycin.*
- **Rat Thyroid cell PrimaCell™ Basal Culture Medium,** (5 × 100 ml) --- *A Modified Ham F12*.
- **Rat Thyroid cell PrimaCell™ Medium Supplements,** (5 × 1 ml): *Rat EGF, Hydrocortisone, Cholera toxin penicillin (100 U/ml), streptomycin and Weymouth medium.*
- **Rat Thyroid PrimaCell™ Serum,** (50 ml): A modified f*etal bovine serum.*
- **Rat Thyroid Tissue Washing Medium,** (1 × 100 ml): *A modified DMED medium with 10µg/ml amphotericin,* 10*µg/ml gentamycin, and 10% FCS.*

1.2 Required materials but NOT included:

- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or rat fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, fresh gelatin-coated plate or culture dishes are recommended (see below for treatment of culture dishes).

2.2 Principle

Separation of the thyroid cell from the thyroid tissue is accomplished by enzymatic digestion using the Thyroid Tissue Dissociation System supplied in this kit. The Thyroid Tissue Dissociation System contains a mixture of collagenase, collagenase I, collagenase III, collagenase IV, Trypsin, and Dexoyribonuclease at the optimal concentrations to gently detach the fragile thyroid layer from the thyroid tissue. The isolated thyroid tissue is then further disrupted to release individual thyroid cell by enzymatic and mechanical agitation. The mixture is then filtered through Cell Strainers and seeded on flasks or specially coated tissue culture plates. The thyroid cells are propagated in growth arrested feeder cells and the corresponding media. Sub-populations of thyroid cells can then be isolated based on their selective attachment to specific basement matrix substrates.

2.3 Rat Thyroid Tissue Preparation

Rat Thyroid: Most thyroid cell from thyroid is obtained from hysterectomy specimens.

1. Thyroid glands were excised from 5- to 6-week-old rats that had been killed by CO2 asphyxiation. Usually, the glands from three to six rats were pooled and used for the primary cultures. All the procedures were performed under sterile conditions.
2. Thyroid specimens were incubated in 5-10 ml 70% ethanol for 1 min, followed by inoculating with 5-10 ml fresh **Rat Thyroid Washing Medium** for 5 min.
3. Tissues were freed from adherent connective tissue, finely minced, and washed four times with **Rat Thyroid Washing Medium**.

2.4 Tissue Dissociation

2.4.1 Rat Thyroid OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the donor used, enzymes, culture mediums and growth supplements. The Rat Thyroid Tissue Dissociation System is suited for optimal dissociation of normal rat thyroid tissues to yield maximum number of single thyroid cell.

2.4.2 Enzyme Compositions

- Collagenase: from *Clostridium Histolyticum*
- Trypsin

2.4.3 System Components

- Rat Thyroid Tissue Dissociation System, Thyroid OptiTDS™, (2 × 1 ml).
- Rat Thyroid OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

4. Prepare fresh enzyme working solution: Add 1ml of the reconstituted tissue dissociation solution to one vial of Rat Thyroid OptiTDS™ Digestion Buffer (9 ml). Warm the diluted Rat Thyroid OptiTDS™ working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 5 ml Rat Thyroid OptiTDS™ working solutions.
5. Discard the **Rat Thyroid Washing Medium,** and float thyroid samples on 5 – 10 ml of Rat Thyroid Tissue Dissociation System working solution (prewarmed to 37℃) to the minced tissue, and transfer the tissue to a sterile glass universal containing a small plastic-coated magnetic stirrer bar. Place the universal on a magnetic stirrer and stir slowly for 30-60 min at 37 ℃. This works particularly well with full-thickness thyroid tissue.
6. Allow the suspension to stand at room temperature for 2-3 min, followed by removing the supernatant cellular mixture, and reincubating intact with 5-10 fresh Rat Thyroid Tissue Dissociation System working solution for 30 min intervals until all fragments were digested.

2.6 Thyroid cells Isolation

Note: Please read section 2.6 for specific information on Cervical Epithelium culture and platting before preceding this section.

7. Combine the supension containing liberated follicles, centrifuge at 200 *g* for 2 min, and the resulting pellet was resuspended in RPMI medium and allowed to sediment with gravity for 1 h.
8. The supernatant (containing single cells) was discarded, and the sedimented follicles were collected, pooled, resuspended in RPMI, and filtered through a 200-mm nylon mesh.
9. Wash follicles twice with **Rat Thyroid PrimaCell™ Basal Culture Medium** and centrifuge at 200 g for 2 min.

2.6 Primary Thyroid cells Culture

2.6.1 Medium Preparation.

Thaw out **Rat Thyroid PrimaCell™ Basal Culture Medium**, **Rat Thyroid cell PrimaCell™ Medium Supplements**, and **Rat Thyroid PrimaCell™ Serum** on ice.

Rat Complete Thyroid PrimaCell™ Culture Medium: To every 100 ml **Rat Thyroid PrimaCell™ Basal Culture Medium**, add 10 ml **Rat Thyroid PrimaCell™ Medium Supplements** and 1 ml **Rat Thyroid PrimaCell™ Serum** mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use.

Rat Complete Thyroid PrimaCell™ Culture Medium/FibrOut: To every 100 ml **Rat Thyroid PrimaCell™ Basal Culture Medium**, add 10 ml **Rat Thyroid PrimaCell™ Medium Supplements,** 1 ml **Rat Thyroid PrimaCell™ Serum**, and 1 ml **Rat Thyroid Fibroblast Growth Inhibitors, Thyroid FibrOut™**,mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use. (**Important:** Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination)

2.6.2 Primary Cell Culture

10 The twice-washed follicles were resuspended with **Rat Complete Thyroid PrimaCell™ Culture Medium/FibrOut**, and distributed into 10-cm Falcon plastic tissue culture dishes at

10^4 to 10^5 cells per dish.Follicles attached to the flask surface, and monolayer cultures developed.

11 Medium was changed every 3–4 days, and cells were passaged on confluent monolayers.

Δ ***Safety Note.*** The rest of the biopsy and all tubes, pipettes, plates, etc., used in the procedure should be treated with hypochlorite before disposal.

2.7 Subculture

Propagating Thyroid cells in culture can be somewhat challenging, especially when Thyroid cells are cultured alone on plastic, however the following methods have worked consistently in many laboratories.

12 Remove culture medium, and wash cells with **Rat Thyroid PrimaCell™ Basal Culture Medium.**
13 Add a small volume of EDTA gently to the cells and remove it immediately.
14 Add sufficient trypsin solution (0.25%) to form a thin layer over the cells.
15 When cells detach, add 5 to 10 ml of **Rat Complete Thyroid PrimaCell™ Culture Medium**, pass the culture very gently in and out of a pipette, and then centrifuge the cells for 10 min at 350 g.
16 Count an aliquot and seed the cells at the chosen concentration.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Skeletal muscle cells cultures derived from preconfluent layers.

17 Trypsinize cells as above, and centrifuge at 100 g for 10 min.
18 Resuspend cells in **Rat Complete Thyroid PrimaCell™ Culture Medium** and count cells.
19 Prepare aliquots of 2×10^6 cells/ml in **Rat Complete Thyroid PrimaCell™ Culture Medium** and 10% glycerol into cryovials.
20 Equilibrate at 4℃ for 1-2 h.
21 Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
22 To recover cells:
   a Thaw cryovials quickly in a 37℃ water bath.
   b Dilute cells tenfold with appropriate **Rat Complete Thyroid PrimaCell™ Culture Medium**.
   c Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in **Rat Complete Thyroid PrimaCell™ Culture Medium.**

IV Characterization

The purity of the thyroid cell population was verified by staining with anti-cytokeratin 18 antibody (a marker for epithelial cells), and quantitated by flow cytometry. Only cultures that were >90% cytokeratin 18 positive were used for experiments.

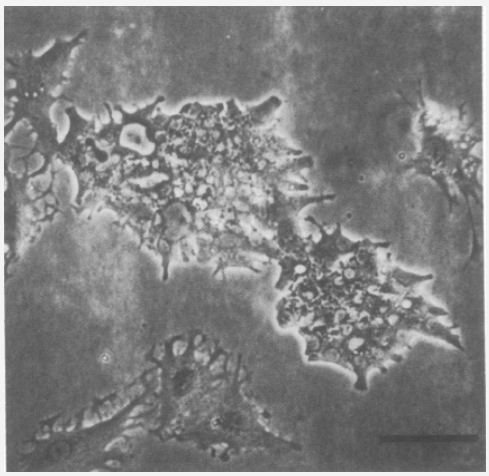


Figure 1. Phase-contrast photomicrograph of isolated FRTL cells in primary culture. [4]

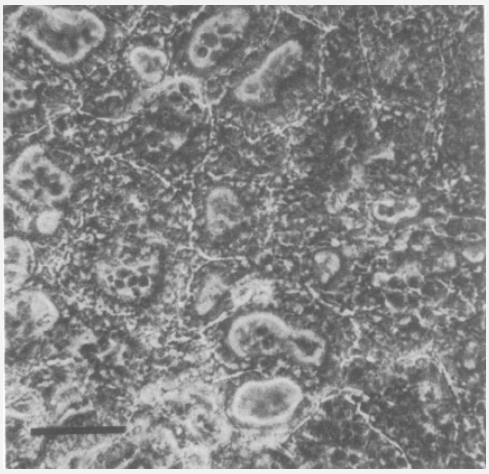


Figure 2. Phase-contrast photomicrograph of a portion of a large colony of FRTL cells at the third passage.[4]

V References

1. Mezosi, E., et al., *Induction and regulation of Fas-mediated apoptosis in human thyroid epithelial cells.* Mol Endocrinol, 2005. **19**(3): p. 804-11.
2. Stein, M.E. and M.J. Stadecker, *Characterization and antigen-presenting function of a murine thyroid-derived epithelial cell line.* J Immunol, 1987. **139**(6): p. 1786-91.
3. Colletta, G., A.M. Cirafici, and G. Vecchio, *Induction of the c-fos oncogene by thyrotropic hormone in rat thyroid cells in culture.* Science, 1986. **233**(4762): p. 458-60.
4. Ambesi-Impiombato, F.S., L.A. Parks, and H.G. Coon, *Culture of hormone-dependent functional epithelial cells from rat thyroids.* Proc Natl Acad Sci U S A, 1980. **77**(6): p. 3455-9.

5. Fusco, A., et al., *A mos oncogene-containing retrovirus, myeloproliferative sarcoma virus, transforms rat thyroid epithelial cells and irreversibly blocks their differentiation pattern.* J Virol, 1985. **56**(1): p. 284-92.
6. Gianoukakis, A.G., et al., *Prostaglandin endoperoxide H synthase expression in human thyroid epithelial cells.* Am J Physiol Cell Physiol, 2001. **280**(3): p. C701-8.
7. Hayashi, I. and G.H. Sato, *Replacement of serum by hormones permits growth of cells in a defined medium.* Nature, 1976. **259**(5539): p. 132-4.

Rat Thyroid PrimaCell™: Thyroid Epithelium

Rat Thyroid Primary Cell Culture

Cat No.	Description	Qt.	Price
2-84904	**Rat** Thyroid **PrimaCell™ system**	**kit**	**$499**
4-26931	Rat Thyroid Tissue Disassociation System, Lung OptiTDS™ (for 500 ml medium)	1 ml	$128
9-48903	Rat Thyroid Epithelial Cells PrimaCell™ Basal Culture Medium	500 ml	$ 61
9-37931	Rat Thyroid Epithelial Cells PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$ 140
7-67931	Rat Thyroid Fibroblast Growth Inhibitors, Thyroid FibrOut™	ea	$146
9-98903	Rat Thyroid Tissue Preparation Buffer Set	ea	$75

Rat Thyroid Epithelial Cells Characterization

6-22611	**Rat Thyroid Epithelial cell Primarker™ Kit**	**kit**	**$220**
6-22612	Rat Thyroid Epithelial cell Primarker™ antibody set	set	$180
6-22613	Rat Thyroid Epithelial cell Primarker™ buffer system	set	$90

CHI SCIENTIFIC

Chapter 4 Human Primary Cell Culture System – Human PrimaCell™

Human Airway PrimaCell™: Bronchial and Tracheal Epithelium
(Cat No. 2-96011)

I. General Description:

This protocol is developed for attachment and growth of normal Human bronchial epithelial (NHBE) cells from newborn or adult Human Airway with Human Airway PrimaCell™ system (Cat No. 2-96011). This system provides an optimal condition of tissue dissociation system, Airway OptiTDS™ that yields 4-7 times of single cells more than most of the tissue dissociation protocols published in the literature. In addition, this system ensures a high viability of the target cells with improved gradient contained in the culture medium. With CHI's proprietary fibroblast inhibitory system, FibrOut™, cells are growing with contamination of minimized amount of the non-epithelial cells.

This procedure involves explanting fragments of large airway tissue in a serum-free medium (LHC-9) in order to initiate and subsequently propagate fibroblast-free outgrowths of NHBE cells; four subculturings and 30 population doublings are routine.

Human Airway PrimaCell™ system applies to all type tissue samples from Human at all age though younger tissue samples are recommended for yielding maximum amount of viable target cells. However, tissue samples contain pathological organism (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Human Airway PrimaCell™ System

- **Human Airway Tissue Washing Medium**, (1 × 100 ml) --- *A mixture of L-15, BSA, penicillin, streptomycin, gentamycin*.
- **Human Airway Tissue Healing Medium (HB Medium),** (5 × 100 ml) --- *A modified formulation based on CMRL 166 medium*.
- **Human Airway Fibroblast Growth Inhibitors, Airway FibrOutTM** ---Airway FibrOut™ (5 × 200 µl) --- *A mixture of collagenase, D-valine, and gentamycin.*
- **Human Airway PrimaCell™ Growth Medium,** (5 × 100ml) --- *A modified LHC-9 medium*.
- **Human Airway PrimaCell™ Growth Medium Supplements with Serum** (5 × 1 ml): *Highly purified and special-treated EGF, Epinephrine, Hydrocortisone sodium succinate, 3,3'5-triiodo-L-thyronine, insulin, Adenine SO4, Thymidine, Lipoic acid, Phosphoethanolamine.*
- **Human Airway Tissue Healing Medium Supplements with Serum** (5 × 1 ml) --- *A mixture of insulin, hydrocortisone, β-retinyl acetate, glutamine, penicillin, stretomycin, gentamucin, fungison, FBS.*
- **Human Airway PrimaCell™ II Culture Dish Coating Solution** --- *A mixture of FN/V/BSA, human fibronectin, collagen, BSA, and LHC basal medium*

1.2 Required Materials but not provided

1. Plastic tissue culture dishes (60 and 100 mm)
2. Surgical scissors
3. Half-curved microdissecting forceps
4. Gloves sterilized with autoclave (Human tissue can be contaminated with biologically

hazardous agents)
5. Controlled atmosphere chamber
6. Rocker platform
7. Phase-contrast inverted microscope

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, culture plate (Corning, NY) must be coated with the provided coating solution.

2.2 Tissue Preparation and Healing

Replicative cultures of NHBE cells can be established from several sources of donated airway specimens, including surgeries and autopsies. Of these, tissue recovered by surgery from noncancerous patients or donors undergoing ‘‘immediate’’ autopsies yield the greatest quantity of culturable cells and are least likely to harbor malignant cells. Cells can also be obtained by biopsy or brushing of airways during bronchoscopy.

2.2.1 Complete Healing Medium Prepration

Thaw out the Human Airway Tissue Healing Medium Supplements with Serum on ice. To every 100 ml Human Airway Tissue Healing Medium, add 1 ml Human Airway Tissue Healing Medium Supplements with Serum, mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use.

2.2.2 Procedures for tissue dissociation and healing

1. Coat a culture dish with 1 ml of the Human Airway PrimaCell™ II Culture Dish Coating Solution per 60-mm dish, and incubate the dish in a humidified CO_2 incubator at 37℃ for at least 2 h (not to exceed 48 h). Vacuum aspirate the mixture and fill the dish with 5 ml of Human Airway Tissue Washing Medium.
2. Aseptically dissected lung tissue from noncancerous donors autopsied within the previous 12 h is placed into ice-cold Human Airway Tissue Washing Medium for transport to the laboratory, where the bronchus is further dissected from the peripheral lung tissues.
3. Before culturing, scratch an area of one square centimeter at one edge of the surface of the 60-mm culture dishes with a scalpel blade.
4. Open the airways (submerged in the Human Airway Tissue Washing Medium) with surgical scissors, and cut (slice, do not saw) the tissue with a scalpel into two pieces, 20 × 30 mm.
5. Using a scooping motion to prevent damage to the epithelium, pick up the moist fragments and place them epithelium side up onto the scratched area of the 60-mm dish. Remove the Human Airway Tissue Washing Medium, and incubate the fragments at room temperature for 3 to 5 min to allow time for them to adhere to the scratched areas of the dishes.
6. Add 3 ml of Human Complete Airway Tissue Healing Medium/FibrOut to each dish and

place them in a controlled-atmosphere chamber. Flush the chamber with a high-O_2 gas mixture and place it on a rocker platform. Rock the chamber at 10 cycles per minute, causing the medium to flow intermittently over the epithelial surface. Incubate rocking tissue fragments at 37℃, changing the medium and atmospheric pressure after Day 1 and again after Day 2 - intervals for 6-8 d. This step improves subsequent explant cultures by reversing any ischemic damage to the epithelium that occurred from time of death of the donor until the tissue was placed in the ice-cold Human Airway Tissue Washing Medium.

2.2.3 Storage:

Reconstituted tissue dissociation systems should occur before use and can only be stored for 2-4 days at 4 ^{o}C. For long-term use, it should be aliquoted and stored at -20 ^{o}C. Avoid repeated freeze-thaw cycles.

2.3 Explant Outgrowth Cultures and Subculture

2.3.1 Medium Preparation.

Thaw out the Human Airway PrimaCell™ Growth Medium Supplements with Serum, Human Airway Fibroblast Growth Inhibitors, Airway FibrOut™ on ice.

Human Complete Airway PrimaCell™ Growth Medium: To every 100 ml Human Airway PrimaCell™ Growth Medium, add 1 ml Human Airway PrimaCell™ Growth Medium Supplements with Serum and 200 µl Human Airway Fibroblast Growth Inhibitors, Airway FibrOut™, mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use. **Important:** Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination.

2.3.2 Procedures For Tissue Explant

7. Before explanting, scratch seven areas of the surface of each 100-mm culture dish with a scalpel. Coat the surfaces of the scratched culture dishes with the Human Airway PrimaCell™ Culture Dish Coating Solution, and aspirate the surplus solution as before.
8. Cut the moist ischemia-reversed fragments into 7 × 7-mm pieces, and explant the pieces epithelium side up on the scratched areas. Incubate the pieces at room temperature without medium for 3-5 min, as before.
9. Add 10 ml of Human Complete Airway PrimaCell™ Growth Medium/FibrOut to each dish, and incubate explants at 37℃ in a humidified 5% air/CO_2 incubator. Replace spent medium with fresh medium every 3 to 4 d.

After 8 to 11 d of incubation, when epithelial cell outgrowths radiate from the tissue explants more than 0.5 cm, transfer the explants to new culture dishes scratched and coated with Human Airway PrimaCell™ Culture Dish Coating Solution to produce new outgrowths of epithelial cells. This step can be repeated up to seven times with high yields of NHBE cells.

2.3.3 Procedures for Dissociation and Subculture of Bronchial Epithelium

10. Incubate the postexplant outgrowth cultures in Human Complete Airway PrimaCell™ Growth Medium for an additional 2 to 4 d before trypsinizing (with the

trypsin/EGTA/PVP solution) for subculture or for experimental use.

11. Aspirate the medium and bathe the culture two times with Human Airway Tissue Washing Medium.
12. Remove the Human Airway Tissue Washing Medium and incubate the culture at room temperature in 0.5 M urea for 5 min.
13. Remove the urea solution by aspiration and bathe the cells in a minimal volume of 0.05mg/mL Tripsin and 0.5mM/ml EGTA, and incubate at room temperature until the cells floats free (usually 5–10 min).
14. Resuspend the cells with Human Airway Tissue Washing Medium and pellet by centrifugation (125 g for 5 min).

Resuspend the cells with Human Complete Airway PrimaCell™ Growth Medium, enumerate the cells and reinoculate at the desired cell density into culture dishes that have been coated with Human Airway PrimaCell™ Culture Dish Coating Solution.

III Cryopreservation

Both dissociated NHBE cells and bronchial tissue fragments can be cryopreserved with good viability using relatively routine procedures, as follows.

15. Suspend the tissue (0.5-cm^3 fragments) or pelleted normal human bronchial epithelial (NHBE) cells ($2–5 \times 10^6$) in 0.5 ml of cold 2 × Human Complete Airway PrimaCell™ Growth Medium in a freezing vial.
16. Add 0.5 ml of DMSO freezing medium to the vial, swirl the mixture, and close the vial.
17. Transfer vial(s) to a controlled-rate freezer and freeze the cells/tissues at 1℃/min according to the manufacturer's directions.
18. Transfer the frozen vials to liquid N2 for storage.
19. Resurrect the cells/tissues by rapidly warming the vial to 37℃.
20. Swab the vial with 70% alcohol, open it, and transfer the cells to 10 ml of Human Complete Airway PrimaCell™ Growth Medium.
21. Pellet the cells, resuspend in Human Complete Airway PrimaCell™ Growth Medium, and inoculate into Human Airway PrimaCell™ Culture Dish Coating Solution-coated culture dishes containing Human Complete Airway PrimaCell™ Growth Medium.

IV Characterization

NHBE are identified by several criteria based on the characteristic structure and function of normal epithelium. In explant outgrowth cultures, polygon-shaped epithelial cells grow out from the periphery of the explant onto the culture dish before the fusiform fibroblast cells. Cytochemical stains can further distinguish epithelial cells and fibroblasts in primary cultures. Squamous epithelial cells stain positively with the immunoperoxidase method for prekeratin and keratin, whereas fibroblasts stain negatively. In some cultures, epithelial cells will stain positively with alcian blue-PAS before and after treatment with diastase, indicating the production of acidic and neutral mucopolysaccharides, two components of mucus. Most cultures, however, will not exhibit positive staining for mucous substances. Epithelial cells in first-passage cultures are similar in appearance to those in explant outgrowth cultures, and they continue to react positively with the keratin antibodies. Scanning electron microscopy of subcultures shows colonies composed of prolate spherical cells covered with varying numbers of microvilli and apposed cell borders. In addition, human bronchial epithelial cells do not form colonies in soft agar when plated at a density of 100,000 per 1 ml, and chromosome analysis shows that the cells retain the normal human

karyotype (2N = 46) throughout the replicative phase. Additionally, these cells are metabolically active and capable of converting xenobiotics to DNA adducts.

V References:

1. Yim HW, Slebos RJ, Randell SH, Umbach DM, Parsons AM, Rivera MP, Detterbeck FC, Taylor JA. *Smoking is associated with increased telomerase activity in short-term cultures of Human bronchial epithelial cells.* Cancer Lett. 2006 Mar 3;
2. Doherty GM, Christie SN, Skibinski G, Puddicombe SM, Warke TJ, de Courcey F, Cross AL, Lyons JD, Ennis M, Shields MD, Heaney LG. *Non-bronchoscopic sampling and culture of bronchial epithelial cells in children.* Clin Exp Allergy. 2003 Sep; 33(9):1221-5.
3. Mattinger C, Nyugen T, Schafer D, Hormann K. *Evaluation of serum-free culture conditions for primary Human nasal epithelial cells.* Int J Hyg Environ Health. 2002 Apr; 205(3):235-8.
4. de Jong PM, van Sterkenburg MA, Kempenaar JA, Dijkman JH, Ponec M. *Serial culturing of Human bronchial epithelial cells derived from biopsies.* In Vitro Cell Dev Biol Anim. 1993 May; 29A(5):379-87.
5. Robbins RA, Koyama S, Spurzem JR, Rickard KA, Nelson KJ, Gossman GL, Thiele GM, Rennard SI. *Modulation of neutrophil and mononuclear cell adherence to bronchial epithelial cells.* Am J Respir Cell Mol Biol. 1992 Jul;7(1):19-29.
6. Lechner JF, Wang Y, Siddiq F, Fugaro JM, Wali A, Lonardo F, Willey JC, Harris CC, Pass HI. *Human lung cancer cells and tissues partially recapitulate the homeobox gene expression profile of embryonic lung.* Lung Cancer. 2002 Jul; 37(1):41-7.
7. Lechnewr JF, & LeVeck MA. *A serum free method for culturing normal Human bronchial epithelial cells at clonal density.* J. Tissue Cult. Methods 9: 43-48.

Human Airway PrimaCell™: Bronchial and Tracheal Epithelium

Human Airway Primary Cell Culture

Cat No.	Description	Qt.	Price
2-96011	**Human Airway PrimaCell™ System**	**kit**	**$550**
7-66011	Bronchial and Tracheal Fibroblast Growth Inhibitors (for 500 ml medium), Airway FibrOut™, Human	1 ml	$146
9-46001	Bronchial and Tracheal PrimaCell™ Basal Medium, Human	500 ml	$70
9-36011	Bronchial and Tracheal PrimaCell™ Growth Medium Supplements (for 500 ml medium), Human	set	$160
4-26011	Human Bronchial and Tracheal Tissue Disassociation system	ea	$128
9-87001	Human Airway Tissue Preparation Buffer Set	ea	$90

Human Airway Primary Cell Characterization

6-32811	**Human Bronchial and Tracheal Epithelium Primarker™ Kit**	**kit**	**$220**
6-32812	Human Bronchial and Tracheal Epithelium Primarker™ antibody set	set	$180
6-32813	Human Bronchial and Tracheal Epithelium Primarker™ buffer system	set	$90

Human Bone Marrow PrimaCell™: Hematopoietic Cells

(Cat No. 2-96021)

I. General Description:

This protocol is developed for Hematopoietic Cells from newborn or adult Human Bone marrow with Human Bone marrow PrimaCell™ system (Cat No. 2-96011). This system provides an optimal condition of tissue dissociation system, Bone Marrow OptiTDS™ that yields 4-7 times of single cells more than most of the tissue dissociation protocols published in the literature. In addition, this system ensures a high viability of the target cells with improved gradient contained in the culture medium. With CHI's proprietary fibroblast inhibitory system, FibrOut™, cells are growing with contamination of minimized amount of the non- Hematopoietic Cells.

Marrow is aspirated into growth medium and maintained as an adherent cell multilayer for at least 12, and up to 30, weeks. Stem cells and maturing and mature myeloid cells are released from the adherent layer into the growth medium. Granulocyte/macrophage progenitor cells can be assayed in soft gels..

Human Bone marrow PrimaCell™ system applies to all type tissue samples from human at all age though younger tissue samples are recommended for yielding maximum amount of viable target cells. However, tissue samples contain pathological organism (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Human Bone marrow PrimaCell™ System

- **Human Bone marrow PrimaCell™ system, Bone marrow OptiTDS™,** (2 x 1 ml) --- *A mixture of collagenase, collagenase I, collagenase I and Human bone marrow OptiTDS™ Reconstitution Buffer.*
- **Human bone marrow OptiTDS™ Digestion Buffer**, (2 × 9 ml).
- **Human Bone Marrow Fibroblast Growth Inhibitors, Bone Marrow FibrOut™** (5 × 200 µl) --- *A mixture collagenase, D-valine, praline and gentamycin*.
- **Human Bone Marrow Tissue Washing Medium**, (1 × 100 ml) --- *HBSS with 5% BSA, 200 U/ml of penicillin, 200 µg/ml of streptomycin, and 50 µg/ml of gentamycin*.
- **Human Bone Marrow PrimaCell™ Basal Culture Medium,** (5 × 100 ml) --- *A modified Fischer's Medium.*
- **Human Bone Marrow PrimaCell™ Medium Supplements**, (5 × 1.0 ml) --- *A mixture of Hydrocortisone, Sodium Succinate, penicillin, streptomycin, and NaHCO3*.
- **Human Bone Marrow PrimaCell™ Serum**, (10 × 10 ml) --- *Highly purified special-treated Horse Serum.*

1.2 Required Materials but not provided:

- 70% sterile ethanol
- Plastic tissue culture dishes (60 and 100 mm)
- Surgical scissors
- Half-curved microdissecting forceps

- Pipettes (10 and 25 ml)
- Gloves sterilized with autoclave (human tissue can be contaminated with biologically hazardous agents)
- Rocker platform

II. Procedures

2.1 Material Preparation

Human primary Hematopoietic Cells were isolated from bone marrow samples from patients who undergo operations under sterile conditions. All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, collagen-coated culture plate (Corning, NY) must be used in this experiment.

2.2 Surgical specimens

The bone marrow specimens should be taken by the pathologist from the operating room and immediately transferred in the provided Human Bone Marrow Tissue Washing Medium on ice. The excised tissue samples should be preceded within 3-h from separation of patient body.

2.3 Tissue Preparation and Dissociation

2.3.1 Human Bone Marrow OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Human Bone Marrow Tissue Dissociation System, OptiTDS™, is suited for optimal dissociation of normal adult and newborn Bone marrow samples to yield maximum number of single primary cells of colonic tissues.

2.3.2 Enzyme Compositions

- Collagenase: from *Clostridium Histolyticum*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase II: from *Clostridium Histolyticum*

2.3.3 System Components

- Human Bone Marrow Tissue Dissociation System, OptiTDS™, 2 x 1.0 ml.
- Human Bone Marrow OptiTDS™ Digestion Buffer, 2 × 9 ml.

2.3.4 Procedures For Tissue Preparation and Dissociation

1. Add 1.0 ml of Human Bone Marrow Tissue Dissociation System, Bone OptiTDS™ to one vial of Human Bone OptiTDS™ Digestion Buffer (9 ml). Warm the diluted enzyme working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 4-5 g tissue samples per 10 ml diluted enzyme working solutions.
2. Human bone marrow was immediately placed in a 100-mm tissue culture dish containing 5-10 ml of diluted enzyme working solutions, followed by incubating 30 min at room temperature.

2.3.5 Storage:

Reconstituted tissue dissociation systems should occur before use and can only be stored for 2-4 days at 4 ^{o}C. For long-term use, it should be aliquoted and stored at -20 ^{o}C. Avoid repeated freeze-thaw cycles.

2.4 Culture of Primary Human Bone Marrow Hematopoietic Cells

2.4.1 Medium Preparation.

Thaw out the Human Bone marrow PrimaCell™ Medium Supplements and Human Bone marrow PrimaCell™ I Serum on ice. To prepare complete Human Bone Marrow Medium, add one vial of Human Bone marrow PrimaCell™ I Medium Supplements and 10 ml Human Bone marrow PrimaCell™ I Serum to every 100 ml Human Bone Marrow PrimaCell™ Basal Culture Medium; Adding one vial of Human Bone marrow Fibroblast Growth Inhibitors, Bone Marrow FibrOut™ to Complete Human Bone Marrow Medium makes Complete Human Bone Marrow Medium/FibrOut. Mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use.

Important: Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination.

2.4.2 Primary Cell Culture

3. Disperse the marrow to a suspension by pipetting the large marrow cores through a 10-ml pipette. There is no need to disaggregate small clumps of cells.
4. Centrifuge cells for 2 min at 580 g at room temperature and remove the supernatant.
5. Dispense 10-ml aliquots of the cell suspension into 25-cm^2 tissue culture flasks by using Complete Human Bone Marrow Medium containing the FibrOut™, swirling the suspension often to ensure an even distribution of the cells in the 10 cultures.
6. Gas the flasks with 5% CO_2 in air and tighten the caps.
7. Incubate the cultures horizontally at 33℃.
8. Feed the cultures weekly:
    a) Agitate the flasks gently to suspend the loosely adherent cells.
    b) Remove 5 ml of growth medium, including the suspension cells; take care not to touch the layer of adherent cells with the pipette.
    c) Add 5 ml of fresh Complete Human Bone Marrow Medium/FibrOut to each flask; to avoid damage, do not dispense the medium directly onto the adherent layer.
    d) Gas the cultures and replace them in the incubator.

2.4.3 Subculture and Propagation

9. Gently rinse the culture dish twice with 0.02% (0.7 mM) EDTA.
10. Add 3 ml of 0.25% trypsin/0.1% (2.5 mM) EDTA, and incubate at 37℃. Examine the dish under phase microscopy every 5 min to detect cell detachment.
11. When most cells have detached, add 12 ml Complete Human Bone Marrow Medium to inactive the trypsin activity.
12. Pipette the contents of the dish to ensure complete hematopoietic cells detachment.

13. Aspirate and centrifuge the cells for 5 min at 350 g.
14. Aspirate the supernatant, resuspend the cells in a Complete Human Bone Marrow Medium, and replate at 2-4 $\times$ 10^4 cells per 100-mm dish.
15. Refeed the culture twice a week with Complete Human Bone Marrow Medium.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample; the best results are reported when cells from preconfluent primary cultures are used.

16. Detach cells as for the subculture, and centrifuge at 100 g for 10 min.
17. Resuspend cells in Complete Human Bone Marrow Medium and count.
18. Dispense aliquots of 2 $\times$ 10^6 cells/ml in Complete Human Bone Marrow Medium with 10% glycerol into cryopreservation tubes.
19. Equilibrate at 4℃ for 1-2 h.
20. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min
21. To recover cells:
    a) Thaw cryotubes quickly in a 37℃ water bath.
    b) Dilute cells tenfold with medium.
    c) Centrifuge cells and resuspend them at an appropriate concentration in the desired Complete Human Bone Marrow Medium, and seed culture vessel.

Human cells can be grown in all media for 4-7 weeks and can be subcultured only 4-5 times.

IV Fibroblast Contamination

There are several techniques have been published in the literature to deal with fibroblast contamination during colorectal primary cell culture. These include: (1) Physically remove a well-isolated fibroblast colony by scraping it with a sterile blunt instrument (e.g., a cell scraper). Care has to be taken to wash the culture up to six times to remove any fibroblasts that have detached in order to prevent them from reseeding and reattaching to the flask. (2) Differential trypsinization can be attempted with the carcinomas. (3) Dispase preferentially (but not exclusively) removes the epithelium during passaging and leaves behind most of the fibroblastic cells attached to the culture vessel. During subculture, cells that have been removed with dispase can be preincubated in plastic petri dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the hematopoietic cells. This technique takes advantage of the fact that fibroblasts in general attach much more quickly to plastic than do clumps of melanocytes, so that a partial purification step is possible. (4) Reduce the concentration of serum to about 2.5-5% if there are heavy concentrations of fibroblastic cells. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and that using any or all of the preceding techniques will eventually push the cells through so many divisions that any fibroblasts will senesce.

Human Bone marrow PrimaCell™ system includes a fibroblast elimination system, the Human Bone marrow Fibroblast Growth Inhibitors, Bone marrow FibrOut™. It contains a mixture of collagenase and gentamycin. This system can effectively eliminate Bone marrow fibroblast contamination while has not affect on the behavior of hematopoietic cells.

V Characterization of Hematopoietic Cells

Because Hematopoietic Cells are very heterogeneous, containing many cell types, many assays have been developed in order to characterize the cell populations that are present in a sample. Upon culture of these cells, the ratio of different populations may change significantly, such that the total cell number generated is not an adequate measure of outcome. Therefore, the use of appropriate assays is critical to determine the success of particular culture technique. Histology, widely used in clinic, is the first method to assay Hematopoietic Cells. These methods utilize spreads of Wright-Giemsa stained cells under oil-immersion microscopy, or automated instruments that have been developed tocarry out these differentials (counting of different cell types). It is most useful for assessing mature cell populations with large numbers and distinctive morphological features. Flow cytometry has been used extensively in the study of Hematopoietic Cells. Antibodies detecting different cell types have been developed. Because of the close relation of many cell types, combinations of antigens are often required to definitively identify a particular cell. CD34, the most utilized antigen which appears to identify all cells from stem through progenitor stage, is stage-specific but not lineage-specific. However, CD34 antigen is not restricted to Hematopoietic Cells. Also, recent controversial data suggest that the most primitive stem cells are $CD34^-$, and that these give rise to more $CD34^+$ population. In order to deal with rarity of stem and progenitor cells, many in vitro biological function assays have been developed, such as colony-forming unit assay (CFU) and long-term culture- initiating cell assay (LTC-IC), . Most of these assays are performed by culturing cells under defined conditions and examining their progeny, both in number and type.

VI References:

1. Yamaguchi M, Hirayama F, Kanai M, Sato N, Fukazawa K, Yamashita K, Sawada K, Koike T, Kuwabara M, Ikeda H, Ikebuchi K.*Serum-free coculture system for ex vivo expansion of human cord blood primitive progenitors and SCID human-reconstituting cells using human bone marrow primary stromal cells.* Exp Hematol. 2001 Feb;29(2):174-82.
2. Koller, M.R.P., B. O.; Masters, J. R. W., *Human Cell Culture: Primary Hematopoietic Cells.* 1999: Springer.
3. Goodrum, F.D., et al., *Human cytomegalovirus gene expression during infection of primary hematopoietic progenitor cells: a model for latency.* Proc Natl Acad Sci U S A, 2002. 99(25): p. 16255-60.
4. Plett, P.A., et al., *Proliferation of human hematopoietic bone marrow cells in simulated microgravity.* In Vitro Cell Dev Biol Anim, 2001. 37(2): p. 73-8.
5. Huss, R., *Isolation of primary and immortalized CD34-hematopoietic and mesenchymal stem cells from various sources.* Stem Cells, 2000. 18(1): p. 1-9.
6. Dao, M.A. and J.A. Nolta, *Cytokine and integrin stimulation synergize to promote higher levels of GATA-2, c-myb, and CD34 protein in primary human hematopoietic progenitors from bone marrow.* Blood, 2007. 109(6): p. 2373-9.

Human Bone Marrow PrimaCell™: Bone Marrow Hematopoietic Cell

Human Bone Marrow Primary Cell Culture

Cat No.	Description	Qt.	Price
2-96021	**Human Bone Marrow PrimaCell™ System**	**kit**	**$550**
7-66022	Human Bone Marrow Fibroblast Growth Inhibitors (for 500 ml medium), Bone Marrow FibrOut™	1 ml	$195
9-46002	Human Bone Marrowl PrimaCell™ Basal Medium	500 ml	$73
9-36022	Human Bone Marrow Hematopoietic cells PrimaCell™ Growth Medium Supplements and serum (for 500 ml medium)	set	$160
4-26022	Human Bone Marrow Tissue Disassociation system	ea	$146
9-86002	Human Bone Marrow Tissue Preparation Buffer Set	ea	$90

Human Bone Marrow Primary Cell Characterization

6-33011	**Human Bone Marrow Hematopoietic Cell Primarker™ Kit**	**kit**	**$220**
6-33012	Human Bone Marrow Hematopoietic Cell Primarker™ antibody set	set	$180
6-33013	Human Bone Marrow Hematopoietic Cell Primarker™ buffer system	set	$90

Human Bone PrimaCell™: Osteoblasts

(Cat No. 2-96151)

I. General Description

Although bone is mechanically difficult to handle, thin slices treated extensively with EDTA/EGTA subsequently digested in bone tissue dissociation system provide in the kit, give rise to cultures of osteoblasts that have some functional characteristics of the tissue. This protocol is developed for attachment and growth of normal Human Bone epithelial cells from newborn or adult Human Bone with Human Bone PrimaCell™ system (Cat No. 2-96151). This system provides an optimal condition of tissue dissociation system, Bone OptiTDS™ that yields 5-6 times of single cells more than most of the tissue dissociation protocols published in the literature. In addition, this system ensures a high viability of the target cells with improved gradient contained in the culture medium. With CHI's proprietary fibroblast inhibitory system, FibrOut™, cells are growing with contamination of minimized amount of the non-epithelial cells.

The Human Bone PrimaCell™ system is suited for culturing Osteoblasts from bone tissues of Human.

1.1 The Human Bone PrimaCell™ system include:

- **Human Bone Tissue Dissociation System, Bone OptiTDS™** (2 × 1.0 ml) --- *collagenase I with a modified reconstitution buffer*.
- **Human Bone OptiTDS™ Digestion Buffer,** (2 × 9 ml).
- **Bone Fibroblast Growth Inhibitors, Bone FibrOut™** (5 × 1.0 ml) --- *A mixture of cis-OH-proline, collagenase, Gentamycin.*
- **Human Bone Tissue Washing Medium**, (1 × 100 ml) --- *A modified HBSS with 5% BSA, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Human Bone Osteoblasts PrimaCell™ Medium Supplements With Serum** (5 × 1.0 ml): *A mixture of highly purified and special treated serum and proprietary osteoblast supplements.*
- **Basal Bone Osteoblasts PrimaCell™ Basal Culture Medium (**5 × 100 ml) --- A modified Ham's F12 culture medium.

1.2 Required Materials but not provided

- Centrifuge tube, 50 ml
- Nylon gauze cell strainer (BD Bioscience)
- Petri dishes, collagenase -I coated, 100 mm (Corning, NY)
- Scalpels, curved forceps
- 70% ethanol, sterile
- PBSA (PBS containing 10% BSA), sterile.
- 9-cm Petri dishes
- 25- or 75-cm2 flasks (Corning, Falcon, Nunc)

II. Procedures

2.1 Procedure Outline and Material Preparation

Bone culture suffers from the inherent problem that the hard nature of the tissue makes manipulation difficult. However, conventional primary explant culture or digestion in collagenase and trypsin releases cells that may be passaged in the usual way. All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, culture plate must be coated with collagen-II.

2.2 Surgical specimens

1. Human Bone: Bone specimens (males and/or females under 30 years of age) are collected by operational scalps. The bone specimens should be doused with 70% ethanol to minimize contamination of the primary cultures, and immediately placed in a 100-mm tissue culture dish containing 10 ml of Human Bone Tissue Washing Medium (each organ type is placed in a separate dish). After all bones were collected, they were transferred to fresh 100-mm dishes containing 10 ml of sterile Human Bone Tissue Washing Medium. Bones were minced into 1-mm cubes using razor blades dipped in 70% ethanol. The minced tissues were transferred into sterile 15-ml conical tubes containing sterile Human Bone Tissue Washing Medium. After allowing the minced tissue pieces to settle, the Human Bone Tissue Washing Medium was aspirated, and the tissues washed once more with sterile PBS.
2. Incubate Bone biopsies sequentially in 10 ml 70% ethanol for 2 min, in 10 ml PBSA for 2 min, and finally transferred to a tube containing 20 ml fresh Bone Tissue Washing Medium.
3. Incubate sliced bone tissues in 10 ml Bone Tissue Preparation Buffer for 20 min. Repeat this procedure if total bone specimens is over 100g. This procedure can effectively prevent various infections. Repeat this procedure if total bone specimens is over 100g.
   (This procedure is not affecting cell viability.)

4. Keep the soaked tissue slices in ice till tissue dissociation procedures.

2.3 Tissue Preparation and Dissociation

2.3.1 Human Bone OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Human Bone Tissue Dissociation System, OptiTDS™, is suited for optimal dissociation of normal adult and newborn Bone biopsies samples to yield maximum number of single primary cells of colonic tissues.

2.3.2 Enzyme Compositions

- Trypsin: from *Bovine Pancreas*
- Dispase: from *Bacillus polymyxa*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase: from *Clostridium Histolyticum*
- DNase I: from *Clostridium Histolyticum*

2.3.3 System Components

- Bone Tissue Dissociation System, OptiTDS™, 2 x 1.0 ml.
- Bone OptiTDS™ Digestion Buffer, (2 x 9 ml).

2.3.4 Procedures For Tissue Preparation and Dissociation

1. Prepare fresh enzyme working solutions: to each vial of Bone Tissue Dissociation System, OptiTDS™, add 1.0 ml of the Bone OptiTDS™ Reconstitution Buffer, Mix well.
2. Add 1.0 ml of the fresh enzyme working solution to one vial of Bone OptiTDS™ Digestion Buffer (9.0 ml). Warm the diluted enzyme working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 5-7 mg tissue samples per 10 ml diluted enzyme working solutions.
3. Rinse surgical instruments in sterile PBS.
4. Mince pre-washed tissue into pieces approximately 1 mm cubes with two scalpel and forceps.
5. Incubate minced tissue with Bone Dissociation System by one of the following steps:

    a. Rapid dissociation: Incubating Bone samples with rocking in Bone Dissociation System solution for 2-3 h min at 37 ℃. This works particularly well also with full-thickness Bone.
    b. Slow dissociation: Incubating the samples with rocking in ice-cold Bone Dissociation System at 4℃ for 15-24 h.

6. Rinse woven cloth 2X in PBS and place into sterile funnel on top of sterile beaker and strain the cell mixture to remove debris (alternatively, a Nylon gauze cell strainer can be used).
7. Tirturate the cell mixture with sterile pipette several times to facilitate a single cell mixture and collect cells by passing the mixture through the woven cloth or Nylon gauze cell strainer
8. The filtration, which contained single cells dissociated from the tissue specimens, were collected into sterile 15-ml conical tubes by centrifugation at 800 g for 5 min. The resulting pellet containing the primary culture cells was resuspended in 5 ml of complete culture medium containing 10% fetal bovine serum and growth supplements. Save the supernatant for repeating tissue dissociation process in step 9.
9. To the remaining pieces of tissue, repeat the tissue dissociation process with the saved Bone Dissociation working solutions from step 8 to yield additional cells. This process can repeat up to 3 times if it is necessary without changing the enzyme working solution. In general, Bones from each 5-6 month old Human should produce six to eight 60-mm dishes of primary culture cells.
10. Count viable cells and plate cells at a density of 1×10^5 cells per cm^2.

2.3.5 Storage:

Reconstituted tissue dissociation systems should occur before use and can only be stored for 2-4 days at 4 ^oC. For long-term use, it should be aliquoted and stored at -20 ^oC. Avoid repeated freeze-thaw cycles.

2.4 Culture of Primary Human Bone Osteoblasts

2.4.1 Medium Preparation.

Thaw out Human Bone PrimaCell™ Basal Culture Medium, Human Bone PrimaCell™ Medium Serum, and Human Bone PrimaCell™ Medium Supplements on ice. To every 100 ml Human Bone PrimaCell™ Basal Culture Medium, add 10 ml Human Bone PrimaCell™ Medium Serum;

and one vial of Human Bone PrimaCell™ Medium Supplements, one vial of Human Bone Fibroblast Growth Inhibitors, Bone FibrOut™, mix thoroughly and warm the complete medium at a 37 °C water bath for 10 min prior use.

2.4.2 Primary Cell Culture

(a) Explant Cultures

Small fragments of tissue are allowed to adhere to the culture flask by incubation in a minimal amount of medium. The adherent explants are then flooded and the outgrowth is monitored.

11. Obtain bone specimens from the operating room.
12. Rinse the tissue several times with Bone Tissue Washing Medium at room temperature with sterile saline.
13. If the bone cannot be used immediately, cover the specimen with Complete Bone PrimaCell™ Culture Medium. The bone may be stored overnight at 4˚C.
14. The next morning, rinse the tissue with Bone Tissue Preparation Buffer solution containing penicillin (100 U/ml) and streptomycin sulfate (100 µg/ml).
15. Place the bone in a Petri dish and with the use of scalpel and forceps, remove the trabeculae and place them in a second Petri dish.
16. Add 10 ml of Bone Tissue Preparation Buffer solution over the excised trabeculae. Rinse the trabeculae several times with Bone Tissue Washing Medium until blood and fat cells are removed.
17. To initiate explant cultures, prepare a 25-cm^2 flask by preincubating it with 2 ml of Bone Tissue Preparation Buffer for 20 min to equilibrate the tissue for dissociation procedures.
18. Cut the trabeculae into fragments of 1-3 mm.
19. Remove the preincubation medium from the flask, and add 2.5 ml of freshly prepared Complete Bone PrimaCell™ Medium to the flask. Transfer between 25 and 40 fragments of trabeculae to the flask.
20. With the flask in an upright position, slide the explant pieces along the base of the flask with the aid of an inoculating loop, and distribute the explant pieces evenly.
21. Permit the flask to remain upright for 15 min at 37˚C.
22. Slowly restore the flask into a normal horizontal position. The explant pieces will stick to the bottom of the flask.
23. Leave the flask in the horizontal position at 37˚C for 5 to 7 d. After this period, check for outgrowth, and replace the medium in the flask with fresh medium. To prevent the explant pieces from becoming detached, lift the flask slowly into the vertical position before carrying it from the incubator to the hood or to the microscope.
24. To maintain cultures, change the medium twice per week.
25. When confluence is reached, the explant pieces are removed, the cell layer is trypsinized, and the cells are isolated by centrifugation and seeded into flasks or wells.

(b) Monolayer Cultures from Disaggregated Cells

Trabecular bone is dissected down to 2-5 mm and digested in collagenase and trypsin. Suspended cells are seeded into flasks in Complete Bone PrimaCell™ Culture Medium.

26. Wash trabecular bone specimens repeatedly with Bone Tissue Washing Medium to remove the fat and blood cells. The trabeculae are excised with scalpel and forceps under sterile conditions.
27. After collecting as much bone as possible, wash the remaining blood and fat cells away by

rinsing the specimens three times with Bone Tissue Washing Medium and cut into 2-5 mm fragments.
28. Wash the cut trabeculae with Bone PrimaCell™ Washing Medium.
29. Place the pieces of bone in a small sterile bottle with a magnetic stirrer, and add 4 ml of digestion solution. (This amount should cover the bone specimens.)
30. Stir the solution containing bone fragments at room temperature for 45 min.
31. Remove the suspension of released cells and discard it, since these cells are most likely to contain fibroblasts.
32. Add a second aliquot of 4 ml of digestion solution to the bone fragments, and stir the mixture at room temperature for 30 min.
33. Collect the digestion solution from bone fragments, and centrifuge it for 2 min at 580 g at room temperature.
34. After removing the supernatant, suspend the cells in 4 ml of Complete Bone PrimaCell™ Culture Medium, and count the cells.
35. Centrifuge the suspension at 580 g for 10 min, and resuspend the cells in 4 ml of complete medium. This suspension will become the inoculums.
36. Preincubate 75-cm^2 flasks for 20 min with 8 ml of Bone Tissue Preparation Buffer to equilibrate the tissue for digestion procedures.
37. Remove the preincubation solution and add 2 ml of Complete Bone PrimaCell™ Culture Medium
38. Add 4 ml of medium containing the cell suspension. The inoculums should contain 6,000-10,000 cells per cm^2 of surface area.
39. Finally, add another 6 ml of Complete Bone PrimaCell™ Culture Medium, to give a total volume of 12 ml.
40. In the interim, add an additional 4 ml of digestion solution to the remaining pieces of bone, and repeat the digestion for 30 min. The released cells are harvested, and, if necessary, the digestion step is repeated several more times. With large amounts of bone, the digestion period can be increased to 1-3 h. Cell counts are performed after each digestion period, and the released cells are used to inoculate a different flask.

2.4.3 Passage of Cells in Culture:

41. Remove the pieces of explant.
42. Remove the medium and rinse the cell layer with PBSA, 0.2 ml/ cm^2.
43. Add trypsin to the flask, 0.1 ml/ cm^2, and incubate at 37˚C until the cells have detached and separated from one another. Monitor cell detachment and separation on the microscope. In general, 10-min incubation is sufficient.
44. Transfer the released cells to a centrifuge tube with an equal volume of Complete Bone PrimaCell™ Culture Medium with 20% fetal calf serum (FCS).
45. Centrifuge the cells at 600 g for 5 min.
46. Discard the supernatant, and resuspend the cells in complete medium by gentle, repeated pipetting.
47. Set one aliquot aside for the determination of cell concentration and another for DNA determination.
48. Inoculate the remaining cells into culture flasks or wells that have previously been equilibrated with medium. The cells should reattach within 24 h.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same

tissue sample; the best results are reported when cells from preconfluent primary cultures are used.

49. Detach cells as for the subculture, and centrifuge at 100 g for 10 min.
50. Resuspend cells in Human Complete Bone PrimaCell™ Culture Medium and count.
51. Dispense aliquots of 2×10^6 cells/ml in Human Complete Bone PrimaCell™ Culture Medium with 10% glycerol into cryopreservation tubes.
52. Equilibrate at 4℃ for 1-2 h.
53. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
54. To recover cells:
    a) Thaw cryotubes quickly in a 37℃ water bath.
    b) Dilute cells tenfold with medium.
    c) Centrifuge cells and resuspend them at an appropriate concentration in the desired Human Complete Bone PrimaCell™ Culture Medium, and seed culture vessel.

IV Fibroblast Contamination

There are several techniques have been published in the literature to deal with fibroblast contamination during colorectal primary cell culture. These include: (1) Physically remove a well-isolated fibroblast colony by scraping it with a sterile blunt instrument (e.g., a cell scraper). Care has to be taken to wash the culture up to six times to remove any fibroblasts that have detached in order to prevent them from reseeding and reattaching to the flask. (2) Differential trypsinization can be attempted with the carcinomas. (3) Dispase preferentially (but not exclusively) removes the epithelium during passaging and leaves behind most of the fibroblastic cells attached to the culture vessel. During subculture, cells that have been removed with dispase can be preincubated in plastic petri dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the osteoblasts. (4) Reduce the concentration of serum to about 2.5-5% if there are heavy concentrations of fibroblastic cells. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and that using any or all of the preceding techniques will eventually push the cells through so many divisions that any fibroblasts will senesce.

Human Bone PrimaCell™ system includes a fibroblast elimination system, the Human Bone Fibroblast Growth Inhibitors, Bone marrow FibrOut™. It contains a mixture of cis-OH-proline, collagenase, and gentamycin. This system can effectively eliminate Bone fibroblast contamination while has not affect on the behavior of osteoblasts.

V Characterization of osteoblasts

In order to characterize the human osteoblasts, various analyses need to be performed. Due to presence of large amount of rough endoplasmic reticulum, cytoplasm of osteoblasts appears to be basophilic via normal HE stain. A large Golgi apparatus is also present in the centre, and the nucleus is spherical and large. These include measurement of cell growth, alkaline phosphatase, and response to PTH by measuring cyclic adenosine monophosphate (cAMP) production. Several genes, such as alkaline phosphatase, osteocalcin, and Cbfa1/Osf2, are known to be regulated during osteoblastic differentiation and are commonly used as “osteoblast markers” for *in vitro* or in *vivo* studies. In addition, osteoblasts can be stained for procollagen type 1 (PICP), alpha 1(I) procollagen, Bone Gla Protein (BGP), Bone Sialoprotein (BSP), etc.

VI References:

1. Günter Lauer, R.S., *Isolation of human osteoblasts and in vitro amplification for tissue engineering and subsequent bone repair.* Int Poster J Dent Oral Med, 2001.
2. Ongphiphadhanakul, B., et al., *Etidronate inhibits the thyroid hormone-induced bone loss in rats assessed by bone mineral density and messenger ribonucleic acid markers of osteoblast and osteoclast function.* Endocrinology, 1993. **133**(6): p. 2502-7.
3. Garcia, T., et al., *Behavior of osteoblast, adipocyte, and myoblast markers in genome-wide expression analysis of human calvaria primary osteoblasts in vitro.* Bone, 2002. **31**(1): p. 205-11.
4. Zhou, H., et al., *In situ hybridization to show sequential expression of osteoblast gene markers during bone formation in vivo.* J Bone Miner Res, 1994. **9**(9): p. 1489-99.

Rat Bone PrimaCell™: Osteoblasts

Rat Bone Primary Cell Culture

Cat No.	Description	Qt.	Price
2-82021	**Rat Bone PrimaCell™ System**	**kit**	**$**499
7-61151	Rat Bone Fibroblast Growth Inhibitors, Bone FibrOut™ (for 500 ml medium)	1 ml	$158
9-41015	Rat Bone Osteoblasts PrimaCell™ Basal Culture Medium	500 ml	$73
9-31151	Rat Bone Osteoblasts PrimaCell™ Medium Supplements With Serum (for 500 ml medium)	set	$240
4-21151	Rat Bone Tissue Dissociation System, Bone OptiTDS™	ea	$133

Rat Bone Primary Cell Characterization

6-24011	**Rat Osteoblasts Primarker™ Kit**	**kit**	**$180**
6-24012	Primary Antibody for Rat Osteoblast Surface Marker, rabbit	50 µl	$120
6-24013	Rat Osteoblast Primarker™ buffer system	set	$90
6-24014	FITC-secondary antibody, goat anti-rabbit	50 µl	$70

Human Brain PrimaCell™ I: Cerebellar Granule Cells
(Cat No. 2-2-96041)

I. General Description

Neurons are extremely fastidious in their choice of substrate. They usually do very poorly on untreated glass or plastic surfaces, but demonstrate neurite outgrowth on collagen and poly-D-lysine. Neurite outgrowth is encouraged by the polypeptide Nerve Growth Factor (NGF) and several other unique growth factors secreted by glial cells. Most tissue culture conditions used for neurons do not support neuronal proliferation, even with neurons isolated from embryonic stages where evidence of mitosis was observed *in vivo*. Recently, however, breakthroughs in techniques have identified methods of propagating neurons in ways which allow proliferation *in vitro* and relocalization of those neurons *in vivo*.

Cerebellar granule cells in culture provide a well characterized neuronal cell population that is well suited for morphological and biochemical studies of cellular and molecular correlates of mechanisms of survival/apoptosis and neurodegeneration/neuroprotection. Cerebellar granule cell is one of the most reliable models for the study of neural development, function and pathology.. The Human Brain PrimaCell kit (Cat No. 2-96041) allows the isolation of cerebellar granule cells from human adult brain specimens. Non-neuronal cells are prevented from interfering with the target cells by the addition of Brain Fibroblast Growth Inhibitors, FibrOut™ to the culture medium.

1.1 Components of the Human Brain PrimaCell™ I System

- **Human Brain Tissue Dissociation System, Brain OptiTDS™** (2 × 1 ml) --- *A mi×ture of collagenase, collagenase I, Trypsin and Human Brain OptiTDS™ Reconstitution Buffer.*
- **Human Brain OptiTDS™ Digestion Buffer,** (2 × 9 ml).
- **Human Brain Fibroblast Growth Inhibitors, Brain FibrOut™** ---Cervi× FibrOut™ (5 x 200 µl) --- *A mi×ture of cis-OH-proline, collagenase and gentamycin.*
- **Human Brain Cerebellar Granule Cells PrimaCell™ Basal Culture Medium,** (5 x 100 ml) --- A *Modified DMEM medium*.
- **Human Brain Cerebellar Granule Cells PrimaCell™ Medium Supplements,** (5 × 1 ml): *Glucose, L-glutamine, KCl, Insulin, P-aminobenzoic acid, and Gentamycin.*
- **Human Brain PrimaCell™ Serum,** (5 x 10 ml) --- *A modified heat inactivated fetal calf serum.*
- **Human Brain Tissue Washing Medium,** (1 x 100 ml): *HBSS with 200U/ml penicillin, 200 µg/ml streptomycin, 50 µg/ml gentamycin and 5% BSA.*
- **Brain PrimaCell™ Culture Dish Coating Solution,** (1 x 100 ml) --- *Poly-L-lysine.*

1.2 Required materials but NOT included

- PBS
- Water bath
- Tissue culture dishes
- 35-mm tissue culture Petri dishes
- Scalpels, scissors, and forceps

- Pasteur pipettes and 10-ml pipettes
- Test tubes, 12 and 50 ml

1.3 Siliconization of Pasteur pipettes

- Prepare a diluted Apuasil solution provided in this kit.
- Dip the pipettes into the solution and flush out the insides of the pipettes.
- Air-dry the pipettes for 24 h, or dry for several minutes at 100° C.
- Sterilize the pipettes by dry heat.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, fresh Poly-L-lysine-coated plate or culture dishes are recommended (see below for treatment of culture dishes).

Treatment of Culture Dishes:

.

4. Add 1 ml of **Human Brain PrimaCell™ Culture Dish Coating Solution** to each of the 35-mm tissue culture dishes.
5. Incubate the tissue culture dishes with the **Human Brain PrimaCell™ Culture Dish Coating Solution** for a minimum of 2 hrs, and aspirate.
6. Air dry tissue culture plates in a hood.

2.2 Procedure Outline

The cerebella from human are cut into small cubes and incubated with the Human Brain Tissue Dissociation System, Brain OptiTDS™. The provided Human Brain OptiTDS™ is diluted 1:10 in the provided Human Brain OptiTDS™ Digestion Buffer and the diluted Human Brain OptiTDS™ is incubated with brain tissue at a ratio of 10 ml diluted Human Brain OptiTDS™ solution per 3-5 g tissue sample for 30 min at 37° C. The cell suspension is seeded in coated culture dishes or flasks prepared above.

2.3 Human Cerebella Tissue Preparation

Human Brain: Brain from 7-8 day rats yield a large number of cells (5-10 × 10^6), with a 30-40% plating efficiency. Rats are sacrificed by CO_2 narcosis or a method that is approved by the home institution. Carefully remove the skin and dissect out the area containing the cerebella.

4 Dissect out the cerebella aseptically and place in Human Brain Tissue Washing Medium at room temperature.
5 Mince the tissue using scalpels into small cubes measuring approximately 0.5 mm^3.
6 Transfer the minced tissue into 15 ml conical tubes and wash the tissue three times with Human Brain Tissue Washing Medium. Allow the tissue to settle to the bottom of the tubes between each washing.

2.4 Cerebella Separation and Tissue Dissociation

2.4.1 Human Brain OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. Human Brain Tissue Dissociation System is suited for optimal dissociation of normal human cerebella tissues to yield maximum number of single Cerebellar Granule Cell (CGC).

2.4.2 Brain OptiTDS™ Compositions

- Collagenase: from *Clostridium Histolyticum*
- Collagenase I: from *Clostridium Histolyticum*
- Trypsin

2.4.3 System Components

- Human Brain Tissue Dissociation System, Brain OptiTDS™, (2 × 1 ml).
- Human Brain OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

7 Reconstitute the Human Brain Tissue Dissociation System stock solution by adding 1 ml Human Brain OptiTDS™ to 9 ml of Human Brain OptiTDS™ Digestion Buffer, mix well. We recommend using 10 ml diluted Brain OptiTDS™ solution per 3-5 g of brain tissue and follow by incubating in a water bath for 30 min at 37° C.

8 Disaggregate the tissue by trituration through a siliconized Pasteur pipette - until a single-cell suspension is obtained.

2.12 Cerebellar Granule Cell Isolation and Culture

2.6.1 Medium Preparation

Thaw out Human Brain Cerebellar Granule Cells PrimaCell™ Medium Supplements, Human Brain PrimaCell™ Serum, and Human Brain Fibroblast Growth Inhibitors, Brain FibrOut™ on ice.

Human Complete Brain PrimaCell™ I Culture Medium: To every 100 ml Human Brain PrimaCell™ Basal Culture Medium, add 10 ml Human Brain Cerebellar Granule Cells PrimaCell™ Medium Supplements, 10 ml Human Brain PrimaCell™ Serum, mix thoroughly and warm the complete medium at a 37 ^{o}C water bath for 10 min prior use.

Human Complete Brain PrimaCell™ I Culture Medium containing FibrOut™: To every 100 ml Human Brain PrimaCell™ Basal Culture Medium, add 10 ml Human Brain Cerebellar Granule Cells PrimaCell™ Medium Supplements, 10 ml Human Brain PrimaCell™ Serum , and one vial of Human Brain Fibroblast Growth Inhibitors, Brain FibrOut™, mix thoroughly and warm the complete medium at a 37 ^{o}C water bath for 10 min prior use. **Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination.*

9 Once all cells have been separated, allow the cell suspension to remain in the 50 ml conical for 3-5 min, allowing the small clumps of tissue to settle to the bottom of the tube. Carefully remove these clumps with a Pasteur pipette.
10 Centrifuge the single-cell suspension at 200 g for 5 min, and carefully aspirate off the supernatant.
11 Re-suspend the pellet in Complete Brain Medium (as described in step 5), and seed the cells at a concentration of 2.5-3.0 × 10^6 cell/dish on previously treated culture plates.
12 After 2-4 days (best results are usually obtained after 2 days), continue to culture the cells with Complete Media containing the Human Brain Fibroblast Growth Inhibitors, Brain FibrOut™ for 24 h. FibrOut™ is used at 1:500 dilution in Complete Media.
13 After 24-48 hrs or culturing, switch to Complete Brain Medium without FibrOut™.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample; the best results are reported when cells from preconfluent primary cultures are used.
14 Detach cells as for the subculture, and centrifuge at 100 g for 10 min.
15 Resuspend cells in Human Complete Brain PrimaCell™ I Culture Medium and count.
16 Dispense aliquots of 2 × 10^6 cells/ml in Human Complete Brain PrimaCell™ I Culture Medium with 10% glycerol into cryopreservation tubes.
17 Equilibrate at 4℃ for 1-2 h.
18 Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
19 To recover cells:
g) Thaw cryotubes quickly in a 37℃ water bath.
h) Dilute cells tenfold with medium.
i) Centrifuge cells and resuspend them at an appropriate concentration in the desired Human Complete Brain PrimaCell™ I Culture Medium, and seed culture vessel.

IV Characterization

Neurons can be identified immunologically using neuron-specific enolase antibodies or by using tetanus toxin as a neuronal marker. It is reported that alpha 6 subunit of gamma-aminobutyric type A receptors is used to detect the purity of cerebellar granule cells. Astrocyte contamination can be assessed by staining cultures with glial fibrillary acidic protein as a marker.

V References

1. Hansen, S.L., et al., *Effects of GABA (A) receptor partial agonists in primary cultures of cerebellar granule neurons and cerebral cortical neurons reflect different receptor subunit compositions.* Br J Pharmacol, 2001. **133**(4): p. 539-49.
2. Sui, Z., et al., *Human immunodeficiency virus-encoded Tat activates glycogen synthase kinase-3beta to antagonize nuclear factor-kappaB survival pathway in neurons.* Eur J Neurosci, 2006. **23**(10): p. 2623-34.
3. New, D.R., et al., *HIV-1 Tat induces neuronal death via tumor necrosis factor-alpha and activation of non-N-methyl-D-aspartate receptors by a NFkappaB-independent mechanism.* J Biol Chem, 1998. **273**(28): p. 17852-8.
4. Yavin, E. and Z. Yavin, *Attachment and culture of dissociated cells from rat embryo cerebral hemispheres on polylysine-coated surface.* J Cell Biol, 1974. **62**(2): p. 540-6.
5. Zhao, L.Z., et al., *Activation of c-Jun and suppression of phospho-p44/42 were involved in*

diphenylhydantoin-induced apoptosis of cultured rat cerebellar granule neurons. Acta Pharmacol Sin, 2003. **24**(6): p. 539-48.
6. Yan, G.M., et al., *Diphenylhydantoin induces apoptotic cell death of cultured rat Cerebellar granule neurons.* J Pharmacol Exp Ther, 1995. **274**(2): p. 983-90.

Human Brain PrimaCell™ I: Cerebellar Granule Cells

Human Brain Primary Cell Culture I

Cat No.	Description	Qt.	Price
2-96041	**Human Brain PrimaCell™ I System**	**kit**	**$599**
7-66042	Human Brain Fibroblast Growth Inhibitors, Brain FibrOut™ (for 500 ml medium)	1 ml	$195
9-46004	Human Brain Cerebellar Granule Cells PrimaCell™ Basal Culture Medium	500 ml	$73
9-36042	Human Brain Cerebellar Granule Cells PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$160
4-26042	Human Brain Tissue Dissociation System, Brain OptiTDS™	ea	$146
9-86004	Human Brain I Tissue Preparation Buffer Set	ea	$90

Human Primary Cerebellar Granule Cells Characterization

6-35011	**Human Cerebellar Granule Cell Primarker™ Kit**	**kit**	**$220**
6-35012	Human Cerebellar Granule Cell Primarker™ antibody set	set	$180
6-35013	Human Cerebellar Granule Cell Primarker™ buffer system	set	$90

Human Brain PrimaCell™ II: Olfactory Bulb Ensheathing Cells
(Cat No. 2-96051)

I. General Description

Olfactory bulb Ensheathing cells have a unique biology and thus are widely used for experiments to study the activity of oncogenes in epithelial neoplasias, and the molecular mechanisms implicated in warts and other brain associated disorders. In addition, several *in vitro* brain models have been developed that accurately mimic the epidermis making it possible to study the brain in a physiologically relevant context. While these cells are extremely useful in the laboratory they are notoriously difficult to isolate and culture. The Human Brain PrimaCell™ II kit (Cat No. 2-96051) is designed for the successful isolation and culture of Olfactory bulb Ensheathing cells from human brain allowing reproducible and dependable results.

1.1 Components of the Human Brain PrimaCell™ I System

- **Human Brain Tissue Dissociation System, Brain OptiTDS™** (2 × 1 ml) --- *A mixture of collagenase, collagenase II, Dexoyribonuclease I and Human Brain OptiTDS™ II Reconstitution Buffer.*
- **Human Brain OptiTDS™ Digestion Buffer,** (2 × 9 ml)
- **Human Brain Tissue Washing Medium,** (1 × 100 ml) --- (1 × 100 ml) --- *A modified Leibowitz L-15 with 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin.*
- **Human Brain Fibroblast Growth Inhibitors, Brain FibrOut™** ---Brain FibrOut™ (5 × 200 μl) --- *A mixture of cis-OH-proline, collagenase and gentamycin.*
- **Human Brain PrimaCell™ II Basal Culture Medium** (5 × 100 ml) --- A *Modified mixture of DMEM and glucose*.
- **Human Brain PrimaCell™ II Medium Supplements** (5 × 1 ml) --- *A mixture of gentamycin, BSA Pathocyte, Glutamine, Bovine pancreas insulin, Transferrin, Progesterone, Putrescine, L-thyroxine, Selenium, and 3,3'5-triiodo-L-thyronine.*
- **Human Brain PrimaCell™ Serum** (5 × 10 ml) --- *A modified Fetal Calf serum.*
- **Human Brain PrimaCell™ II Culture Dish Coating Solution** (3 × 10 ml): A modified buffer with Poly-L-lysine.

1.2 Required materials but NOT included:

- DMEM (Invitrogen Cat No. 10313-021)
- Fetal Bovine Serum (FBS, Sigma-Aldrich)
- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or human fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, fresh coated plate or culture dishes are recommended (see below for treatment of culture dishes). Culture plastics and glass coverslips with diluted **Human Brain PrimaCell™ II Culture Dish Coating Solution** (incubate for at least 1 h to a maximum of 24 h), wash the plastics and coverslips once with PBSA, and air dry them prior to use. A greater yield of cells is obtained from adult human.

2.2 Principle

The protocol describes a rapid method for purifying a population of glial cells from the olfactory bulb by using cell adhesion character.

2.3 Human Brain Tissue Preparation

22. The tissue biopsy for culture should be taken by the pathologist and transferred immediately to a Petri dish containing a 5-10 ml 70% ethanol for 1 min, followed by inoculating with 5-10 ml fresh **Human Brain Tissue Washing Medium** for 5 min.
23. Using a sterile scalpel blade, chop the olfactory bulbs into small pieces.

2.4 Epidermal Separation and Tissue Dissociation

2.4.1 Human Brain OptiTDS™ II

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Human Brain Tissue Dissociation System II is suited for optimal dissociation of normal newborn human brain tissues to yield maximum number of single Olfactory bulb Ensheathing cells.

2.4.2 Enzyme Compositions

- Trypsin: from *Bovine Pancreas*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase II: from *Clostridium Histolyticum*
- Dexoyribonuclease I

2.4.3 System Components

- Human Brain Tissue Dissociation System II, Brain OptiTDS™ II, (2 × 1 ml).
- Human Brain OptiTDS™ II Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

24. Prepare fresh enzyme working solution: Add 1ml of **Human Brain Tissue Dissociation System, Brain OptiTDS™** to one vial of **Human Brain OptiTDS™ Digestion Buffer** (9 ml). Warm the diluted **Human Brain Tissue Dissociation System** working solution at 37

℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 5 ml **Human Brain Tissue Dissociation System** working solution.

25. Place the pieces into a small vial containing 5 ml of **Human Brain Tissue Dissociation System** working solution.
26. Incubate the pieces of olfactory bulbs at 37℃ for 30 - 45 min.

2.13 Olfactory bulb Ensheathing cells Isolation

27. Centrifuge the resultant suspension at 1,000 rpm for 5 min and remove the supernatant.
28. Resuspend the pelleted bulb tissue in 5 ml of **Human Brain Tissue Washing Medium**.
29. Glial cells are fragile; therefore, to produce a single-cell suspension, the olfactory bulb tissue must be dissociated gently, taking care not to produce air bubbles. Dissociate the tissue through 19G hypodermic needle followed by a 23G hypodermic needle.
30. Add 5 ml of **Human Brain Tissue Washing Medium**, and strain the cell mixture through a sterile cell strainer (70-100μm) into a centrifuge tube to remove debris.

2.6 Primary Olfactory bulb Ensheathing cells Culture

2.6.1 Medium Preparation.

Thaw out **Human Brain PrimaCell™ II Basal Culture Medium**, **Human Brain PrimaCell™ II Medium Supplements**, and **Human Brain PrimaCell™ II Serum** on ice.

Human Complete Brain PrimaCell™ II Culture Medium: To every 100 ml **Human Brain PrimaCell™ II Basal Culture Medium**, add 1 ml **Human Brain PrimaCell™ II Medium Supplements** and 10 ml **Human Brain PrimaCell™ II Serum** mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

Human Complete Brain PrimaCell™ II Culture Medium containing FibrOut™: To every 100 ml **Human Brain PrimaCell™ II Basal Culture Medium**, add 1 ml **Human Brain PrimaCell™ II Medium Supplements** and 10 ml **Human Brain Serum**, and 200 μl **Human Brain Fibroblast Growth Inhibitors, Brain FibrOut™** mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use. (Important: *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*)

2.6.1 Primary Cell Culture

31. Spin the suspension again at 1,000 rpm for 10 min.
32. Remove the supernatant and resuspend the cells in **Human complete Brain PrimaCell™ II Basal Culture Medium containing FibrOut** yield a concentration of 8.5×10^5 cells/ml.
33. The cell suspension was seeded into uncoated tissue culture plate or flask, and incubated at 37°C and 5% CO2 for 18 hours. Most of the fibroblasts attached during this first incubation period since fibroblasts will settle within one hour of seeding.
34. The supernatants from the step 12 were poured into uncoated tissue culture plate or flask, and incubated at 37°C and 5% CO2 to allow for the attachment of astrocytes. Primary olfactory bulb ensheathing cells do not attach to uncoated slides for 96–120 hours.
35. After 36 hours of incubation, most of the ensheathing cells remained in the supernatant.

This supernatant was used to seed poly-L-lysine-coated tissue culture plate or flask. After seeding, the ensheathing cells attached within 48 hours and neurons do not survive this culture environment.

36. Cells were maintained in an incubator (37°C, 5% CO_2) for 8 days, and the **Human complete Brain PrimaCell™ II Culture Medium/FibrOut** was changed every 2 days.

2.14 Subculture

Propagating Olfactory bulb Ensheathing cells in culture can be somewhat challenging, however the following methods have worked consistently in many laboratories.

37. Remove complete **Human complete Brain PrimaCell™ II Culture Medium** and wash cells in 0.1% trypsin two times. Place just enough trypsin (0.1 % trypsin without EDTA) to moisten cell layer (200-500 µl depending on size of the dish).
38. Incubate for 1-5 min at 37°C. Gently pipette cells and resuspend in **Human complete Brain PrimaCell™ II Culture Medium** (FibrOut™ is not necessarily needed at this step) for counting and replatting on collagen coated tissue culture plates.
39. Gently pipette cells and resuspend in **Human complete Brain PrimaCell™ II Culture Medium** for counting and replatting on tissue culture plates.

III Cryopreservation

40. Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Olfactory bulb Ensheathing cells cultures derived from preconfluent layers.
    - **(a)** Trypsinize cells as above, and centrifuge at 100 g for 10 min.
    - **(b)** Resuspend cells in **Human complete Brain PrimaCell™ II Culture Medium** and count cells.
    - **(c)** Prepare aliquots of 2×10^6 cells/ml in **Human complete Brain PrimaCell™ II Culture Medium** and 10% glycerol into cryovials.
    - **(d)** Equilibrate at 4℃ for 1-2 h.
    - **(e)** Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
    - **(f)** To recover cells:
        (i) Thaw cryovials quickly in a 37℃ water bath.
        (ii) Dilute cells tenfold with appropriate **Human complete Brain PrimaCell™ II Culture Medium**.
        (iii) Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in **Human complete Brain PrimaCell™ II Culture Medium**.

IV Characterization

Primary cultures of olfactory bulb ensheathing cells obtained from human olfactory bulb display a defined phenotype in vivo and in vitro at both ultrastructure and immunocytochemcal levels. Vimetin is the major constituent of ensheathing glia intermediate filamediate filaments in the adult olfactory bulb. Besides vimentin, ensheathing cells express glial fibrillary acidic protein (GFAP) in filaments. The membrane of ensheathing cells contain proteins involved in cell adhesion and axonal growth. These molecules, such as L1, laminin, and neural-cell adhesion molecule (N-CAM), mainly expressed at the contact sites with olfactory axons. Ensheathing cells also express

p75 NGF receptor along their membrane except the areas contacting olfactory axons. Olfactory bulb ensheathing cells can be characterized based on their specific for their glia phenotype to exclude contamination by neuronal cells which were anti-neuron specific enolase (NSE) positive. The source of tissue (embryonic, new-born, adult olfactory bulb) as well as the culture conditions could influence the cell phenotype. The purity is determined by using glial fibrillary acidic protein (GFAP), p75 NGF receptor antibodies or cytoplastic S-100 antibodies the for the olfactory bulb ensheathing cells.

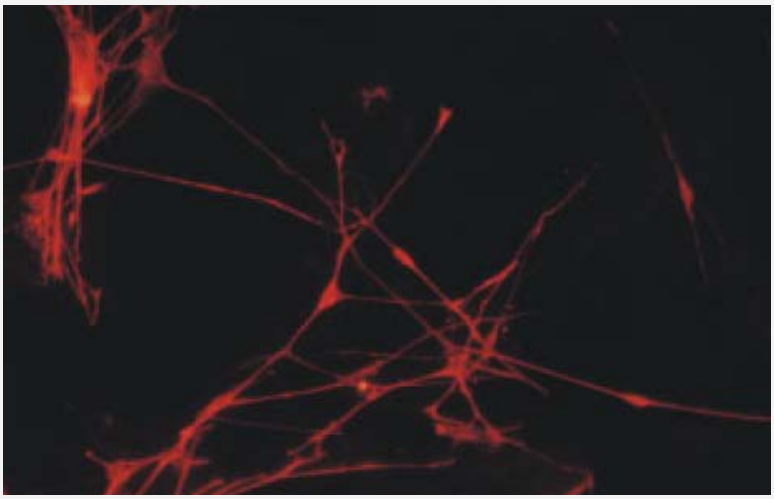


Figure 11. S100-positive staining multipolar OECs (3).

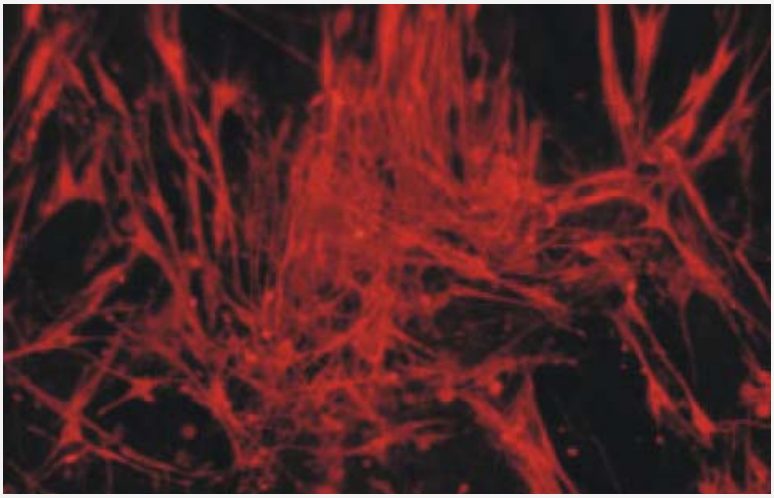


Figure 12. Dense network of S100-positive OECs (3).

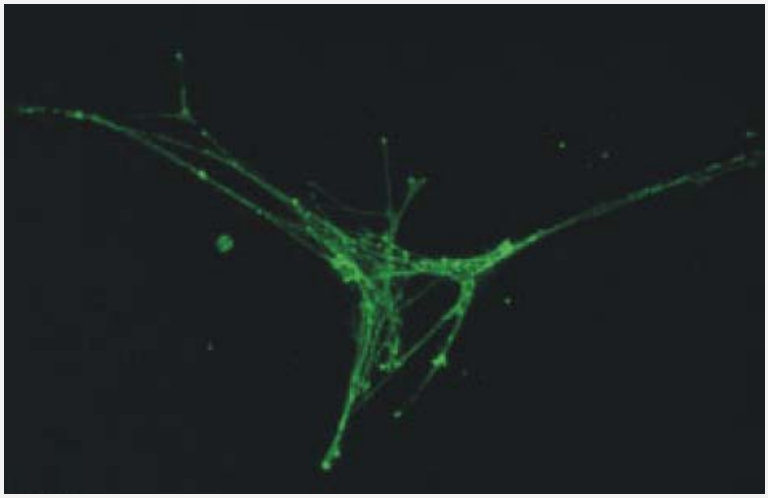


Figure 13. Bi-and tripolar p75-LNGFR-positive OECs (3).

V References:

1. Ramon-Cueto, A. and F. Valverde, *Olfactory bulb ensheathing glia: a unique cell type with axonal growth-promoting properties.* Glia, 1995. **14**(3): p. 163-73.
2. Bakardjiev, A., *Biosynthesis of carnosine in primary cultures of rat olfactory bulb.* Neurosci Lett, 1997. **227**(2): p. 115-8.
3. Miedzybrodzki, R., et al., *The olfactory bulb and olfactory mucosa obtained from human cadaver donors as a source of olfactory ensheathing cells.* Glia, 2006. **54**(6): p. 557-65.

Human Brain PrimaCell™ II: Olfactory Bulb Ensheathing Cells

Human Brain Primary Cell Culture II

Cat No.	Description	Qt.	Price
2-96051	**Human Brain PrimaCell™ II system**	**kit**	**$599**
4-26052	Human Brain Tissue Dissociation System, Brain OptiTDS™ (for 500 ml medium)	1 ml	$146
9-46005	Human Brain PrimaCell™ II Basal Culture Medium	500 ml	$85
9-36052	Human Brain PrimaCell™ II Medium Supplements with Serum (for 500 ml medium)	set	$160
7-66052	Human Brain Fibroblast Growth Inhibitors, Brain FibrOut™	ea	$195
9-86005	Human Brain II Tissue Preparation Buffer Set	ea	$90

Human Primary Olfactory Bulb Ensheathing Cells Characterization

6-36011	**Human Olfactory Bulb Ensheathing Cell Primarker™ Kit**	**kit**	**$220**
6-36012	Human Olfactory Bulb Ensheathing Cell Primarker™ antibody set	set	$180
6-36013	Human Olfactory Bulb Ensheathing Cell Primarker™ buffer system	set	$90

Human Breast PrimaCell™: Mammary Epithelium
(Cat No. 2-96031)

I. General Description:

This protocol is developed for attachment and growth of normal Human Breast epithelial cells from 4-7 mm^3 biopsies with Human Breast PrimaCell™ system (Cat No. 2-96031). This system provides an optimal condition of tissue dissociation system, Breast OptiTDS™ that yields 5-7 times of single cells more than most of the tissue dissociation protocols published in the literature. In addition, this system ensures a high viability of the target cells with improved gradient contained in the culture medium. With CHI's proprietary fibroblast inhibitory system, Breast FibrOut™, cells are growing with contamination of minimized amount of the non-epithelial cells.

The preparation of tissue specimens for cell culture is usually started within 1-2 h of removal from the patient. If this is impossible, fine cutting of the tissue into small pieces (1-2 mm) with scalpels and storage overnight at 4℃ in washing medium (see below) can also prove successful.

Human Breast PrimaCell™ system applies to all types of normal adult Human biopsies samples. Biopsies samples contain pathological organism (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Human Breast PrimaCell™ System

- **Human Breast Tissue Dissociation System, Breast OptiTDS™,** (2 x 1 ml) --- *A mixture of collagenase I, collagenase III, collagenase IV, collagenase, and trypsin*.
- **Human Breast OptiTDS™ Digestion Buffer**, (2 x 9 ml).
- **Human Breast Tissue Washing Medium**, (1 x 100 ml) --- *Basal Breast PrimaCell™ Culture Medium with 5% FCS, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Human Breast Fibroblast Growth Inhibitors, Breast FibrOut™** (5 x 200 μl) --- *A mixture of cis-OH-proline, toxin ricin, gentamycin and formulated serum substitutes*.
- **Human Breast PrimaCell™ Basal Culture Medium**, (5 x100 ml) --- *Modified formulation based on RPMI 1640 and DMEM medium*.
- **Human Breast PrimaCell™ Medium Supplements**, (5 x 1.0 ml) --- A mixture of *EGF, Insulin, Hydrocortisone, Cholera toxin, penicillin, and streptomycin*.
- **Human Breast PrimaCell™ Serum**, (1 x 50 ml) --- H*eat-inactivated and special-treated Fetal Calf Serum.*

1.2 Required Materials but not provided

- Pasteur pipettes
- Collagen I-coated Culture dishes
- Scalpels, scissors, and forceps
- Pasteur pipettes and 10-ml pipettes
- Test tubes, 12 and 50 ml

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, collagen I-coated plate (Corning, NY) MUST be pre-treated with 5 ml Coating Solutions for 5 min. Aspirate the coating solutions; let the dishes be air-dry in the ventilated cell culture hood for 5-10 min.

2.2 Surgical specimens

Biopsies of about 4-6 mm^3 are taken with biopsy forceps to portions of mammary glands. All surgical specimens should be immediately placed in Human Breast Tissue Washing Medium, transported on ice to the laboratory within 1 h and worked up immediately. With autoclaved scalpels, scissors, and forceps, carefully remove muscle and fat from specimens followed by washing procedures. Place specimens in a 10 ml falcon tube contain 5-10 ml fresh Breast Tissue Washing Medium followed by inoculating for 10 min at the room temperature. For large tissue specimens, 50 ml falcon tube and more wash medium is needed to ensure thoroughly washing. Aspirate the washing medium and repeat the washing procedures with fresh washing medium two more time. Washing tissue specimens sequentially in 70% ethanol for 1 min at the room temperature, in PBS for 5 min, and in fresh Breast Tissue Washing Medium for 10 min. Collecting tissue specimen by centrifugation prior to tissue dissociation procedures (see below).

2.3 Tissue Preparation and Dissociation

2.3.1 Human Breast OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Breast Tissue Dissociation System, OptiTDS™, is suited for optimal dissociation of normal adult Human biopsies samples to yield maximum number of single primary cells of Breast tissues.

2.3.2 Enzyme Compositions

- Collagenase I: from *Clostridium Histolyticum*
- Collagenase III: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*
- Collagenase: from *Clostridium Histolyticum*
- Trypsin: from *Bovine Pancreas*

2.3.3 System Components

- Breast Tissue Dissociation System, OptiTDS™, (2 x 1 ml).
- Breast OptiTDS™ Digestion Buffer, (2 x 9 ml).

2.3.4 Procedures For Tissue Preparation and Dissociation

1. Prepare fresh enzyme working solutions: add 1.0 ml of Breast Tissue Dissociation System, OptiTDS™, to one vial of Breast OptiTDS™ Digestion Buffer (9.0 ml). Warm the diluted enzyme working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 4-5 g tissue samples per 10 ml diluted enzyme working solutions.
2. Mince pre-washed tissue into pieces approximately 0.2-0.5 mm^2 in diameter with two scalpel and forceps.
3. Incubate minced tissues with the enzyme working solutions by incubating minced tissue samples (up to 5 g) in 10 ml fresh enzyme working solutions with slow magnetic stirring for 30 min at 37 ℃.
4. At the end of tissue dissociation period, gentle triturating tissue with a 10 ml pipette, constitutes filling the empty the barrel at a rate of 2-3 ml per second. Repeat this procedure for 5-6 times.
5. Collecting cells by filtration the mixture through a cell strainer to remove fatty layer and larger undigested tissues followed by centrifugation at 1 x 100 g. Carefully collect enzyme working solutions and resuspend the cell pellet with 1.0 ml complete culture medium.
6. Count the cells and seed cells in 3-4 T-25 collagen I-coated flasks (**Important:** pre-treat the flask with the provided Coating Solutions for 5 min and air-dry in hood prior to use) upon the density of viable cells (2.5-5 x 10^5 Cells/flask).
7. The undigested tissues can be repeated for digestion process by inoculating the tissue with the saved enzyme working solutions from step 5 for another 30 min, to obtain additional cells.

2.3.5 Storage:

Reconstituted tissue dissociation systems should occur before use and can only be stored for 2-4 days at 4 ^oC. For long-term use, it should be aliquoted and stored at -20 ^oC. Avoid repeated freeze-thaw cycles.

2.4 Culture of primary Human Mammary Epithelial cells

2.4.1 Medium Preparation.

Preparation of complete culture medium: thaw out one vial of **Human Mammary Epithelium PrimaCell™ Medium Supplements** and one vial of **Human Breast PrimaCell™ Serum**, one vial of **Human Breast Fibroblast Growth Inhibitors**, **Breast FibrOut™** on ice. To every 100 ml **Human Mammary Epithelium PrimaCell™ Basal Culture Medium**, add one vial of **Human Mammary Epithelium PrimaCell™ Medium Supplements**; one vial **Human Breast PrimaCell™ Serum**; and one vial of **Human Breast Fibroblast Growth Inhibitors, Breast FibrOut™**, mix thoroughly and warm the complete medium at a 37^oC water bath for 10 min prior use. (**Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*).

2.4.2 Treatment of Culture Dishes.

To enhance cell attachment to the culture dishes, collagen I-coated plate (Corning, NY) MUST be freshly pre-treated with the provided Coating Solutions by adding appropriate volume of Coating Solutions (enough to cover the whole cell-growth area) and incubate for 5 min. Aspirate the

medium; let the dishes be air-dry in the ventilated cell culture hood for 5-10 min prior to use.

2.4.3 Standard primary culture conditions.

Inoculate epithelial tubules and clumps of cells derived from tissue specimens into T-25 flasks coated with collagen type I with pre-treatment of basal culture medium as described above at 37℃ in a 5%-CO_2 incubator with 4 ml of complete culture medium prepared in 2.4.1. Change the culture medium twice weekly. The tubules and cells start to attach to the substratum, and epithelial cells migrate out within 1-2 days. Most of the tubules and small clumps of epithelium attach within 4 days.

2.4.4 Alternative Primary Culture conditions.

The attachment of epithelium during primary culture and subculture is more reproducible and efficient when cells are inoculated onto collagen-coated flasks, and significantly better growth is obtained with 3T3 feeders than without. When the epithelial cells expand several passages, they become less dependent on 3T3 feeders, and no further addition of feeders is necessary. All medium and solutions described in the standard culture condition are applicable in this culture method.

2.4.5 Subculture and Propagation

Most mammary epithelium primary cultures cannot at present be passaged by routine trypsin / EDTA procedures. Disaggregation to single cells of the cultured Breast cells with 0.1% trypsin in 0.25 mM (0.1%) EDTA will result in extremely poor or even zero growth, so Dispase is used instead. One of the advantage using dispase is that dispase can only detach epithelial cell but not the fibroblast, thus increase the purity of epithelium.

8. Add 0.5% Dispase (Sigma, w/v) to the cell monolayer, just enough to cover the cells (~2.5 ml/25-cm^2 flask), and leave the solution to stand for 40-60 min for primary cultures and 20-40 min for cell lines.
9. Once the epithelial layers begin to detach (they do so as sheets rather than single cells), pipette to help detachment and disaggregation into smaller clumps.
10. Wash and replate the cells under standard culture conditions. It may take several days for clumps to attach, so replace the medium carefully when feeding.

III Fibroblast Contamination

There are several techniques have been published in the literature to deal with fibroblast contamination during colorectal primary cell culture. These include: (1) Physically remove a well-isolated fibroblasts by scraping it with a sterile blunt instrument (e.g., a cell scraper). Care has to be taken to wash the culture up to six times to remove any fibroblasts that have detached in order to prevent them from reseeding and reattaching to the flask. (2) Differential trypsinization can be attempted with the carcinomas. (3) Dispase preferentially (but not exclusively) removes the epithelium during passaging and leaves behind most of the fibroblastic cells attached to the culture vessel. During subculture, cells that have been removed with dispase can be preincubated in plastic Petri dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the epithelium. Clumps of epithelial cells still floating can be transferred to new flasks under standard culture conditions. This technique takes advantage of the fact that fibroblasts in general attach much more quickly to plastic than do clumps of epithelial

cells, so that a partial purification step is possible. (4) Reduce the concentration of serum to about 2.5-5% if there are heavy concentrations of fibroblastic cells. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and that using any or all of the preceding techniques will eventually push the cells through so many divisions that any fibroblasts will senesce.

Human Breast PrimaCell™ includes a fibroblast elimination system, the Human Breast Fibroblast Growth Inhibitors, Breast FibrOut™. It contains a mixture of cis-OH-proline, toxin ricin, gentamycin and formulated serum substitutes. This proprietary mixture kills contaminating fibroblasts, but shows no signs of toxicity toward the epithelium, whether derived from an adenoma, a carcinoma or normal Breast tissues.

IV References:

1. Blatchford DR, Quarrie LH, Tonner E, McCarthy C, Flint DJ, Wilde CJ. *Influence of microenvironment on mammary epithelial cell survival in primary culture.* J Cell Physiol. 1999 Nov; 181(2):304-11.
2. Ethier SP, Mahacek ML, Gullick WJ, Frank TS, Weber BL. *Differential isolation of normal luminal mammary epithelial cells and breast cancer cells from primary and metastatic sites using selective media.* Cancer Res. 1993 Feb 1; 53(3):627-35.
3. Michaelson JS, Cho S, Browning B, Zheng TS, Lincecum JM, Wang MZ, Hsu YM, Burkly LC. *Tweak induces mammary epithelial branching morphogenesis.* Oncogene. 2005 Apr 14;24(16):2613-24.
4. Zhang H, Herbert BS, Pan KH, Shay JW, Cohen SN. *Disparate effects of telomere attrition on gene expression during replicative senescence of human mammary epithelial cells cultured under different conditions.* Oncogene. 2004 Aug 19; 23(37):6193-8.
5. Goepfert TM, McCarthy M, Kittrell FS, Stephens C, Ullrich RL, Brinkley BR, Medina D. *Progesterone facilitates chromosome instability (aneuploidy) in p53 null normal mammary epithelial cells.* FASEB J. 2000 Nov; 14(14):2221-9.
6. Klevjer-Anderson P, Buehring GC. *Effect of hormones on growth rates of malignant and nonmalignant human mammary epithelia in cell culture.* In Vitro. 1980 Jun; 16(6):491-501.

Human Breast PrimaCell™: Mammary Epithelium

Human Breast Primary Cell Culture

Cat No.	Description	Qt.	Price
2-96031	**Human Breast PrimaCell™ system**	**kit**	**$499**
4-26031	Human Breast Tissue Dissociation System, Breast OptiTDS™ (for 500 ml medium)	1 ml	$127
9-46003	Human Mammary Epithelium PrimaCell™ Basal Culture Medium	500 ml	$60
9-36031	Human Mammary Epithelium PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$160
7-66031	Human Breast Fibroblast Growth Inhibitors, Breast FibrOut™	ea	$146
9-86003	Human Breast Tissue Preparation Buffer Set	ea	$90

Human Primary Mammary Epithelium Characterization

6-37011	**Human Mammary Epithelium Primarker™ Kit**	**kit**	**$220**
6-37012	Human Mammary Epithelium Primarker™ antibody set	set	$180
6-37013	Human Mammary Epithelium Primarker™ buffer system	set	$90

Human Cartilage PrimaCell™: Articular Cartilage
(Cat No. 2-96061)

I. General Description

Articular cartilage is a unique tissue where hydrostatic pressure is a significant component of the mechanical loading environment. Articular cartilage is sparsely populated by chondrocytes immobilized in the extracellular matrix. The chondrocytes, or cells of articular cartilage, represent 1% of the matrix volume, are responsible for secreting matrix molecules such as type II collagen and glycosaminoglycan (GAG) to maintain the correct size and mechanical properties of the tissue. Chondrocytes are highly specialized cells of mesenchymal origin that are responsible for synthesis, maintenance, and degradation of the cartilage matrix.Chondrocytes live in an unusual and constantly changing physicochemical environment. They receive signals during the loading of the tissue and produce, through a balance between macromolecular synthesis and degradation, a mechanically resilient extracellular matrix influenced by changes to the intracellular composition, such as cell volume, pH and ionic content. Chondrocytes have a unique biology and thus are widely used for experiments to study the molecular mechanisms implicated in cartilage associated disorders. A great deal of research in the field of rheumatology has been focused on understanding the mechanisms that induce metabolic changes in articular chondrobytes during osteoarthritis and rheumatoid arthritis. While these cells are extremely useful in the laboratory they are notoriously difficult to isolate and culture. They rapidly divide, become fibroblastic, and lose their biochemical characteristics The Human Cartilage PrimaCell™ kit (Cat No. 2-96061) is designed for the successful isolation and culture of chondrocytes from human cartilage allowing reproducible and dependable results.

1.1 Components of the Human Cartilage PrimaCell™ System

- **Human Cartilage Tissue Dissociation System , Cartilage OptiTDS™** (2 × 1 ml) --- *A mixture of collagenase I, collagenase II, Hyaluronidase I, trypsin and Human Cartilage OptiTDS™ Reconstitution Buffer,* (2 × 1 ml).
- **Human Cartilage OptiTDS™ Digestion Buffer,** (2 × 9 ml)
- **Human Cartilage PrimaCell™ Washing Medium** (1 × 100 ml) --- *A modified Ham's F12 with netilmycin, Vancomycin, ceftazidim, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin.*
- **Human Cartilage Fibroblast Growth Inhibitors, Cartilage FibrOut™** ---Cartilage FibrOut™ (5 × 200 μl) --- *A mixture of D-valine, collagenase and gentamycin.*
- **Human Cartilage Carilage Basic Culture Medium** (5 × 100 ml) --- A *Modified formulation based on Ham's F12 medium*.
- **Human Cartilage PrimaCell™ Medium Supplements** (5 × 1 ml): *gentamycin.*
- **Human Cartilage PrimaCell™ Washing Medium** (1 × 100 ml) --- *A modified Ham's F12 with netilmycin, Vancomycin, and ceftazidim.*
- **Human Cartilage PrimaCell™ Serum** (5 × 10 ml) --- *A modified Fetal Calf serum.*
- **Human Cartilage PrimaCell™ Gelation Solution** (5 × 10 ml) --- *A modified mixture of HEPES and $CaCl_2$.*
- **Human PrimaCell™ Solubilization Solution** (4 × 10 ml) --- *A modified mixture of HEPES and EDTA.*
- **Human PrimaCell™ Alginate Solution** (5 × 1 ml) --- *A modified mixture of HEPES Sodium alginateand, and NaCl.*

❖ **Human PrimaCell™ Alginate Washing Solution** (5 × 10 ml) --- *NaCl.*

1.2 Required materials but NOT included:

- DMEM (Invitrogen Cat No. 10313-021)
- Fetal Bovine Serum (FBS, Sigma-Aldrich)
- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or human fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, fresh gelatin-coated plate or culture dishes are recommended (see below for treatment of culture dishes).

2.2 Principle

Among the various methods explored for maintaining the phenotype of chondrocytes, culture in alginate beads appears the most promising, since it has been shown that this culture system leads to the formation of a matrix similar to that of native articular cartilage. The system maintains the expression of the differentiated phenotype and is also able to restore it in dedifferentiated chondrocytes. Another advantage over other three-dimensional methods is that cells can easily be recovered after the culture is completed, allowing protein and gene expression studies.

2.3 Human Cartilage Tissue Preparation

Human Cartilage: Prepare cultures from knee, shoulder, and hip joints. Fetal or young donors are preferable to adults, as they provide higher quantities of cells and take longer to senesce.

1 Wash tissue pieces thoroughly with **Human Cartilage PrimaCell™ Washing Medium** before dissection. Dissection should begin without delay. Remove skin, muscle, and tendons from joints. Carefully take cartilage fragments from articulations that are free of connective tissue.
2 Using crossed scalpels, mince cartilage slices into 1-mm^3 pieces.
3 Transfer cartilage fragments into a 30-ml flat-bottomed vial.
4 Incubate tissue specimens with 5-10 ml 70% ethanol for 1 min, followed by inoculating with 5-10 ml fresh **Human Cartilage PrimaCell™ Dissection Medium** for 5 min.

2.4 Articular carilage Separation and Tissue Dissociation

2.4.1 Human Cartilage OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Human Cartilage Tissue Dissociation System is suited for optimal dissociation of normal newborn human cartilage tissues to yield maximum number of single chondrocyte.

2.4.2 Enzyme Compositions

- Trypsin: from *Bovine Pancreas*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase II: from *Clostridium Histolyticum*
- Hyaluronidase I

2.4.3 System Components

- Human Cartilage Tissue Dissociation System, Cartilage OptiTDS™, (2 × 1 ml).
- Human Cartilage OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

5 Prepare fresh enzyme working solution: Add 1ml of **Human Cartilage Tissue Dissociation System, Cartilage OptiTDS™** to one vial of **Human Cartilage OptiTDS™ Digestion Buffer** (9 ml). Warm the diluted **Human Cartilage Tissue Dissociation System** working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 5 ml **Human Cartilage Tissue Dissociation System** working solution.

6 Discard the **Human Cartilage PrimaCell™ Dissection Medium,** and add 5 – 10 ml of **Human Cartilage Tissue Dissociation System** working solution (prewarmed to 37℃) and incubate the fragments with moderate magnetic agitation for 30-60 min at 37 ℃ in a sealed vial at room temperature.

2.5 Chondrocytes Isolation

Note: Please read section 2.6 for specific information on chondrocytes culture and platting before preceding this section.

7 Transfer the cell suspension into a 50-ml centrifuge tube and mix on a vortex mixer for a few seconds.

8 Remove residual material left after digestion by passing the digested material through a 70-μm nylon filter.

9 Centrifuge the filtrate at 400 g for 10 min.

10 Resuspend the cell pellet in 20 ml of **Human Cartilage PrimaCell™ Dissection Medium**, and count the cells with a hemocytometer.

2.6 Primary chondrocytes Culture

2.6.1 Medium Preparation.

Thaw out **Human Cartilage PrimaCell™ II Basal Culture Medium**, **Human Cartilage PrimaCell™ II Medium Supplements**, and **Human Cartilage Serum** on ice.

Human Complete Cartilage PrimaCell™ II Culture Medium: To every 100 ml **Human Cartilage PrimaCell™ II Basal Culture Medium**, add 1 ml **Human Cartilage PrimaCell™ II Medium Supplements** and 10 ml **Human Cartilage PrimaCell™ II Serum** mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

Human Complete Cartilage PrimaCell™ II Culture Medium/FibrOut: To every 100 ml **Human Cartilage PrimaCell™ II Basal Culture Medium**, add 1 ml **Human Cartilage PrimaCell™ II Medium Supplements** and 10 ml **Human Cartilage Serum**, and 200 µl **Human Cartilage Fibroblast Growth Inhibitors, Cartilage FibrOut™** mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

2.6.2 Primary Cell Culture

(Important: *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*)

11 Centrifuge the cells at 400 g for 10 min.
12 Resuspend the cell pellet in 1 ml of Cartilage PrimaCell™ Alginate Solution, and then dilute the suspension progressively in more Cartilage PrimaCell™ Alginate Solution, until a cellular density of 2×10^6 cells/ml is reached. This progressive dilution is necessary to obtain a homogeneous cell suspension in alginate.
13 Express the cell suspension in drops through a 21 G needle into the Cartilage PrimaCell™ Gelation Solution with moderate magnetic stirring, and allow the alginate to polymerize for 10 min to form beads.
14 Wash the beads 3 times in 5 vol Cartilage PrimaCell™ Alginate Washing Solution.
15 Distribute the beads, 5 ml (1×10^7 cells), into 75-cm^2 flasks containing 20 ml of **Human Complete Cartilage PrimaCell™ Culture Medium/FibrOut**.
16 Incubate the culture at 37˚C in a humidified atmosphere of 5% CO_2, and 95% air.

2.7 Recovery

17 Discard the medium.
18 Add 2 vol of Cartilage PrimaCell™ Solubilization Solution to the beads.
19 Incubate the culture 15 min at 37˚C.
20 Centrifuge the cells at 400 g for 10 min.
21 Resuspend the cell pellet in **Human Complete Cartilage PrimaCell™ Culture Medium/FibrOut** containing 0.06% collagenase.
22 Incubate the cells for 30 min at 37˚C in a humidified atmosphere of 5% CO_2, and 95% air.
23 Centrifuge the cells at 400 g for 10 min.
24 Resuspend the cell pellet in **Human Cartilage PrimaCell™ Basal Culture Medium**, and count the cells with a hemocytometer.
25 Centrifuge the cells at 400 g for 10 min.
26 Repeat steps 28, 29, without counting the cells.

2.8 Subculture

Propagating chondrocytes in culture can be somewhat challenging, especially when chondrocytes are cultured alone on plastic, however the following methods have worked consistently in many laboratories. Remove complete Human Chondrocytes Culture Medium and wash chondrocytes in 0.1% trypsin two times. Place just enough trypsin (0.1 % trypsin without EDTA) to moisten articular carilage layer (200-500 µl depending on size of the dish). Incubate for 1-5 min at 37°C.

27 Remove complete Human Chondrocytes Culture Medium and wash chondrocytes in 0.1% trypsin two times. Place just enough trypsin (0.1 % trypsin without EDTA) to moisten articular carilage layer (200-500 µl depending on size of the dish).
28 Incubate for 1-5 min at 37°C. Gently pipette chondrocytes and resuspend in complete Human Chondrocytes Culture Medium (FibrOut™ is not necessarily needed at this step) for counting and replatting on collagen coated tissue culture plates.
29 Gently pipette chondrocytes and resuspend in **Human Complete Cartilage PrimaCell™ Culture Medium** (FibrOut™ is not necessarily needed at this step) for counting and replatting on tissue culture plates.

III Cryopreservation

30 Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Chondrocytes cultures derived from preconfluent layers.
   (a) Trypsinize cells as above, and centrifuge at 100 g for 10 min.
   (b) Resuspend cells in **Human Complete Cartilage PrimaCell™ Culture Medium** and count cells.
   (c) Prepare aliquots of 2×10^6 cells/ml in **Human Complete Cartilage PrimaCell™ Culture Medium** and 10% glycerol into cryovials.
   (d) Equilibrate at 4℃ for 1-2 h.
   (e) Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
   (f) To recover cells:
      (i) Thaw cryovials quickly in a 37℃ water bath.
      (ii) Dilute cells tenfold with appropriate **Human Complete Cartilage PrimaCell™ Culture Medium**.
      (iii) Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in **Human Complete Cartilage PrimaCell™ Culture Medium**.

IV Characterization

The cells of articular cartilage live in an unusual and constantly changing physicochemical environment. Chondrocytes, the only cells found in cartilage, produce and maintain the cartilaginous matrix. Classic markers of a chondrocytic phenotype are—Sox9, collagen II, and aggrecan. Sox9 is the major regulator of the chondrocytic phenotype. It is a potent promoter of collagen II gene expression, a molecule which is produced almost exclusively in the chondrocyte. The proteoglycan aggrecan is also a characteristic gene product of chondrocytes. Furthermore, there are various markers for differentiation of chondrocytes: 11-fibrau, a reliable and sensitive marker of chondrocyte phenotype, and a useful marker to characterize chondrocyte differentiation stage. Monoclonal antibody 11-fibrau: a useful marker to characterize chondrocyte differentiation

stage.were reported by van Osch GJ, van der Veen SW, Marijnissen WJ, and Verhaar JA; Annexin VI. Cartilage oligomeric matrix protein (COMP), a sensitive marker for the differentiation state of articular primary chondrocytes; Cathepsin B, a marker of the dedifferentiated chondrocyte phenotype; cartilage matrix protein (CMP, or Matrilin-1), a mature chondrocyte marker; Collagen X and Collagen IX; etc. Chondrocyte expressed protein-68 (CEP-68), a member of a new protein family harbouring an EGF-like calcium-binding domain, is a novel human marker gene for cultured chondrocytes. Its expression can be assayed by flow cytometry, northern blotting or real time PCR. In addition, the Chondrocyte expressed protein-68 (CEP-68), a novel human marker gene was shown to be specifically for cultured chondrocytes.

V References:

1. Ikenoue, T., et al., Mechanoregulation of human articular chondrocyte aggrecan and type II collagen expression by intermittent hydrostatic pressure in vitro. J Orthop Res, 2003. 21(1): p. 110-6.
2. Ho, Y.C., et al., Highly efficient baculovirus-mediated gene transfer into rat chondrocytes. Biotechnol Bioeng, 2004. 88(5): p. 643-51.
3. Hauselmann, H.J., et al., Phenotypic stability of bovine articular chondrocytes after long-term culture in alginate beads. J Cell Sci, 1994. 107 (Pt 1): p. 17-27.
4. Hall, A.C., E.R. Horwitz, and R.J. Wilkins, The cellular physiology of articular cartilage. Exp Physiol, 1996. 81(3): p. 535-45.
5. Loeser, R.F., et al., Human chondrocyte expression of growth-arrest-specific gene 6 and the tyrosine kinase receptor axl: potential role in autocrine signaling in cartilage. Arthritis Rheum, 1997. 40(8): p. 1455-65.
6. Loeser, R.F., Jr. and R. Wallin, Vitamin K-dependent carboxylation in articular chondrocytes. Connect Tissue Res, 1991. 26(3): p. 135-44.
7. Magne, D., et al., The new IL-1 family member IL-1F8 stimulates production of inflammatory mediators by synovial fibroblasts and articular chondrocytes. Arthritis Res Ther, 2006. 8(3): p. R80.
8. Lotz, M., I. Clark-Lewis, and V. Ganu, HIV-1 transactivator protein Tat induces proliferation and TGF beta expression in human articular chondrocytes. J Cell Biol, 1994. 124(3): p. 365-71.
9. Guicheux, J., et al., Primary human articular chondrocytes, dedifferentiated chondrocytes, and synoviocytes exhibit differential responsiveness to interleukin-4: correlation with the expression pattern of the common receptor gamma chain. J Cell Physiol, 2002. 192(1): p. 93-101.

Human Cartilage PrimaCell™: Articular Cartilage

Human Cartilage Primary Cell Culture

Cat No.	Description	Qt.	Price
2-96061	**Human Cartilage PrimaCell™ system**	**kit**	**$550**
4-26062	Human Cartilage Tissue Dissociation System, Cartilage OptiTDS™ (for 500 ml medium)	1 ml	$146
9-46006	Human Cartilage PrimaCell™ Basal Culture Medium	500 ml	$73
9-36062	Human Cartilage PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$160
7-66062	Human Cartilage Fibroblast Growth Inhibitors, Cartilage FibrOut™	ea	$195
9-86006	Human Cartilage Tissue Preparation Buffer Set	ea	$90

Human Primary Articular Cartilage Characterization

6-38011	**Human Articular Cartilage Primarker™ Kit**	**kit**	**$220**
6-38012	Human Articular Cartilage Primarker™ antibody set	set	$180
6-38013	Human Articular Cartilage Primarker™ buffer system	set	$90

Human Cervix PrimaCell™: Cervical Epithelium
(Cat No. 2-96071)

I. General Description

The physiologic and pathologic conditions that affect the uterine cervix are of considerable contemporary interest. Human cervical epithelial cells have been cultured for studies on the relationship between human papillomavirus (HPV) and carcinoma of the lower genital tract in females. Primary cervical epithelia are crucial target for pharmacotherapy. While these cells are extremely useful in the laboratory, they are notoriously difficult to isolate and culture.
The Human Cervix PrimaCell™ kit (Cat No. 2- 96071) is designed for the successful isolation and culture of cervical epithelia from human cervix tissue allowing reproducible and dependable results.

1.1 Components of the Human Cervix PrimaCell™ System

- **Human Cervix Tissue Dissociation System, Cervix OptiTDS™** (2 × 1 ml) --- *A mixture of collagenase, collagenase I, collagenase III, collagenase IV, Trypsin, Dexoyribonuclease and Human Cervix OptiTDS™ Reconstitution Buffer.*
- **Human *Cervix OptiTDS™ Digestion Buffer,*** (2 × 9 ml)
- **Human Cervix Fibroblast Growth Inhibitors, Cervix FibrOut™**---Cervix FibrOut™ (5 x 200 µl) --- *A mixture of D-valine, collagenase and gentamycin.*
- **Human Cervical Epithelium PrimaCell™ Basal Culture Medium,** (5 × 100 ml) --- *A Modified Weymouth medium*.
- **Human Cervical Epithelium PrimaCell™ Medium Supplements,** (5 × 1 ml): *Human EGF, Hydrocortisone, Cholera toxin penicillin (100 U/ml), streptomycin and Weymouth medium.*
- **Human Cervix PrimaCell™ Serum,** (50 ml): A modified f*etal bovine serum.*
- **Human Cervix Tissue Washing Medium,** (1 × 100 ml): *A modified DMED medium with 10µg/ml amphotericin,* 10*µg/ml gentamycin, and 10% FCS.*

1.2 Required materials but NOT included:

- DMEM (Invitrogen Cat No. 10313-021)
- Fetal Bovine Serum (FBS, Sigma-Aldrich)
- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or human fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

1.3 Preparation of Swiss 3T3 Fibroblasts (optional):

(a) A large master stock of cells should be prepared and frozen in individual ampules of 1×10^6 cells. Cells should not be used for more than 20 passages.
   (i) Grow 3T3s in DMEM/10% calf serum in 175-cm^2 tissue culture flasks. Inoculate cells at 1.5×10^4 cells/cm^2. Change the medium after 2d. Subculture every 4-5 d.
   (ii) To avoid low-level contamination, maintain one master flask of cells on antibiotic-free medium; these cells are then used at each passage to inoculate the flasks required for that week's feeder cells.

(b) Feeder layers are inactivated by irradiation with 60 Gy (6,000 rad), either from an X-ray or 60Co source. Irradiated cells (XR-3T3) may be kept at 4℃ for 3-4 d.

(c) In the absence of a source of irradiation, inactivate feeder cells with mitomycin C.
   (i) Expose 3T3 cells growing in monolayer to 400 μg/ml of mitomycin C for 1 h at 37℃.
   (ii) Trypsinize the treated cells, resuspend and wash the cell pellet twice with fresh medium with serum, resuspend the cells at a suitable concentration in complete medium, and use.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, fresh gelatin-coated plate or culture dishes are recommended (see below for treatment of culture dishes).

2.2 Principle

Separation of the cervical epithelium from the cervix tissue is accomplished by enzymatic digestion using the Cervix Tissue Dissociation System supplied in this kit. The Cervix Tissue Dissociation System contains a mixture of collagenase, collagenase I, collagenase III, collagenase IV, Trypsin, and Dexoyribonuclease at the optimal concentrations to gently detach the fragile cervix layer from the cervix tissue. The isolated cervix tissue is then further disrupted to release individual cervical epithelium by enzymatic and mechanical agitation. The mixture is then filtered through Cell Strainers and seeded on flasks or specially coated tissue culture plates. The cervical epithelia are propagated in growth arrested feeder cells and the corresponding media. Sub-populations of cervical epithelia can then be isolated based on their selective attachment to specific basement matrix substrates.

2.3 Human Cervix Tissue Preparation

Human Cervix: Most cervical epithelium from cervix is obtained from hysterectomy specimens.

1. The cervical biopsy for culture should be taken by the pathologist and transferred immediately to Human Cervix Tissue Washing Medium. Rinse cervical biopsy two to three times with 5 ml of cold **Human Cervix Tissue Washing Medium**.
2. Place the biopsy, epithelial surface down, on a sterile culture dish.
3. Using surgical blade, cut and scrape away as much of the muscle and stroma as possible, leaving a thin, opaque epithelial strip.
4. Mince the epithelial strip finely with curved iris scissors.
5. Incubate tissue specimens with 5-10 ml 70% ethanol for 1 min, followed by inoculating with 5-10 ml fresh **Human Cervix Tissue Washing Medium** for 5 min.

2.4 Cervix Separation and Tissue Dissociation

2.4.1 Human Cervix OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the donor used, enzymes, culture mediums and growth supplements. The Human Cervix Tissue Dissociation System is suited for optimal dissociation of normal human cervix tissues to yield maximum number of single cervical epithelium.

2.4.2 Enzyme Compositions

- Collagenase: from *Clostridium Histolyticum*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase III: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*
- Trypsin
- Dexoyribonuclease

2.4.3 System Components

- Human Cervix Tissue Dissociation System, Cervix OptiTDS™, (2 × 1 ml).
- Human Cervix OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

6. Prepare fresh enzyme working solution: Add 1ml of the reconstituted tissue dissociation solution to one vial of Human Cervix OptiTDS™ Digestion Buffer (9 ml). Warm the diluted Human Cervix OptiTDS™ working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 5 ml Human Cervix OptiTDS™ working solutions.
7. Discard the **Human Cervix Tissue Washing Medium,** and float cervix samples on 5 – 10 ml of Human Cervix Tissue Dissociation System working solution (prewarmed to 37℃) to the epithelial mince, and transfer the tissue to a sterile glass universal containing a small plastic-coated magnetic stirrer bar.
8. Place the universal on a magnetic stirrer and stir slowly for 30-60 min at 37 ℃. This works particularly well with full-thickness cervix. Monitor the separation of the cervix carefully when using the rapid dissociation method.
9. Allow the suspension to stand at room temperature for 2-3 min
10. Remove the supernatant containing single cells to a 50-ml centrifuge tube, and spin down the cell mixture at 1000 rmp (800g) for 5 min. Collect the supernatant dissociation system working solution which can be reused, and add 10 ml of **Human Complete Cervix PrimaCell™ Culture Medium** to resuspend cells.
11. Add a further 5-10 ml of warm Human Cervix Tissue Dissociation System working solution to the fragments in the universal, and repeat the steps 8-10.

2.6 Cervical Epithelia Isolation

Note: Please read section 2.6 for specific information on Cervical Epithelium culture and platting before preceding this section.

12. Combine the supension containing single cells, and strain the cell mixture through a sterile cell strainer (70-100μm) into a 50-ml centrifuge tube to remove debris. Cell strainers fit perfectly in 50 ml conical and are very convenient for this procedure. If cell strainers are not available, nylon gauze can be used after rinsed twice in PBS and placed at the opening of a 50 ml conical.
13. Centrifuge the strained mixture at 1000 rmp (800 g) for 5 min at 4°C.
14. Remove the supernatant; add 10 ml of complete medium to the pellet, resuspend the cells vigorously to give a single-cell suspension, and count the cells with a hemocytometer. Assess cell viability with trypan blue exclusion.

2.6 Primary Cervical Epithelia Culture

2.6.1 Medium Preparation.

Thaw out **Human Cervix PrimaCell™ Basal Culture Medium**, **Human Cervical Epithelium PrimaCell™ Medium Supplements**, and **Human Cervix PrimaCell™ Serum** on ice.

Human Complete Cervix PrimaCell™ Culture Medium: To every 100 ml **Human Cervix PrimaCell™ Basal Culture Medium**, add 10 ml **Human Cervix PrimaCell™ Medium Supplements** and 1 ml **Human Cervix PrimaCell™ Serum** mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

Human Complete Cervix PrimaCell™ Culture Medium/FibrOut: To every 100 ml **Human Cervix PrimaCell™ Basal Culture Medium**, add 10 ml **Human Cervix PrimaCell™ Medium Supplements,** 1 ml **Human Cervix PrimaCell™ Serum**, and 1 ml **Human Cervix Fibroblast Growth Inhibitors, Cervix FibrOut™**,mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

2.6.2 Primary Cell Culture

(Important: Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination)

15. Primary Cervical Epithelia Primary Cervical Epithelia can be cultured alone or on a layer of feeder cells depending on the nature of the experiments.

    - Cervical Epithelium Culture with feeder layer cells:
        - Prepare feeder layers by culturing 3T3 cells 3 days.When the cells have reached confluence irradiate the 3T3 at 30 Gy
        - Dilue the cervical cell suspension with **Human Complete Cervix PrimaCell™ Culture Medium/FibrOut**, and plate cells out at 2×10^4 cells/cm^2 together with 1×10^5 cells/cm^2 of lethally inactivated 3T3 cells (i.e., for 1×10^5 cervical cells, 5×10^5 XR-3T3/50-mm dish).
        - Incubate the cultures at 37℃ in 5% CO_2.
    - Cervical Epithelium Culture without feeder layer:
        - Spin the suspension at 1,000 rpm (800 g) for 5 min. Remove the supernatant and resuspend the cells in 10 ml of **Human Cervix Tissue Washing Medium**.
        - Spin the suspension again and wash the cells once more with **Human**

Cervix Tissue Washing Medium. Resuspend the cells in 10 ml of **Human Complete Cervix PrimaCell™ Culture Medium/FibrOut**, and seed into culture dishes or flasks at a density of 2 $\times 10^5$ cells/cm2 (10^6 cells/50-mm Petri dish, 4 $\times 10^6$ /90-mm Petri dish).

- Incubate the cultures at 37℃ in 5% CO2

16. Seventy-two hours after the initial platin, replace the medium with a **Human Complete Cervix PrimaCell™ Culture Medium/FibrOut**. Check the cultures microscopically to ensure that the feeder layer is adequate. Add further feeder cells if necessary. Change the **Human Complete Cervix PrimaCell™ Culture Medium/FibrOut** twice weekly; keratinocyte colonies become visible on the microscope by days 9-12 and should be visible to the naked eye by days 14-16. Change **Human Complete Cervix PrimaCell™ Culture Medium/FibrOut** to **Human Complete Skeletal Muscle PrimaCell™ Culture Medium** after 3-5 cycles or an acceptable level of fibroblast cell contamination is observed. Cultures should be subcultured at this time.

Δ ***Safety Note.*** The rest of the biopsy and all tubes, pipettes, plates, etc., used in the procedure should be treated with hypochlorite before disposal.

2.7 Subculture

17. Propagating Cervical Epithelia in culture can be somewhat challenging, especially when Cervical Epithelia are cultured alone on plastic, however the following methods have worked consistently in many laboratories.

(a) Cervical Epithelia grown on a feeder layer:
- (i) Spin the suspension at 1,000 rpm (80 g) for 5 min. Remove the supernatant and resuspend the cells in 10 ml of **Human Cervix Tissue Washing Medium**.
- (ii) Spin the suspension again and wash the cells once more with **Human Cervix Tissue Washing Medium**. Resuspend the cells in **Human Complete Skeletal Muscle PrimaCell™ Culture Medium** and plate them onto culture dishes at 10^5 cells/cm^2 (5×10^5 cells/50-mm Petri dish, 2 $cm^2 \times 10^6$ cells/90-mm Petri dish).
- (iii) Cellsmay also be frozen at this stage for recovery at a later date

(b) Cultures in complete Human Cervical epithelium Culture Medium:
- (i) Remove the medium from the cell layer, and remove the feeders by rinsing rapidly with 0.01% EDTA. Wash twice with **Human Cervix Tissue Washing Medium.**
- (ii) To each culture dish, add enough prewarmed trypsin/EDTA to cover the cell sheet. Leave the cultures at 37℃ until the keratinocytes have detached; check for detachment with a microscope. Do not leave the cells in trypsin for more than 20 min.
- (iii) Remove the cell suspension from the plate and transfer it to a sterile centrifuge tube.
- (iv) Rinse the growth surface with **Human Complete Cervix PrimaCell™ Culture Medium** and add to the suspension. Mix and dispense the suspension with a 10-ml pipette.
- (v) Spin the cells at 1,000 rpm for 5 min, remove the supernatant, add 10 ml of **Human Complete Cervix PrimaCell™ Culture Medium**, and resuspend the cells vigorously with a 10-ml pipette to achieve a single-cell suspension.
- (vi) Count the cells with a hemocytometer.
- (vii)Cells may be replated on inactivated 3T3 cells and grown as just described or

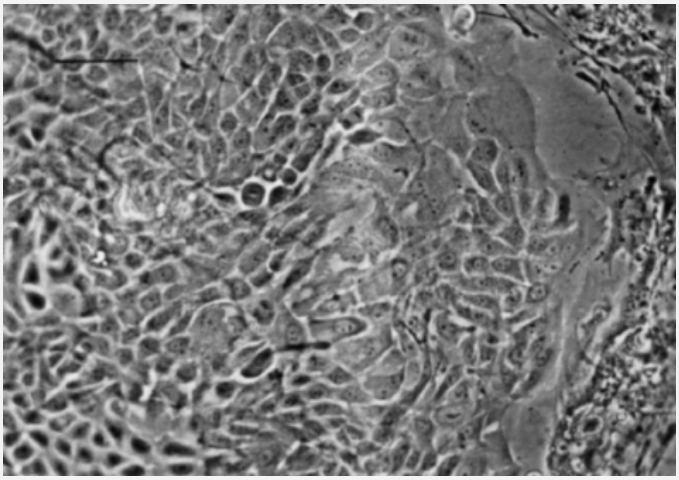
frozen for later recovery.

III Cryopreservation

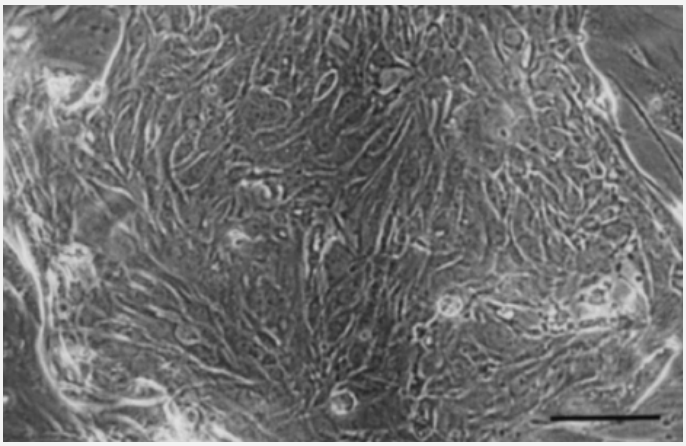
18. Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Cervical Epithelia cultures derived from preconfluent layers.
    (a) Trypsinize cells as above, and centrifuge at 100 g for 10 min.
    (b) Resuspend cells in **Human Complete Cervix PrimaCell™ Culture Medium** and count cells.
    (c) Prepare aliquots of 2 x 10^6 cells/ml in **Human Complete Cervix PrimaCell™ Culture Medium** and 10% glycerol into cryovials.
    (d) Equilibrate at 4℃ for 1-2 h.
    (e) Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
    (f) To recover cells:
        (i) Thaw cryovials quickly in a 37℃ water bath.
        (ii) Dilute cells tenfold with **Human Complete Cervix PrimaCell™ Culture Medium**.
        (iii) Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in **Human Complete Cervix PrimaCell™ Culture Medium**.

IV Characterization

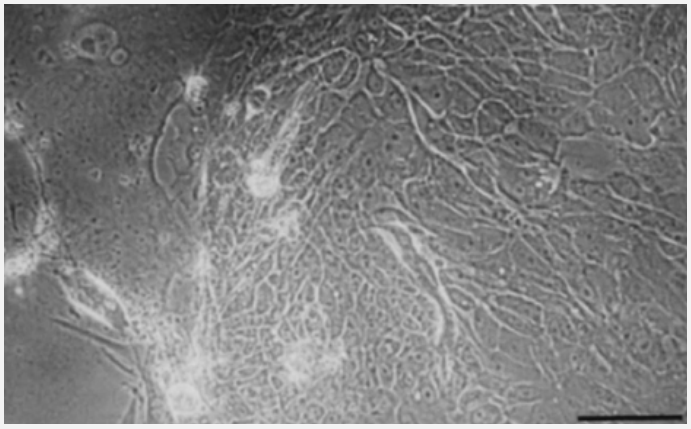
Studies on primary cultures of cervical epithelia have identified differences in morphology and gene expression between cells derived from endocervical and ectocervical regions. However, cervical epithelia have been relatively underinvestigated with regard to their in vitro growth characteristics, immune functions, and susceptibility to toxic agents and mechanisms of infection by pathogens other than HPV. Specific differentiation markers of value for the characterization of cultured cervical keratinocytes include keratin intermediate filaments, involucrin, and intracytoplasmic glycogen. LSCM was used to determine the cytokeratin (such as cytokeratin 4, 13, 15, and 16) expression pattern of the primary cervical cell monolayers with respect to the tissue from which they were derived.



Ectocervical epithelia have a characteristic polygonal morphology and grow in small, tight colonies, the edge of the colony displacing the surrounding 3T3 feeder cells. Mitotic figures are evident. [2, 3]



After 10 days in primary culture, endocervical epithelial cells have a heterogeneous morphology and are frequently elongated with protrusions and extensions[2, 4]



After 24 days in primary culture, endocervical epithelial cells have undergone a change in morphology and have the appearance of small keratinocytes, particularly at the edge of the colonies[2, 4]

V References

1. Fichorova, R.N., J.G. Rheinwald, and D.J. Anderson, *Generation of papillomavirus-immortalized cell lines from normal human ectocervical, endocervical, and vaginal epithelium that maintain expression of tissue-specific differentiation proteins.* Biol Reprod, 1997. **57**(4): p. 847-55.
2. Freshney, R.I.F.a.M.G., *CULTURE OF EPITHELIAL CELLS*, ed. 2. Vol. 5. 2002: Wiley-Liss, Inc.
3. Stanley, M.A. and E.K. Parkinson, *Growth requirements of human cervical epithelial cells in culture.* Int J Cancer, 1979. **24**(4): p. 407-14.
4. Dixon, I.S. and M.A. Stanley, *Immunofluorescent studies of human cervical epithelia in vivo and in vitro using antibodies against specific keratin components.* Mol Biol Med, 1984. **2**(1): p. 37-51.
5. Edwards, J.L., et al., *Neisseria gonorrhoeae elicits membrane ruffling and cytoskeletal rearrangements upon infection of primary human endocervical and ectocervical cells.* Infect Immun, 2000. **68**(9): p. 5354-63.

Human Cervix PrimaCell™: Cervical Epithelium

Human Cervix Primary Cell Culture

Cat No.	Description	Qt.	Price
2-96071	**Human Cervix PrimaCell™ system**	**kit**	**$550**
4-26072	Human Cervix Tissue Dissociation System, Cervix OptiTDS™ (for 500 ml medium)	1 ml	$146
9-46007	Human Cervical Epithelium PrimaCell™ Basal Culture Medium	500 ml	$73
9-36072	Human Cervical Epithelium PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$160
7-66072	Human Cervix Fibroblast Growth Inhibitors, Cervix FibrOut™	ea	$195
9-86007	Human Cervix Tissue Preparation Buffer Set	ea	$90

Human Primary Cervical Epithelium Cell Characterization

6-39011	**Human Cervical Epithelium Primarker™ Kit**	**kit**	**$220**
6-39012	Human Cervical Epithelium Primarker™ antibody set	set	$180
6-39013	Human Cervical Epithelium Primarker™ buffer system	set	$90

Human Colon PrimaCell™: Colorectal Epithelium
(Cat No. 2-96081)

I. General Description:

The Human Colon PrimaCell™ kit (Cat No. 2-9608) is developed to isolate and encourage the growth of normal human colonic epithelial cells derived from 1-3 mm of human biopsy tissues. This kit includes the Colon OptiTDS™ Tissue Dissociation System that provides the optimal enzymatic conditions for isolating viable cells from whole tissue and has been proven to yield 4-7 times more cells than most of the protocols published in the literature. The Human Colon PrimaCell™ kit also contains the optimal media and supplements that ensure the isolated colonic epithelial cells remain viable in tissue culture. The specific growth of colonic epithelial cells is further ensured by including Colon FibrOut™, a proprietary blend of enzymes and chemical reagents that inhibits the growth of aberrant fibroblasts that usually overtake primary cell cultures and crowd out the growth of colonic epithelial cells.

The Human Colon PrimaCell™ kit can be used to isolate primary colonic epithelial cells from normal adult human tissue samples. The results obtained from biopsy samples that may contain pathological organisms (virus, parasites, etc.) or malignancies may not yield optimal results using this system. Tissue specimens used for colon epithelial cells should ideally be used within 1-2 hrs of biopsy and, therefore, tissue removal from the patient. If this is not feasible, the biopsy sample can be cut into small pieces (1-2 mm) and stored at 4° C in washing media (provided in this kit) overnight as described in the protocol below.

1.1 Components of Human Colon PrimaCell™ System

- **Colonic Tissue Dissociation System, Colon OptiTDS™,** (2 aliquots) --- *A* proprietary *mixture of collagenase I, collagenase III, collagenase IV, collagenase, and trypsin*.
- **Colon OptiTDS™ Reconstitution Buffer**, (2 x 1 ml).
- **Colon OptiTDS™ Digestion Buffer**, (2 x 9 ml).
- **Colonic Tissue Washing Medium**, (5 x 10 ml) --- *Basal Colon PrimaCell™ Culture Medium with 5% FBS, 200 u/ml of penicillin, 200 µg/ml of streptomycin, and 50 µg/ml of gentamycin*.
- **Human Colon Fibroblast Growth Inhibitors, Colon FibrOut™** (5 x 200 µl) --- *A* proprietary *mixture of* anti-*Thy-1 monoclonal antibody, D-valine, collagenase, and gentamycin*.
- **Human Colon PrimaCell™ Basal Culture Medium**, (5 x 100 ml) --- *Modified formulation based on NCTC 168 and Weymouth medium*.
- **Human Colon PrimaCell™ Medium Supplements**, (5 x 1.0 ml) --- A mixture of *ethanolamine, phosphoethanolamine, hydrocortisone, ascorbic acid, transferring, insulin, epidermal growth factor, pentagastrin, and deoxycholic acid*.
- **Human Colon PrimaCell™ Serum**, (1 x 50 ml) --- H*eat-inactivated and special-treated Fetal-bovine serum.*
- **Coating Solution**, (5 x 10 ml) --- *Basal growth medium containing 10 µg/ml BSA.*

1.2 Required Materials NOT included in the kit

- 70% sterile ethanol
- Pasteur pipettes, 10 ml pipettes
- Collagen I pre-coated tissue culture dishes
- Scalpels, scissors, and forceps
- Falcon Conicals, 15 and 50 ml

II. Procedures

2.1 Preparation of Tissue Culture Plates

All materials used in this experiment must be sterile to prevent contamination. To enhance cell attachment to tissue culture dishes, collagen I pre-coated plates (Corning, NY) MUST be further pre-treated with the Coating Solution included in this kit for 5 min. Aspirate the Coating Solution and allow the dishes to air-dry in a ventilated cell culture hood for 5-10 min before use.

2.2 Surgical specimens

Once isolated, tissue specimens should be placed immediately in 5-10 ml Colonic Tissue Washing Medium, transported on ice to the laboratory and worked up within 1 hr of isolation. Colon tissue specimens (1-3 mm^3) are further dissected to isolate the mucosal layer away from the muscle layer. With autoclaved scalpels, scissors, and forceps, carefully remove muscle and fat from tissue specimens. Place tissue in a 50 ml conical tube (Falcon) containing 5 ml fresh Colonic Tissue Washing Medium and incubate while shaking at room temperature for 10 min. For large tissue specimens, use a 50 ml conical tube (Falcon) and a larger volume of Colonic Tissue Washing Medium to ensure thorough washing. Aspirate the washing medium and repeat the washing procedures using fresh washing medium two more times. After the initial washes, incubate tissue in 70% ethanol for 1 min at room temperature, followed by incubating in 5-10 ml fresh Colonic Tissue Washing Medium for 5 min. Collect tissue pieces by gentle centrifugation and proceed to dissociation.

2.3 Tissue Preparation and Dissociation

2.3.1 Human Colon OptiTDS™

The isolation of primary cells is confounded by several important factors that can greatly affect yield and cell viability. The Colon Tissue Dissociation System, Colon OptiTDS™, is developed to produce the optimal conditions that allow for the dissociation of colonic epithelial cells from normal adult human tissue samples. This system uses a defined proprietary ratio of specific enzymes to yield the maximum number of single primary cells that remain viable in tissue culture.

2.3.2 Enzyme Compositions

- Collagenase I: from *Clostridium Histolyticum*
- Collagenase III: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*
- Hyaluronidase I: from *Bovine Testes*

2.3.3 System Components

- Colonic Tissue Dissociation System, OptiTDS™: 2 vials

- Colon OptiTDS™ Reconstitution Buffer: (2 x 1 ml)
- Colon OptiTDS™ Digestion Buffer: (2 x 9 ml)

2.3.4 Procedures For Tissue Preparation and Dissociation

15. Add 1.0 ml of the Colon OptiTDS™ Reconstitution Buffer to a vial of Colonic Tissue Dissociation System OptiTDS™ and mix well (Do not Vortex) with hand. It is important that the Tissue Dissociation System OptiTDS is freshly prepared and is provided in aliquots for your convenience.
16. Transfer the 1.0 ml of freshly prepared enzyme solution from step 1, to a vial containing 9.0 ml of Colon OptiTDS™ Digestion Buffer. Warm this enzyme solution at 37 ℃ for 10 min, just prior to use. For optimal results, we recommend a ratio of 100-150 mg tissue per 10 ml of enzyme solution.
17. Mince the pre-washed tissue into pieces measuring approximately 0.2-0.5 mm^2 in diameter with scalpel and forceps or scissors.
18. Incubate the minced tissue with the diluted enzyme solution with gentle stirring for 30 min at 37 ℃. Up to 5 mg of minced tissue can be incubated with 10 ml of the diluted enzyme solution.
19. Gently pipette sample with a 10 ml pipette, filling the barrel of the pipette at a rate of 2-3 ml per second. Repeat this for 5-6 times.
20. Filter the cell mixture through a cell strainer (100μM). Centrifuge the strained mixture at 100 g. Carefully remove the supernatant and resuspend the cell pellet with1.0 ml of Complete Colon Culture Medium (See 2.4.1 for preparation).
21. Count and seed the cells in 3 or 4 T-25 collagen I-coated flasks containing 4 ml of Complete Colon Culture Medium (**Important:** Collagen coated plates must be pre-treated with the provided Coating Solution, see section 2.1). This procedure will give you approximately 2.5-5 x 10^5 Cells/per T-25 flask.

2.3.5 Storage of Tissue Dissociation System:

Colonic Tissue Dissociation System, Colon OptiTDS™ should only be reconstituted when needed for cell preparation and can be stored for 2-4 days at 4 ^{o}C. For long-term use, it should be aliquoted and stored at -20 ^{o}C. Avoid repeated freeze-thaw cycles.

2.4 Culture of primary human colon cells

2.4.1 Medium Preparation

Thaw the Human Colon PrimaCell™ Medium Supplements and Human Colon PrimaCell™ Serum on ice. To prepare Complete Colon Culture Media dilute one vial of Human Colon PrimaCell™ Medium Supplements into 100 ml Human Colon PrimaCell™ Basal Culture Medium. Add 10 ml Human Colon PrimaCell™ Serum and one vial of Colon FibrOut™ (Human Colon Fibroblast Growth Inhibitors). Mix thoroughly and warm the Complete Colon Media in a 37^{o}C water bath for 10 min prior use.

2.6.1 Treatment of Culture Dishes

To facilitate primary colon cell attachment to the tissue culture plates, the plates should be pre-coated with collagen I (available from Corning, NY) and MUST be pre-treated with the provided Coating Solution (Basal Growth Medium containing 10 μg/ml BSA). Cover the entire surface

area of tissue culture plates with the Coating Solution and incubate for 5 min. Aspirate the Coating Solution and allow the dishes to air-dry in a ventilated cell culture hood for 5-10 min prior to use.

2.6.1 Standard primary culture conditions

Seed epithelial tubules and cell clumps isolated from colon tissues (from 2.3.4) into prepared T-25 flasks containing 4 ml of complete media and incubate a 37° C, 5%-CO_2 incubator. The Complete Colon Culture Medium should be changed twice a week. The tubules and cells will attach to the matrix on the plates and the colon epithelial cells will begin to migrate out within 1-2 days. Most of the tubules and cell clumps of epithelium will attach within 7 days, however, larger organoids can take up to 6 weeks to attach. It is important to note that the cells remain viable during this time. (**Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*).

2.6.1 Alternative Primary Culture conditions

Primary colon epithelium requires a substratum to efficiently attach during tissue culture propagation. While this can be achieved using pre-coated Collagen I plates, the growth of primary colon epithelium is significantly better when a layer of 3T3 feeder cells are used. As the initial colon epithelial colonies expand to several hundred cells per colony, the cells become less dependent on the 3T3 feeder cells, and can be cultured without the feeder cells. All media and solutions supplied in this kit can be used whether cells are grown on collagen coated plates or 3T3 feeder cells.

2.4.5 Subculture and Propagation

Most colon primary cells cannot be passaged by routine procedures using trypsin/EDTA. Colon epithelial cells can be de-attached using a mild dissociation enzyme, dispase as this treatment leads to more successful passaging. A further advantage of using dispase to passage cells is that dispase can only detach epithelial cell but not fibroblast. Thus this property decreases fibroblast contamination of the colon epithelial cells with each successive passage.

8. Add 0.5% Dispase (Sigma, w/v) to the cell monolayer. Use just enough dispase to cover the cells (~2.5 ml/25-cm^2 flask), and incubate for 40-60 min for primary colon cultures and 20-40 min for primary colon cell lines.
9. Once the epithelial layers begin to detach (they do so as sheets rather than single cells), pipette to encourage the detachment and dis-aggregation into smaller clumps.
10. Wash and replate the cells under standard culture conditions. It may take several days for clumps to attach, so take special care when changing the media and feeding the cells.

III Fibroblast Contamination

Several techniques have been published that help prevent fibroblast contamination of primary colon cell cultures. These include: (1) Physically removing isolated fibroblast colonies by scraping off the fibroblast colonies with a sterile cell scraper. This method requires extensive washing to ensure that all the lifted fibroblast that remain are washed away and not allowed to reattach and repopulate the primary colon cultures. (2) Differential trypsinization can be attempted with the carcinomas but may not work well with normal primary colon cells. (3) Using dispase to lift off colon epithelial cells preferentially but not exclusively during routine passaging.

In addition, cells can be seeded on mock plates for 4-6 hrs so that the fibroblasts attach, and floating colon cells can be transferred to collagen coated plates under standard culture conditions. This technique takes advantage of the fact that fibroblasts in general attach much more quickly to plastic than do clumps of epithelial cells. (4) Reducing the concentration of serum to about 2.5-5% to eliminate heavy concentrations of fibroblastic cells. The above methods can be used in combination knowing that normal primary fibroblasts have a finite growth span *in vitro* and if these measures are used persistently the fibroblasts will eventually be forced into senesce.
Important Note: The Human Colon PrimaCell™ kit includes a fibroblast elimination system Colon FibrOut™, an effective mixture of Human Colon Fibroblast Growth Inhibitors. It contains a mixture of anti-Thy-1 monoclonal antibody, toxin ricin and formulated serum substitutes. The principle behind the using this system is that Thy-1 antigen is present only on colorectal fibroblasts, but not colorectal epithelial cells. Using this approach, therefore, fibroblasts are eliminated without added toxicity to epithelial cells.

IV References:

1. Youngman KR, Simon PL, West GA, Cominelli F, Rachmilewitz D, Klein JS, Fiocchi C: *Localisation of intestinal interleukin 1 activity and protein and gene expression to lamina propria cells.* Gastroenterology 1993, 104:749-758.
2. Gibson PR, van de Pol E, Maxwell LE, Gabriel A, Doe WF: *Isolation of colonic crypts that maintain structural and metabolic viability in vitro.* Gastroenterology 1989, 96:283-291.
3. Whitehead RH, Brown A, Bhathel PS: *A method for the isolation and culture of human colonic crypts in collagen gels.* In Vitro 1986, 23:436-442.
4. Knoll N, Weise A, Claussen U, Sendt W, Marian B, Glei M, Pool-Zobel BL. *2-Dodecylcyclobutanone, a radiolytic product of palmitic acid, is genotoxic in primary human colon cells and in cells from preneoplastic lesions.*
5. Buset M, Winawer S, Friedman E. *Defining conditions to promote the attachment of adult human colonic epithelial cells.*

Human Colon PrimaCell™: Colorectal Epithelium

Human Colon Primary Cell Culture

Cat No.	Description	Qt.	Price
2-96081	**Human Colon PrimaCell™ system**	**kit**	**$550**
4-26082	Human Colon Tissue Dissociation System, Colon OptiTDS™ (for 500 ml medium)	1 ml	$146
9-46008	Human Colorectal Epithelium PrimaCell™ Basal Culture Medium	500 ml	$73
9-36082	Human Colorectal Epithelium PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$160
7-66082	Human Colon Fibroblast Growth Inhibitors, Colon FibrOut™	ea	$195
9-86008	Human Colon Tissue Preparation Buffer Set	ea	$90

Human Primary Colorectal Epithelium Characterization

6-31011	**Human Colorectal Epithelium Primarker™ Kit**	**kit**	**$220**
6-31012	Human Colorectal Epithelium Primarker™ antibody set	set	$180
6-31013	Human Colorectal Epithelium Primarker™ buffer system	set	$90

Human Endothelium PrimaCell™: Vascular Endothelial Cells
(Cat No. 2-96111)

I. General Description:

The Human Endothelium PrimaCell kit (Cat No. 2-92111) allows the isolation and tissue culture growth of normal human vascular endothelial cells from the human vascular endothelial tissues. This system provides the optimal conditions for endothelial cell culture and yields 5-7 times more cells than most available protocols published in the literature. In addition, this system ensures high cell viability and a pure endothelial cell population that is obtained using CHI's proprietary fibroblast inhibitory system, FibrOut™.

Endothelial cells exist in a single cell layer lining the inner surface of all blood vessels. Historically, the vessels most commonly used to obtain cultured endothelial cells are derived from bovine and human aorta, bovine adrenal capillaries, rat and human brain capillaries, human umbilical veins, and human dermal and adipose capillaries. Although all endothelia share some common properties, significant differences exist between the endothelial cells of large and small blood vessels.

The Human Vascular Endothelial PrimaCell™ kit can be used to isolate most types of endothelial cells from mice ranging from E16 through 3 weeks. E20-22 or 2-3 weeks are only recommended for convenience and obtaining a maximum yield. The preparation of aorta, capillaries or blood vessels for cell culture is usually started within 1-2 h of removal from the animal. If this is impossible, cut the vessels into fine pieces of about 10-15 mm and store overnight at 4° C in washing medium (see below). Endothelial samples containing pathological organism (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Human Vascular Endothelial PrimaCell™ System

- **Vascular Endothelial Tissue Dissociation System, Vascular Endothelial OptiTDS™, (**2 aliquots) --- *A proprietary mixture of Collagenase, Collagenase I, Collagenase IV, Elastase, Dispase, Trypsin, Soybean Trysin Inhibitor*
- **Vascular Endothelial Tissue OptiTDS™ Reconstitution Buffer, (**2 x 1 ml)
- **Vascular Endothelial Tissue OptiTDS™ Digestion Buffer, (**2 x 9 ml)
- **Vascular Endothelial Tissue Washing Medium**, (1 x 100 ml) --- *Basal Vascular Endothelial PrimaCell™ Culture Medium with 5% FBS, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Human Vascular Endothelial Fibroblast Growth Inhibitors, Vascular Endothelial FibrOut™, (**5 x 200 μl) --- *A proprietary mixture of cis-OH-proline, collagenase, D-valine, and special serum substitutes*.
- **Human Vascular Endothelial PrimaCell™ Basal Culture Medium,** (5 x100 ml) --- *Modified formulation based on medium 199 and DMEM medium*.
- **Human Vascular Endothelial PrimaCell™ Medium Supplements with Serum,** (5 x 10 ml) --- *A mixture of EGF, VEGF and Heparin, and CHI's proprietary human serum extracts*.
- **Vascular Endothelial PrimaCell™ I Culture Dish Coating Solution,** (5 x 10 ml) --

- 1.5% gelatin solution, 0.5% bovine serum albumin in PBS.

1.2 Required Materials NOT provided

- 70% sterile ethanol
- Tissue culture dishes
- Pasteur pipettes and 10-ml pipettes
- Falcon Conicals: 15 and 50 ml
- Two clamps or hemostats, 25 mm
- Sharp scissors, 50 mm

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, fresh gelatin-coated plate or culture dishes are recommended (see below for treatment of culture dishes).

2.2 Treatment of Culture Dishes

1. Tissue culture ware used to seed endothelial cells has to be pretreated using Vascular Endothelial PrimaCell™ I Culture Dish Coating Solution.
2. Incubate the plates or dishes with 2-3 ml (enough to cover the medium reaching areas) Vascular Endothelial PrimaCell™ I Culture Dish Coating Solution overnight at room temperature in the tissue culture hood.
3. Remove the Vascular Endothelial PrimaCell™ I Culture Dish Coating Solution (without washing the surface), immediately add complete Human Vascular Endothelial PrimaCell™ Medium (see 2.4.1 for Medium Preparation) and incubate the medium until the cells are ready for platting.

(Note: *Pre-coated gelatin plates or dishes must be used within 72 after preparation.*)

2.3 Vascular Endothelial Tissue Preparation

4. Mice at ages of E20-22 mice or 2-3 weeks are recommended for convenient of procedures and yielding maximum amount of viable target cells. Mice are sacrificed by CO_2 narcosis.
5. Aseptically isolated blood vessels, preferably in 10-15 mm sections, approximately 5 mm in diameter. If asepsis cannot be guaranteed, clamp both ends of the blood vessel.
6. Incubate blood vessels for up to 10 min in Vascular Endothelial Tissue Washing Medium to prevent infection. (This procedure will not affect endothelial cells viability.)
7. Incubate tissues in 10 ml 70% sterile ethanol for 30 sec.
8. Rinse tissue twice in Vascular Endothelial Tissue Washing Medium for 5 min each and kept on ice.

2.3 Tissue Dissociation

2.3.1 Human Vascular Endothelial OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Human Vascular Endothelial Tissue Dissociation System, OptiTDS™, is suited for optimal dissociation of normal adult Vascular Endothelial tissues to yield maximum number of single endothelial cells.

2.3.2 Enzyme Compositions

- Dispase: from *Bacillus polymyxa*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase II: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*

2.3.3 System Components

- Vascular Endothelial Tissue Dissociation System, OptiTDS™, 2 vials.
- Vascular Endothelial OptiTDS™ Reconstitution Buffer, (2 x 1 ml).
- Vascular Endothelial OptiTDS™ Digestion Buffer, (2 x 9 ml).

2.3.4 Procedures For Tissue Preparation and Dissociation

9. Add 1.0 ml of Vascular Endothelial Tissue Dissociation System, OptiTDS™ to one vial of Vascular Endothelial OptiTDS™ Digestion Buffer (9.0 ml). Warm the diluted Vascular Endothelial Tissue OptiTDS™ digestion solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 10 ml diluted Vascular Endothelial OptiTDS™ working solutions.
10. Ligate one end of a 10-cm section of blood vessel 2-10 mm in diameter to a 5-ml plastic syringe.
11. Run the Vascular Endothelial Tissue OptiTDS™ digestion solution through the blood vessel until it appears at the bottom end, clamp that end with a hemostat, and incubate the vessel containing the fresh enzyme working solution at room temperature for 30 min with rocking.
12. Cut the vessel above the clamp with sharp scissors, and collect the Vascular Endothelial Tissue OptiTDS™ digestion solution in a 10-cm Petri dish.
13. Rinse the lumen of the vessel with 10 ml of Vascular Endothelial Tissue Washing Medium, and add this to the Vascular Endothelial Tissue OptiTDS™ digestion solution collected from step 4.
14. Repeat procedure 3-5 until finish all blood vessels and collecting all enzymes digestion solution containing cells.
15. Collect cells by centrifugation at 350 g, washing cells with 10 ml PBS or complete culture medium twice. At the end of washing process, collect cells and dilute cell in 0.5-1.0 ml Complete Human Vascular Endothelial PrimaCell™ I Culture Medium (see 2.4.1 for Medium Preparation).
16. Count viable cells.
17. Seed cells at 37℃ in Complete Human Vascular Endothelial PrimaCell™ I Culture Medium at desired densities (see 2.4.1 for Medium Preparation).

Alternative Method: The vessels can be cut along the length to reveal the inner layer of endothelial cells. The vessels can be attached to a Styrofoam board with the interior of the vessel facing up. Then the entire board revealing the exposed vessel lumen can be inverted

into the Vascular Endothelial Tissue OptiTDS™ digestion solution to dissociate the endothelial cells.

2.3.5 Storage:

Diluting tissue dissociation systems should occur before use and can only be stored for 2-4 days at 4 ^{o}C. For long-term use, it should be aliquoted and stored at -20 ^{o}C. Avoid repeated freeze-thaw cycles.

2.4 Culture of Vascular Endothelial Cells

2.4.1 Medium Preparation.

Thaw out the Vascular Endothelial PrimaCell™ Medium Supplements with Serum on ice. To every 100 ml Human Vascular Endothelial PrimaCell™ Basal Culture Medium, add 10 ml of Vascular Endothelial PrimaCell™ Medium Supplements with Serum; and one vial of Human Vascular Endothelial Fibroblast Growth Inhibitors, Vascular Endothelial FibrOut™, mix thoroughly and warm the Complete Vascular Endothelial Media in a 37^{o} C water bath for 10 min prior use. (**Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*).

2.6.1 Primary Culture Protocols.

18. Resuspend the final pellet collected from last step described in 2.3.4 in Complete Vascular Endothelial Media, and seed the cells into fresh prepared gelatin-coated dishes or flasks, with approximately 5 x 10^5 cells derived from one 10-15 cm section of blood vessel, 5 mm in diameter, per 72-cm flask or 10-cm-diateter dish.
19. Subculture by conventional trypsinization method.

2.4.3 Subculture and Propagation

20. Gently rinse the culture dish twice with 1X PBS.
21. Add 3 ml of 0.25% trypsin/0.1% (2.5 mM) EDTA, and incubate at 37℃. Examine the dish under phase microscopy every 5 min to detect cell detachment.
22. When most cells have detached, add 10 ml Complete Vascular Endothelial Media to inactive the trypsin activity.
23. Pipette the contents of the dish to ensure complete endothelial cell detachment.
24. Aspirate and centrifuge the cells for 5 min at 350 g.
25. Aspirate the supernatant, resuspend the cells in a complete growth medium, and re-plate at 2-4 × 10^5 cells per 100-mm dish.
26. Feed the culture twice a week with Complete Vascular Endothelial Media.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample; the best results are reported when cells from preconfluent primary cultures are used.

27. Detach cells as for the subculture, and centrifuge at 350 g for 10 min.
28. Resuspend cells in complete culture medium with serum, and count.

29. Dispense aliquots of 2 x 10^6 cells/ml in Complete Vascular Endothelial Media with additional 15% FCS and 10% glycerol into cryopreservation tubes.
30. Equilibrate at 4℃ for 1-2 h.
31. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
32. To recover cells:
    (i) Thaw cryotubes quickly in a 37℃ water bath.
    (ii) Dilute cells tenfold with medium.
    (iii) Centrifuge cells and resuspend them at an appropriate concentration in the desired culture medium, and seed culture vessel.

Human Vascular Endothelial cells can be grown in all media for 4-6 weeks and can be subcultured only 4-5 times.

IV Fibroblast Contamination

Human Vascular Endothelial PrimaCell™ system includes a fibroblast elimination system, the Human Vascular Endothelial Fibroblast Growth Inhibitors, Vascular Endothelial FibrOut™. It contains a mixture of cis-OH-proline, collagenase, D-valine, and formulated serum substitutes. This system can effectively eliminate Vascular Endothelial fibroblast contamination while has not affect on the behavior of endothelial cells. (**Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*).

V Confirmation of Vascular Endothelial Cells

Vascular endothelial cells are typically identified by the production of factor VIII, angiotensin-converting, the uptake of acetylated low-density lipoprotein, the presence of Weibel-Palade bodies, and the expression of endothelial-specific cell surface antigens.

VI References:

1. Zetter BR. The endothelial cells of large and small blood vessels. Diabetes. 1981;30(Suppl 2):24-8.
2. Kern PA, Knedler A, Eckel RH. *Isolation and culture of microvascular Vascular Endothelial from human adipose tissue. J Clin Invest. 1983 Jun;71(6):1822-9.*
3. Davison PM, Bensch K, Karasek MA. *Isolation and long-term serial cultivation of endothelial cells from the microvessels of the adult human dermis. In Vitro. 1983 Dec;19(12):937-45.*
4. Jaffe EA, Nachman RL, Becker CG, Minick CR. *Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest. 1973 Nov;52(11):2745-56*
5. Folkman J, Haudenschild CC, Zetter BR. *Long-term culture of capillary endothelial cells. Proc Natl Acad Sci U S A. 1979 Oct;76(10):5217-21.*
6. Bowman PD, Betz AL, Ar D, Wolinsky JS, Penney JB, Shivers RR, Goldstein GW. *Primary culture of capillary Vascular Endothelial from rat brain. In Vitro. 1981 Apr;17(4):353-62.*
7. Booyse FM, Sedlak BJ, Rafelson ME Jr. *Culture of arterial endothelial cells: characterization and growth of bovine aortic cells.* Thromb Diath Haemorrh. 1975 Dec

15;34(3):825-39.
8. Kobayashi M, Inoue K, Warabi E, Minami T, Kodama T. *A simple method of isolating human aortic endothelial cells.* J Atheroscler Thromb. 2005;12(3):138-42.

Human Endothelium PrimaCell™: Vascular Endothelial Cells

Human Endothelium Primary Cell Culture

Cat No.	Description	Qt.	Price
2-96111	**Human Endothelium PrimaCell™ system**	**kit**	**$ 550**
4-26112	Human Vascular Endothelial Tissue Dissociation System, Vascular Endothelial OptiTDS™ (for 500 ml medium)	1 ml	$ 146
9-46011	Human Vascular Endothelial PrimaCell™ Basal Culture Medium	500 ml	$ 85
9-36112	Human Vascular Endothelial PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$ 160
7-66112	Human Vascular Endothelial Fibroblast Growth Inhibitors, Vascular Endothelial FibrOut™	ea	$ 195
9-86009	Human Endothelium Tissue Preparation Buffer Set	ea	$90

Human Primary Vascular Endothelium Cell Characterization

6-31111	**Human Vascular Endothelium Primarker™ Kit**	**kit**	**$220**
6-31112	Human Vascular Endothelium Primarker™ antibody set	set	$180
6-31113	Human Vascular Endothelium Primarker™ buffer system	set	$90

Human Eye PrimaCell™: Corneal Epithelial Cells
(Cat No. 2-96091)

I. General Description:

This protocol is developed for attachment and growth of normal human corneal epithelial cells from adult human eye with Human Eye PrimaCell™ system (Cat No. 2-96091). This system provides an optimal condition of tissue dissociation system, Human Eye OptiTDS™ that yields 6-7 times of single cells more than most of the tissue dissociation protocols published in the literature. In addition, this system ensures a high viability of the target cells with improved gradient contained in the culture medium. With CHI's proprietary fibroblast inhibitory system, FibrOut™, cells are growing with contamination of minimized amount of the non-epithelial cells.

Human Eye PrimaCell™ system applies to all type tissue samples from human at all age though adult tissue samples are recommended for yielding maximum amount of viable target cells. However, tissue samples contain pathological organism (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Human Eye PrimaCell™ System

- **Human Eye Tissue Dissociation System, Eye OptiTDS™,** (2 x 1 ml) --- *A mixture of Collagenase II, Collagenase III, Collagenase IV, Elastase, Hyaluronidase I, Trypsin, Papain.*
- **Human Eye OptiTDS™ Digestion Buffer**, (2 x 9 ml).
- **Human Eye Tissue Washing Medium**, (1 x 100 ml) --- *Corneal Epithelial Cells PrimaCell™ Basal Culture Medium with 5% FBS, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Human Eye Fibroblast Growth Inhibitors, Eye FibrOut™** (5 x 200 μl) --- *A mixture of cis-OH-proline, collagenase, D-valine, and formulated serum substitutes*.
- **Human Corneal Epithelial Cells PrimaCell™ Basal Culture Medium**, (5 x100 ml) --- *Modified formulation based on Weymouth medium*.
- **Human Corneal Epithelial Cells PrimaCell™ Medium Supplements**, (5 x 1.0 ml) --- A mixture of *Human EGF, Insulin, Hydrocortisone, bovine pituitary extract, penicillin, and streptomycin*.
- **Human Eye PrimaCell™ Serum**, (5 x 10 ml) --- *Highly purified special-treated Fetal-bovine serum.*

1.2 Required Materials but not provided

- PBS containing 5% sterilized BSA
- 70% sterile ethanol
- Trypsin-EDTA: Trypsin, 0.05%, EDTA, 0.5 mM
- Rat-tail collagen, type I-coated 6-well plate, (Becton Dickinson)
- Fibronectin-collagen-coated (FNC) culture dishes, 60 mm and 100 mm (Becton Dickinson)
- Scalpels No. 1621 (Becton Dickinson)

- Pipettes (10 and 25 ml)
- Gloves sterilized with autoclave (human tissue can be contaminated with biologically hazardous agents)
- Controlled atmosphere chamber
- Phase-contrast inverted microscope

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, culture plate (Corning, NY) must be coated with the provided coating solution.

2.2 Surgical specimens

- Carefully remove eye tissue from donor in a way that is minimized damages to the whole corneal area.
- Incubate the eye tissue sequentially in 70% sterile ethanol for 1 min; in Eye Tissue Washing Medium for 10 min. (This procedure will not affect cell viability.)
- Carefully separate the corneas from connective tissues.
- Place corneas epithelial side up on a sterile surface (e.g., a regular cell culture dish), and cut them into 12 triangular shaped wedges, using a single cut of the scalpel and avoiding any sawing motion.

2.3 Tissue Preparation and Dissociation

2.3.1 Human Eye OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Human Eye Tissue Dissociation System, OptiTDS™, is suited for optimal dissociation of normal adult cornea samples to yield maximum number of single primary cells of eye tissues.

2.3.2 Enzyme Compositions

- Collagenase II: from *Clostridium Histolyticum*
- Collagenase III: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*
- Elastase: from *Porcine Pancreas*
- Hyaluronidase I: from *Bovine Testes*
- Trypsin: from *Bovine Pancreas*
- Papain: from *Bovine Testes*

2.3.3 System Components

- Human Eye Tissue Dissociation System, OptiTDS™, (2 x 1 ml)
- Human Eye OptiTDS™ Digestion Buffer, (2 x 9 ml).

2.3.4 Procedures For Tissue Preparation and Dissociation

1. Prepare Human Eye OptiTDS™ Digestion solution: Add 1.0 ml of Human Eye Tissue Dissociation System, OptiTDS™ to one vial of Human Eye OptiTDS™ Digestion Buffer (9.0 ml).
2. Prepare complete tissue dissociation solution: Mix equal volume of Human Eye OptiTDS™ Digestion solution and Human Corneal Epithelial Cells PrimaCell™ Basal Culture Medium (without serum, the kit supplied with extra 50 ml basal culture medium).
3. Warm the complete tissue dissociation solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 10 ml complete tissue dissociation solution.
4. Turn each corneal segment epithelial side down, and place four segments in each well of a six-well tray (precoated with rat-tail collagen, type I, Biocoat, Becton Dickinson).
5. Press each segment down gently with forceps to ensure good contact between the tissue and the tissue culture surface. Allow the tissue to dry for 20 min.
6. Place one drop of complete tissue dissociation solution from step 3 carefully upon each segment. The solution should be applied to fully cover but not to spread over the cornea segments.
7. Incubate the culture for 30 min at 37℃ in 5% CO_2.

2.3.5 Storage:

Reconstituting tissue dissociation solution should occur before use and can only be stored for 2-4 days at 4 ^{o}C. For long-term use, it should be aliquoted and stored at -20 ^{o}C. Avoid repeated freeze-thaw cycles.

2.4 Culture of Primary Human Keratinocytes

2.4.1 Medium Preparation.

Thaw out the Human Corneal Epithelial Cells PrimaCell™ Medium Supplements and one vial of Human Eye Fibroblast Growth Inhibitors, Human Eye FibrOut™ on ice. To every 100 ml Human Corneal Epithelial Cells PrimaCell™ Basal Culture Medium, add one vial of Human Corneal Epithelial Cells PrimaCell™ Medium Supplements; and one vial of Human Eye Fibroblast Growth Inhibitors, Human Eye FibrOut™, mix thoroughly and warm the complete culture medium at a 37^{o}C water bath for 10 min prior use. (**Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*).

2.6.1 Primary Culture Conditions.

8. At the end of 30 min incubation, carefully remove the complete tissue dissociation solution remained on each segment by pipetting.
9. Add 1.5 ml of complete culture medium to each well and continue culturing for overnight at 37℃ in 5% CO_2.
10. Next day, exam cells under microscope. When cells are observed to emigrate only from the limbal region of the cornea. Asparate medium and washing cell once with

1X PBS.

11. Carefully remove the tissue segment with forceps and add 3 ml of complete culture medium. After removal of the donor tissue, adherent cells remained in the plate continuing to proliferate, and within 2 weeks from the time of establishment of the culture, confluent monolayers form, displaying the typical cobblestone morphology associated with epithelia. The yield is approximately 7.5-8 $\times 10^6$ cells/cornea.

2.4.3 Subculture and Propagation

12. Following the initial outgrowth period, feed the cultures twice per week.
13. At 70-80% confluence, rinse the cells in Dulbecco's phosphate-buffered saline (PBS), and release with trypsin/EDTA (0.05% trypsin, 0.53 mM EDTA) for 4 min at 37℃.
14. Stop the reaction with 10% FBS in PBS.
15. Wash the cells (centrifugation followed by resuspension in complete culture medium), count them, and plate at 1×10^4 cells/cm^2 onto tissue culture surfaces coated with FNC.
16. Incubate the culture at 37℃ in 95% air and 5% CO_2.
17. Exchange the culture medium with fresh complete culture medium 1 d after trypsinization and reseeding. Immediately after passage, cells appear more spindle shaped, are refractile, and are highly migratory. Within 6-8 d, control cultures become 70-80% confluent, continue to display a cobblestone morphology, and, if allowed to become postconfluent, retain the ability to stratify in discrete areas.
18. Although corneal epithelial cultures can be subcultured up to five times (approximately 9-10 population doublings), most of the proliferation occurs between the first and third passages. Approximate yields are 2.5-3.0 $\times 10^6$ cells/cornea. Senescence always ensues by P5.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample; the best results are reported when cells from preconfluent primary cultures are used.

19. Detach cells as for the subculture, and centrifuge at 100 g for 10 min.
20. Resuspend cells in complete culture medium with serum, and count.
21. Dispense aliquots of 2 x 10^6 cells/ml in complete growth medium with additional 10% FBS and 10% glycerol into cryopreservation tubes.
22. Equilibrate at 4℃ for 1-2 h.
23. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
24. To recover cells:
    (i) Thaw cryotubes quickly in a 37℃ water bath.
    (ii) Dilute cells tenfold with medium.
    (iii) Centrifuge cells and resuspend them at an appropriate concentration in the desired culture medium, and seed culture vessel.

Human corneal epithelial cells can be grown in the complete culture media for 6-8 weeks and can be subcultured only 5-7 times.

IV Fibroblast Contamination

There are several techniques have been published in the literature to deal with fibroblast contamination during cornea primary cell culture. These include: (1) Physically remove a well-isolated fibroblast colony by scraping it with a sterile blunt instrument (e.g., a cell scraper). Care has to be taken to wash the culture up to six times to remove any fibroblasts that have detached in order to prevent them from reseeding and reattaching to the flask. (2) Differential trypsinization can be attempted with the carcinomas. (3) Dispase preferentially (but not exclusively) removes the epithelium during passaging and leaves behind most of the fibroblastic cells attached to the culture vessel. During subculture, cells that have been removed with dispase can be preincubated in plastic petri dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the epithelium. This technique takes advantage of the fact that fibroblasts in general attach much more quickly to plastic than do clumps of epithelial cells, so that a partial purification step is possible. (4) Reduce the concentration of serum to about 2.5-5% if there are heavy concentrations of fibroblastic cells. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and that using any or all of the preceding techniques will eventually push the cells through so many divisions that any fibroblasts will senesce.

Human Eye PrimaCell™ system includes a fibroblast elimination system, the Human Eye Fibroblast Growth Inhibitors, Human Eye FibrOut™. It contains a mixture of of cis-OH-proline, collagenases, D-valine, and formulated serum substitutes. This system can effectively eliminate Eye fibroblast contamination while has not affect on the behavior of cornea epithelial cells.

V References:

1. Iwata M, Kiritoshi A, Roat MI, Yagihashi A, Thoft RA. *Regulation of HLA class II antigen expression on cultured corneal epithelium by interferon-gamma.* Invest Ophthalmol Vis Sci. 1992 Aug;33(9):2714-21.
2. He YG, McCulley JP. *Growing human corneal epithelium on collagen shield and subsequent transfer to denuded cornea in vitro.* Curr Eye Res. 1991 Sep;10(9):851-63.
3. Ebato B, Friend J, Thoft RA. *Comparison of limbal and peripheral human corneal epithelium in tissue culture.* Invest Ophthalmol Vis Sci. 1988 Oct;29(10):1533-7.
4. Maldonado BA, Furcht LT. *Epidermal growth factor stimulates integrin-mediated cell migration of cultured human corneal epithelial cells on fibronectin and arginine-glycine-aspartic acid peptide.* Invest Ophthalmol Vis Sci. 1995 Sep;36(10):2120-6.
5. Ohji M, Mandarino L, SundarRaj N, Thoft RA. *Corneal epithelial cell attachment with endogenous laminin and fibronectin.* Invest Ophthalmol Vis Sci. 1993 Jul;34(8):2487-92.
6. Trocme SD, Hallberg CK, Gill KS, Gleich GJ, Tyring SK, Brysk MM. *Effects of eosinophil granule proteins on human corneal epithelial cell viability and morphology.* Invest Ophthalmol Vis Sci. 1997 Mar;38(3):593-9.

Human Eye PrimaCell™: Corneal Epithelial Cells

Human Eye Primary Cell Culture

Cat No.	Description	Qt.	Price
2-96091	**Human Eye PrimaCell™ system**	**kit**	**$550**
4-26092	Human Eye Tissue Dissociation System, Eye OptiTDS™ (for 500 ml medium)	1 ml	$146
9-46009	Human Corneal Epithelial Cells PrimaCell™ Basal Culture Medium	500 ml	$79
9-36092	Human Corneal Epithelial Cells PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$160
7-66092	Human Eye Fibroblast Growth Inhibitors, Eye FibrOut™	ea	$195
9-87009	Human Eye Tissue Preparation Buffer Set	ea	$90

Human Primary Corneal Epithelial Cells Characterization

6-31211	**Human Corneal Epithelial Cell Primarker™ Kit**	**kit**	**$220**
6-31212	Human Corneal Epithelial Cell Primarker™ antibody set	set	$180
6-31213	Human Corneal Epithelial Cell Primarker™ buffer system	set	$90

Human Fat PrimaCell™: Adipose Cells
(Cat No. 2-96101)

I. General Description

Fat cells, found in adipose tissue, also called adipose cells (adipocytes), are terminally differentiated specialized cells whose primary physiological role hss classically been described as an energy reservoir for the body. The cytoplasm usually being compressed into a thin envelope, with the nucleus at one point in the periphery. The chief chemical constituents of this fat are the neutral glycerol esters of stearic, oleic, and palmitic acids. The size of adipose cells can vary considerably from fat depot to fat depot. Abnormalities in adipose tissue can contribute directly to the pathogenesis of common dieases such as diabetes, hypertension and obesity. Adipocytes are crucial target for pharmacotherapy. While these cells are extremely useful in the laboratory, they are notoriously difficult to isolate and culture. The protocol described here can be scaled up or down as needed and has been used to provide insulin-responsive cells suitable for DNA transfer by electroporation. The Human Fat PrimaCell™ kit (Cat No. 2- 96101) is designed for the optimal isolation and culture of adipocytes from human adipose tissue allowing reproducible and dependable results.

1.1 Components of the Human Fat PrimaCell™ System

- **Human Fat Tissue Dissociation System, Fat OptiTDS™** (2 × 1 ml) --- *A mixture of collagenase, collagenase I and .Human Fat OptiTDS™ Reconstitution Buffer.*
- **Human *Fat OptiTDS™ Digestion Buffer,*** (2 × 9 ml)
- **Human Fat Fibroblast Growth Inhibitors, Fat FibrOut™**---Fat FibrOut™ (5 x 200 µl) ---- *A mixture of cis-OH-proline, collagenase and gentamycin.*
- **Human Adipose Cells PrimaCell™ Basal Culture Medium,** (5 × 100 ml) --- A *Modified DMEM.*
- **Human Adipose Cells PrimaCell™ Medium Supplements with Serum,** (5 × 1 ml): *Glucose, Glutamine, ®-N6-(1-methyl-2-phenylethyl) adenosine, antibiotics (penicillin, 100 U/ml), streptomycin. Gentamycin, BSA and HEPES.*
- **Human Adipose Washing Medium,** (1 × 100 ml): *Basal Kerbs-Ringer medium with 10mM NaHCO3, 30mM HEPES, 200nM adenosine and 5% BSA.*

1.2 Required materials but NOT included:

- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or human fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, fresh gelatin-coated plate or culture dishes are recommended (see below for treatment of culture dishes).

2.2 Principle

Separation of the adipocytes from the adipose tissue is accomplished by enzymatic digestion using the Fat Tissue Dissociation System supplied in this kit. The Fat Tissue Dissociation System contains a mixture of collagenases at the optimal concentrations to gently detach the fragile adipose layer from the adipose tissue. The isolated adipose is then further disrupted to release individual adipocytes by enzymatic and mechanical agitation. The mixture is then filtered through Cell Strainers and seeded on specially coated tissue culture plates. The Adipocytes are propagated in serum-free, low-calcium media or growth arrested feeder cells and the corresponding media. Sub-populations of adipocytes can then be isolated based on their selective attachment to specific basement matrix substrates.

2.3 Human Adipose Tissue Preparation

Human Adipose: Samples of subcutaneous adipose tissues can be used. Each human adipose yield large number of cells (5-10 x 10^6/adipose), with a 30-40% plating efficiency.

11. The fat tissue should be taken by the pathologist.
12. Transport tissue to 70% ethanol and incubate for 1 min, followed by inoculating with 5-10 ml fresh **Human Adipose Washing Medium** for 5 min
13. Add 4 g of fat pads (approximately equivalent to 8 fat pads) to a 30-ml low-density polypropylene vial containing **Human Adipose Washing Medium**. Mince fat pads into pieces approximately 2 mm in diameter with scissors.

2.4 Adipose Separation and Tissue Dissociation

2.4.1 Human Fat OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Human Fat Tissue Dissociation System is suited for optimal dissociation of normal human adipose tissues to yield maximum number of single adipocytes.

2.4.2 Enzyme Compositions

- Collagenase: from *Clostridium Histolyticum*
- Collagenase I: from *Clostridium Histolyticum*

2.4.3 System Components

- Human Fat Tissue Dissociation System, Fat OptiTDS™, (2 × ml).
- Human Fat OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Dissociation

14. Prepare fresh enzyme working solution: Add 1ml of the reconstituted tissue dissociation solution to one vial of Human Cervix OptiTDS™ Digestion Buffer (9 ml). Warm the diluted Human Fat OptiTDS™ working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-4 g tissue samples per 5 ml Human Fat OptiTDS™ working solutions.
15. Discard the Human Fat Washing Medium, and add 5 ml of Human Fat Dissociation System working solution to the vial containing the minced fat pads, and incubate the pieces in a shaking water bath at 37˚C for approximately 1 h, until the cell mixture takes on a creamy consistency.
16. After Human Fat Dissociation System digestion, add 10 ml of Human Adipose Washing Medium at 37˚C to the vial.
17. Mix cells in the vial by swirling, and gently pass the cells through a 250-µm nylon mesh filter into a 50-ml conical tube.

2.6 Adipocytes Isolation

Note: Please read section 2.6 for specific information on Adipocytes culture and platting before preceding this section.

18. Wash the cells by adding 30 ml of **Human Adipose Washing Medium** buffer at 37˚C to the tube and centrifuging briefly at 200 g in a tabletop centrifuge. Remove infranatant with a pipette. Note that adipose cells will be floating on top of the aqueous buffer.
19. Repeat the washing of cells by adding 40 ml of **Human Adipose Washing Medium**, centrifuging, and removing infranatant two additional times.
20. Wash cells twice with 40 ml of **Human Adipose Cells PrimaCell™ Basal Culture Medium** at 37˚C.
21. After the final wash, resuspend the cells from the surface of the medium in **Human Complete Adipose PrimaCell™ Culture Medium/FibrOut** at a cytocrit of approximately 40%.

2.6 Primary Adipocytes Culture

2.6.1 Medium Preparation.

Thaw out **Human Adipose Cells PrimaCell™ Basal Culture Medium**, **Human Adipose Cells PrimaCell™ Medium Su**pplements with Se**rum** on ice.

Human Complete Adipose Cells PrimaCell™ Culture Medium: To every 100 ml **Human Adipose Cells PrimaCell™ Basal Culture Medium**, add 1 ml **Human Adipose Cells PrimaCell™ Medium Supplements with Serum**, mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use.

Human Complete Adipose Cells PrimaCell™ Culture Medium/FibrOut: To every 100 ml **Human Adipose Cells PrimaCell™ Basal Culture Medium**, add 1 ml **Human Adipose Cells PrimaCell™ Medium Supplements with Serum** and 1 ml **Human Fat Fibroblast Growth Inhibitors, Fat FibrOut™**, mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use.

2.6.2 Primary Cell Culture

(Important: Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination)

20. Transfer 2 ml of the 40% cytocrit **Human Complete Adipose PrimaCell™ Culture Medium/FibrOut** suspension, using 200-µl wide-bore pipette tips, into one 60-mm tissue culture dish (Becton Dickinson).
21. Place the cells in a humid incubator at 37˚C with 5% CO_2 for 1.5 h.
22. Add 5 ml of **Human Complete Adipose PrimaCell™ Culture Medium/FibrOut** to each dish. Change the **Human Complete Adipose PrimaCell™ Culture Medium/FibrOut** every 2-3 days;

Δ ***Safety Note.*** The rest of the biopsy and all tubes, pipettes, plates, etc., used in the procedure should be treated with hypochlorite before disposal.

2.7 Subculture

Propagating adipocytes in culture can be somewhat challenging, especially when adipocytes are cultured alone on plastic, however the following methods have worked consistently in many laboratories.

23. The primary cultures grew exponentially until confluency was attained.
24. Remove the medium from the cell layer, and wash twice with **Human Adipose Tissue Washing Medium.**
25. To each culture dish, add enough prewarmed 0.01% EDTA/0.1% trypsin. to cover the cell sheet. Leave the cultures at 37℃ until the adipocytes have detached; check for detachment with a microscope. Do not leave the cells in trypsin for more than 20 min.
26. Remove the cell suspension from the plate and transfer it to a sterile centrifuge tube.
27. Rinse the growth surface with **Human Complete Adipose PrimaCell™ Culture Medium** and add to the suspension. Mix and dispense the suspension with a 10-ml pipette.
28. Spin the cells at 1,000 rpm for 5 min, remove the supernatant, add 10 ml of **Human Complete Adipose PrimaCell™ Culture Medium**, and resuspend the cells vigorously with a 10-ml pipette to achieve a single-cell suspension.
29. Count the cells and plate them onto culture dishes at 10^5 cells/cm^2.
30. Cells may be replated and grown as just described or frozen for later recovery.

III Cryopreservation

31. Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Adipocytes cultures derived from preconfluent layers.
    - **(a)** Trypsinize cells as above, and centrifuge at 100 g for 10 min.
    - **(b)** Resuspend cells in **Human Complete Cervix PrimaCell™ Culture Medium** and count cells.
    - **(c)** Prepare aliquots of 2 x 10^6 cells/ml in **Human Complete Cervix PrimaCell™ Culture Medium** and 10% glycerol into cryovials.
    - **(d)** Equilibrate at 4℃ for 1-2 h.

(e) Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.

(f) To recover cells:

(i) Thaw cryovials quickly in a 37℃ water bath.

(ii) Dilute cells tenfold with **Human Complete Cervix PrimaCell™ Culture Medium**.

(iii) Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in **Human Complete Cervix PrimaCell™ Culture Medium**.

IV Characterization

Morphologically, pre-adipocytes are distinguishable from other primary fibroblasts. There are no biochemical arkers unique to pre-adipocytes in routine use. Morphological changes in pre-adipocytes are apparent when cells start to differentiate. Cells "round up" and lose their fibroblastic morphology, and retain only sparse and tenuous cell-cell contact. As differentiation progresses, intra-cytoplasmic lipid droplets form. It is easy to detect under phase-contrast microscopy, and stain with Oil red-O or Nile red, a simple means of assessing intracytoplasmic lipid accumulation, the classical morphological marker of differentiation. As differentiation continues, more droplets form such that the cytoplasm increases in size and becomes packed with small lipid. Cells tend to differentiate in clumps, with those cells that are refractory to differentiation reverting to a more fibroblastic morphology. The viability of the cells can be checked by glucose uptake assay. Adipocyte markers such as aP2 and adipsin are used in biochemical assays. Glucose transporter GLUT4, mediates insulin-stimulated glucose uptake in adipocytes by rapidly moving from intracellular storage sites to the plasma membrane, is a common used adipocyte specific marker. Lipoprotein lipase (LPL) and pOb24 are widely used as an early marker, and Glycerol 3-phosphate dehydrogenase (G3PDH), along with adipsin and leptin is expressed much later in differentiation. Gene expression assays, such as Northern bloting, real time PCR, etc., are used to detect early or late markers of differentiation.

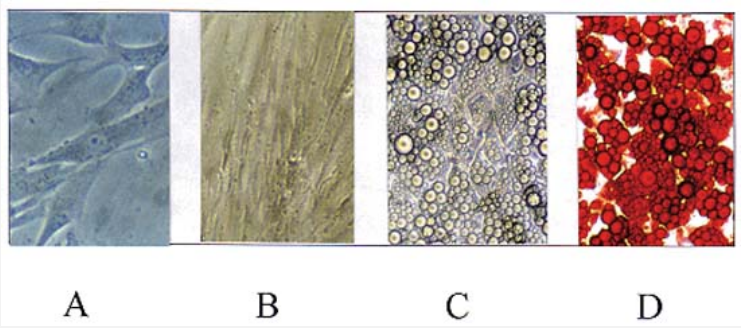


Figure 1 Photomicrographs of Adipocytes cells. Shown here are before and after differentiation (A) Undifferentiated cells during the proliferation phase (magnification 200-fold). (B) Undifferentiated cells in confluent state (magnification 100-fold). (C) Differentiated cells on day 20 (magnification 100-fold). (D) Differentiated cells on day 25, Oil Red Staining (magnification 100-fold) [7]

IV References

1. Freshney, R.I., *Culture of Animal Cells: A Manual of Basic Technique, 4th Edition* 4ed. 2001: Cold Spring Harbor Laboratory Press. 600.
2. Salans, L.B., S.W. Cushman, and R.E. Weismann, *Studies of human adipose tissue. Adipose cell size and number in nonobese and obese patients.* J Clin Invest, 1973. **52**(4): p. 929-41.
3. Abel, E.D., et al., *Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver.* Nature, 2001. **409**(6821): p. 729-33.
4. Park, S.Y., et al., *Calorie restriction improves whole-body glucose disposal and insulin resistance in association with the increased adipocyte-specific GLUT4 expression in Otsuka Long-Evans Tokushima fatty rats.* Arch Biochem Biophys, 2005. **436**(2): p. 276-84.
5. Dani, C., et al., *Expression and regulation of pOb24 and lipoprotein lipase genes during adipose conversion.* J Cell Biochem, 1990. **43**(2): p. 103-10.
6. Tong, Q., et al., *Function of GATA transcription factors in preadipocyte-adipocyte transition.* Science, 2000. **290**(5489): p. 134-8.
7. Wabitsch, M., et al., *Characterization of a human preadipocyte cell strains with high capacity for adipose differentiation.* Int J Obes Relat Metab Disord, 2001. **25**(1): p. 8-15.
8. Bjorntorp, P., et al., *Differentiation and function of rat adipocyte precursor cells in primary culture.* J Lipid Res, 1980. **21**(6): p. 714-23.

Human Fat PrimaCell™: Adipose Cells

Human Fat Primary Cell Culture

Cat No.	Description	Qt.	Price
2-96101	**Human Fat PrimaCell™ system**	**kit**	**$550**
4-26102	Human Fat Tissue Dissociation System, Fat OptiTDS™ (for 500 ml medium)	1 ml	$146
9-46010	Human Adipose Cells PrimaCell™ Basal Culture Medium	500 ml	$73
9-36102	Human Adipose Cells PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$160
7-66102	Human Fat Fibroblast Growth Inhibitors, Fat FibrOut™	ea	$195
9-86010	Human Fat Tissue Preparation Buffer Set	ea	$90

Human Fat Primary Cell Characterization

6-31311	**Human Adipose Cell Primarker™ Kit**	**kit**	**$220**
6-31312	Human Adipose Cell Primarker™ antibody set	set	$180
6-31313	Human Adipose Cell Primarker™ buffer system	set	$90

Human Kidney PrimaCell™ I: Kidney Epithelium
(Cat No. 2-96121)

I. General Description

The Human Kidney PrimaCell™ kit (Cat No. 2-96121) allows the isolation and growth of human kidney epithelial cells from newborn or adult mice with typical yields of 6-7 times more cells than most protocols published in the literature. In addition, the specially formulated media in the Human Kidney PrimaCell™ kit ensures high cell viability and the proprietary Kidney Fibroblast Growth Inhibitors, Kidney FibrOut™ minimizes fibroblast contamination of epithelial cultures.

The preparation of tissues is usually started within 1-2 hrs of removal from donor. If this is impossible, tissue can be cut into 1 mm cubes with sterile scalpels and stored overnight at 4°C in the provided washing medium. The Human Kidney PrimaCell™ system can be used to isolate cells from human kidneys or new-born or adult. Kidney samples containing pathological organisms (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Human Kidney PrimaCell™ System

- **Kidney Tissue Dissociation System**, **Kidney OptiTDS™,** (2 x 1 ml) --- A proprietary mixture of collagenase, collagenase I, collagenase II, collagenase IV, Soybean Trypsin Inhibitor, Dexoyribonuclease I, Trypsin, and Protease.
- **Kidney OptiTDS™ Digestion Buffer,** (2 x 9 ml)
- **Kidney Tissue Washing Medium, (**1 x 100 ml) --- *Basal Kidney PrimaCell™ Culture Medium with 5% FBS, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Kidney Fibroblast Growth Inhibitors, Kidney FibrOut™,** (5 x 200 μl) --- *A proprietary mixture of cis-OH-proline, collagenase, D-valine, and formulated serum substitutes*.
- **Human Kidney Epithelium PrimaCell™ Basal Culture Medium, (**5 x100 ml) --- *Modified formulation based on medium DMEM and F-12*.
- **Human Kidney PrimaCell™ Medium Serum, (**1 x 50 ml) --- *Highly purified and special-treated Fetal-bovine serum.*
- **Human Kidney Epithelium PrimaCell™ Medium Supplements with Serum**, (5 x 10 ml) --- A mixture of insulin, sodium selenite, transferring, 3,3'5-triiodothyronine, highly purified bovine serum albumin, and glutamine, dexamethasone, antibiotics (penicillin, streptomycin), and charcoal-stripped Fetal Bovine Serum.

1.2 Required Materials NOT provided

- Falcon Conicals, 50 ml
- Nylon gauze cell strainer (70-100 μm)
- Plastic tissue culture dishes, collagenase -I coated, 100 mm (Corning, NY)
- Scalpels, curved forceps
- 70% ethanol
- 0.05% EDTA (pH 7.4), sterile

- 0.25% trypsin/0.1% (2.5 mM) EDTA, sterile
- PBSA (PBS containing 10% BSA), sterile

II. Procedures

2.1 Procedure Overview and Materials Preparation

Tissue fragments are excised from the outer cortex of the kidney, minced, washed, and incubated with agitation in Kidney OptiTDS™ solution. The Kidney Dissociation System provides the most optimal conditions to isolate kidney epithelial cells. The kidney epithelium is further dispersed by additional incubations and mechanical disruption. The cells are filtered and platted onto specially treated tissue culture plates. All materials and equipment used in these experiments should be sterilized and rinsed with PBSA prior use.

2.2 Surgical specimens

1. Kidneys are removed from donors using scissors and forceps, immersed in 70% ethanol for 1 min, and immediately placed in a 100-mm tissue culture dish containing 10 ml of Kidney Tissue Washing Medium (each kidney is placed in a separate dish). After all the kidneys are removed, transfers into fresh 100-mm dishes containing 10 ml of Kidney Tissue Washing Medium. Whole kidneys are minced into 1 mm cubes using sterile razor blades. The minced tissues are transferred into sterile 15 ml conical tubes containing Kidney Tissue Washing Medium. After allowing the minced tissue to settle, aspirate off the Kidney Tissue Washing Medium, and wash once more with Kidney Tissue Washing Medium.
2. Incubate kidney pieces sequentially in 10 ml 70% ethanol for 1 min, in 10 ml PBSA for 2 min, and in 20 ml Kidney Tissue Washing Medium for 10 min. These steps reduce the risk of infection and do not interfere with cell viability.
3. Keep tissues on ice until ready to proceed with tissue dissociation.

2.3 Tissue Preparation and Dissociation

2.3.1 Human Kidney OptiTDS™

Several important factors can affect the yield and viability of primary cells, including the tissue type, species, and age of the donor, enzymes, culture media and growth supplements. The Human Kidney Tissue Dissociation System, OptiTDS™, is optimized for the efficient dissociation of kidney epithelial cells from normal adult and newborn human kidneys yielding the maximum number of single primary cells.

2.3.2 Enzyme Compositions

- Collagenase: from *Clostridium Histolyticum*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase II: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*
- Soybean Trysin Inhibitor: from *Glycine Max*
- Dexoyribonuclease I: from *Bovine Pancreas*
- Trypsin: from *Bovine Pancreas*

- Protease: from *Staph Aureus*

2.3.3 System Components

- **Kidney Tissue Dissociation System, OptiTDS™,** (2 x 1 ml).
- **Kidney OptiTDS™ Digestion Buffer,** (2 x 9 ml).

2.3.4 Procedures For Tissue Preparation and Dissociation

4. Prepare fresh enzyme working solutions: Add 1.0 ml of Kidney Tissue Dissociation System, OptiTDS™ to 9 ml of Kidney OptiTDS™ Digestion Buffer. Warm this diluted enzyme solution at 37° C for 10 min prior to use. For optimal results we recommend using 10 ml diluted enzyme solution per 2-3 g of tissue.
5. Mince pre-washed tissue into pieces measuring approximately 1 mm with scalpels or sterile razors.
6. Incubate minced tissue with the fresh Kidney Dissociation enzyme working solution (the diluted enzyme solutions prepared in step 1) by one of the following steps:

    a. Rapid dissociation: Incubate kidney tissue in Kidney Dissociation enzyme working solution with rocking for 2-3 hrs at 37° C. This works particularly well with thicker/older kidneys.
    b. Slow dissociation: Incubate kidney tissue in ice-cold Kidney Dissociation enzyme working solution with rocking at 4° C for 15-24 hrs.

7. Prepare Nylon cell strainers by placing inside a 50 ml conical tube. Alternatively, rinse sterile woven cloth 2X in sterile PBSA and place into sterile funnel on top of sterile beaker and use to strain the cell mixture in order to remove debris. Save the Kidney Dissociation enzyme working solution.
8. Tirturate the cell mixture with a sterile pipette several times to facilitate a single cell mixture. Collect the cells by passing the mixture through the Nylon cell strainers into a 50 ml conical.
9. The collected cells are centrifuged at 800 g for 5 min. The resulting pellet contains the primary kidney epithelial cells. This pellet can be resuspended in 5 ml of Complete Kidney Epithelium Medium (see medium preparation below 2.4.1). Save the supernatant for repeating tissue dissociation process in step 4.
10. To the remaining pieces of tissue, repeat the tissue dissociation process with the saved Kidney Dissociation working solutions from step 4 to yield additional cells. This process can repeat up to 3 times if it is necessary without changing the enzyme working solution. In general, kidneys from each 5-6 month old human should produce six to eight 60-mm dishes of primary culture cells.
11. Count viable cells and plate cells at a density of 1×10^5 cells per 100-mm collagenase -I coated culture dish.

Note: Kidneys can be pooled for this preparation; however, it is very important that the concentration of the dissociation solutions be kept constant at 10 ml /2-3 g kidney tissues.

2.3.5 Storage:

The tissue dissociation systems should be reconstituted just before use and can only be stored for 2-4 days at 4^{o} C. For long-term use, the reconstituted tissue dissociation system solutions

should be aliquoted and stored at -20° C. Avoid repeated freeze-thaw cycles.

2.4 Culture of Primary Human Kidney Epithelium

2.4.1 Medium Preparation

Thaw out Human Kidney Epithelium PrimaCell™ Basal Culture Medium, 1 ml Human Kidney Epithelium PrimaCell™ Medium Supplements, and 1 ml Human Kidney Fibroblast Growth Inhibitors, Kidney FibrOut™ on ice. To every 100 ml Human Kidney Epithelium PrimaCell™ Basal Culture Medium, add 1 ml of Human Kidney PrimaCell™ Medium Supplements, 1 ml of Human Kidney Fibroblast Growth Inhibitors, Kidney FibrOut™, mix thoroughly and warm the Complete Kidney Media at a 37° C water bath for 10 min prior use. (**Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*).

2.6.1 Primary Cell Culture

Primary cultures are incubated at 37 °C, 5% CO^2 incubator for 24 hrs to allow cells to adhere. After 24 hrs the cells should be washed twice with sterile PBS to remove non-adherent cells (i.e. non epithelial cells) and tissue fragments. Return cells to 37° C incubator in Complete Kidney Media described above for another 2–3 days, or until they reach 60–80% confluency. At this time, cells from the same organ are trypsinized, combined, and split into the number of 60-mm tissue-culture dishes required for a single experiment (usually,17–24 dishes). Cells usually require an additional 3-4 days of growth before the appropriate density is reached for experimentation. Please note that individual kidneys can be kept separate or pooled if the concentrations of reagents are scaled up as described per kidney.

2.4.3 Subculture and Propagation

12. Gently rinse the culture dish twice with 0.02% (0.7 mM) EDTA.
13. Add 3 ml of 0.25% trypsin/0.1% (2.5 mM) EDTA, and incubate at 37° C. Examine the dish under a microscopy every 5 min to detect cell detachment.
14. When most cells have detached, add 10 ml Complete Kidney Media to inactive the trypsin.
15. Pipette the contents of the dish to ensure all the cells have detached.
16. Collect the cells and centrifuge at 350 g for 5 min.
17. Aspirate the supernatant; resuspend the cell pellet in a Complete Kidney Media, and plate at a density of 1×10^5 cells per cm^2.
18. Feed the cultures twice a week with Complete Kidney Media.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample; the best results are obtained from preconfluent primary cultures.

19. Trypsinize cells using method used above, and centrifuge cells at 100 g for 10 min.
20. Resuspend the cell pellet in Complete Kidney Media with serum, and count cells.
21. Dispense aliquots of 2 x 10^6 cells/ml in Complete Kidney Medium supplemented with an additional 10% FBS and 10% glycerol into cryopreservation vials.
22. Equilibrate at 4° C for 1-2 hrs.

23. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of one degree per min.

To recover cells:

(i) Thaw cryovials quickly in a 37° C water bath.
(ii) Dilute cells tenfold with medium.
(iii) Centrifuge cells; resuspend them at an appropriate density in Complete Kidney Media and plate.

Human cells can be grown for several weeks and can be subcultured only 4-6 passages, in Complete Kidney Media.

IV Fibroblast Contamination

Several techniques have been published in the literature to address fibroblast contamination of primary kidney primary cell cultures. These include (1) Physically removing well-isolated fibroblast colonies by scraping and following with several careful washes to remove any fibroblasts that have detached (2) Differential trypsinization of carcinomas (3) Using dispase to preferentially (but not exclusively) to remove epithelial cells during passaging. During subculture, cells that have been removed with dispase can be pre-incubated in plastic culture dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the epithelium. This technique takes advantage of the fact that fibroblasts in general attach much more quickly to plastic than do clumps of epithelial cells, so that a partial purification step is possible. (4) Reduce the concentration of serum between 2.5-5% to discourage fibroblast growth. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and that using any or all of the preceding techniques will eventually push fibroblasts through enough divisions to allow the fibroblasts to senesce.

The Human Kidney PrimaCell™ I system includes a unique fibroblast elimination system, the Human Kidney Fibroblast Growth Inhibitors, Kidney FibrOut™. It contains a proprietary mixture of cis-OH-proline, collagenase, D-valine, and formulated serum substitutes that most effectively eliminate kidney fibroblast contaminations and does not interfere with the propagation and biology of kidney epithelial cells.

V References:

1. Kwak S, Jung JE, Jin X, Kim SM, Kim TK, Lee JS, Lee SY, Pian X, You S, Kim H, Choi YJ. *Establishment of immortal swine kidney epithelial cells.* Anim Biotechnol. 2006;17(1):51-8.
2. Orosz DE, Woost PG, Kolb RJ, Finesilver MB, Jin W, Frisa PS, Choo CK, Yau CF, Chan KW, Resnick MI, Douglas JG, Edwards JC, Jacobberger JW, Hopfer U. *Growth, immortalization, and differentiation potential of normal adult human proximal tubule cells.* In Vitro Cell Dev Biol Anim. 2004 Jan-Feb;40(1-2):22-34.
3. Baer PC, Tunn UW, Nunez G, Scherberich JE, Geiger H. *Transdifferentiation of distal but not proximal tubular epithelial cells from human kidney in culture.* Exp Nephrol. 1999 Jul-Aug;7(4):306-13.
4. Williams JM, Boyd B, Nutikka A, Lingwood CA, Barnett Foster DE, Milford DV, Taylor CM. *A comparison of the effects of verocytotoxin-1 on primary human renal cell cultures.* Toxicol Lett. 1999 Mar 8;105(1):47-57.
5. Granot Y, Van Putten V, Przekwas J, Gabow PA, Schrier RW. *Intra- and extracellular proteins in human normal and polycystic kidney epithelial cells.* Kidney Int. 1990 May;37(5):1301-9.

6. Merlet D, Merlet JP, Mellado M, Cambar J. *Morphologic and enzymatic characteristics of healthy human kidney epithelial tubular cells in culture.* C R Seances Soc Biol Fil. 1989;183(4):349-57.

Human Kidney PrimaCell™ Kidney Epithelium

Human Kidney Primary Cell Culture

Cat No.	Description	Qt.	Price
2-96121	**Human Kidney PrimaCell™ system**	**kit**	**$550**
4-26122	Human Kidney Tissue Dissociation System, Kidney OptiTDS™ (for 500 ml medium)	1 ml	$ 146
9-46012	Human Kidney Epithelium PrimaCell™ Basal Culture Medium	500 ml	$79
9-36122	Human Kidney Epithelium PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$ 160
7-66122	Human Kidney Fibroblast Growth Inhibitors, Kidney FibrOut™	ea	$ 195
9-86012	Human Kidney Tissue I PrepaHumanion Buffer Set	ea	$90

Human Primary Kidney Epithelium Characterization

6-31411	**Human Kidney Epithelium Primarker™ Kit**	**kit**	**$220**
6-31412	Human Kidney Epithelium Primarker™ antibody set	set	$180
6-31413	Human Kidney Epithelium Primarker™ buffer system	set	$90

Human Liver PrimaCell™: Hepatocytes
(Cat No. 2-96131)

I. General Description:

The basic two-step perfusion procedure can be used to isolate hepatocytes from the livers of mice, rabbits, guinea pigs, or woodchucks, by proportionally adapting the volume and flow rate of the solutions to the size of the liver. The technique used for human livers involves perfusion of the whole liver or a biopsy sample (15-30 ml/min, depending on the size of the tissue sample). Complete isolation of hepatocytes into a single-cell suspension can be obtained by an additional incubation at 37℃ with collagenase under gentle stirring for 10- 20 min (especially for human liver). Fish hepatocytes can be isolated by cannulation of the intestinal vein and incision of the heart to avoid excessive pressure. Perfusion is performed at room temperature at a flow rate of 12-15 ml per min.

This protocol has been developed for the attachment and growth of normal human liver hepatocytes from adult human liver tissue using the Human Liver PrimaCell™ system (Cat No. 2-96131). This system provides the optimal tissue dissociation system, Liver OptiTDS™ that yields 4-7 times the single cells than most tissue dissociation protocols published in the literature. CHI's proprietary fibroblast inhibitory system, Liver FibrOut™, allows for minimal to no contamination of the hepatocyte cultures by non-epithelial cells types. In addition, the media and supplements provided have been supplied to ensure a robust culture of hepatocytes for experimentation.

The preparation of tissue specimens for cell culture should be started within 1-2 h of organ removal or sacrifice of the animal. If this is impossible, store the tissue overnight at 4℃ in washing medium (see below) as this has also given satisfactory results.

Human Liver PrimaCell™ system is well suited for use in normal adult human liver samples. Livers containing pathological organisms (virus, parasites, etc.) or tumor may not be suitable for use with this system.

1.1 Components of Human Liver PrimaCell™ System

- **Human Liver Tissue Dissociation System, Liver OptiTDS™,** (2 x 1 ml) --- *A proprietary mixture of collagenase, collagenase I, collagenase II, collagenase IV, Hyaluronidase I, and Dispase*
- **Human Liver OptiTDS™ Digestion Buffer**, (4 x 100 ml)
- **Human Liver Tissue Washing Medium**, (2 x 100 ml) --- *Basal Liver PrimaCell™ Culture Medium with NaCl, KCl, Na2HPO4.12H2O, HEPES, and 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*
- **Human Liver Fibroblast Growth Inhibitors, Liver FibrOut™** (5 x 200 μl) --- *A mixture of D-valine, collagenase, and formulated serum substitutes*
- **Human Liver PrimaCell™ Basal Culture Medium**, (5 x100 ml) --- *Modified formulation based on Ham's F12 and Weymouth medium*
- **Human Liver PrimaCell™ Medium Supplements with Serum**, (5 x 1.0 ml) --- A mixture of *Basal culture medium containing bovine albumin (grade V), bovine Insulin,*

1.2 Required Materials not provided in the kit

- Tygon tube (ID, 3.0 mm; OD, 5.0mm)
- Disposable scalp vein infusion needles, 20G
- Sewing thread for cannulation
- Graduated bottles and Petri dishes
- Surgical instruments (Sharp, straight, and curved scissors and clips)
- 2 × 1-ml disposable syringes
- Collagen I-coated plate (Corning, NY)
- Chronometer
- Peristaltic pump (10 to 200 rpm)
- Water bath

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, collagen I-coated plates (Corning, NY) MUST be pre-treated with the Human Liver PrimaCell™ Basal Culture Medium and incubated for 5 min. After 5 min aspirate the Human Liver PrimaCell™ Basal Culture Medium and allow the dishes to air-dry in a ventilated cell culture hood for 5-10 min.

2.2 Surgical specimens

Perfusion of liver tissue must be performed within 2 hours of the liver excision or biopsy to ensure a smooth and complete perfusion. Immediately after the human liver is transported to the laboratory, place a loosely tied ligature around the portal vein (approximately 5 mm from the liver) insert the cannula to the liver, and ligate. Briefly dip the liver tissue in 70% ethanol for 1 min, followed by a rinse in 10 ml PBS, and 10 ml Liver Tissue Washing Medium. Initiate the perfusion with 100 ml Liver Tissue Washing Medium, followed by 100 ml of Liver OptiTDS™ Digestion Buffer, and finally by 300 ml 100 ml of Liver OptiTDS™ Digestion working solution (see below for details).

2.3 Tissue Preparation and Dissociation

2.3.1 Human Liver OptiTDS™

Several important factors can affect yield and viability of primary cell culture, including tissue type, origin of species, tissue age, enzymes, culture medium and growth supplements. The Liver Tissue Dissociation System, OptiTDS™, is suited for optimal dissociation of whole liver or a biopsy sample of a normal adult human liver to yield the maximum number of single primary hepatocytes.

2.3.2 Enzyme Compositions

- Collagenase: from *Clostridium Histolyticum*

- Collagenase I: from *Clostridium Histolyticum*
- Hyaluronidase I: from *Bovine Testes*
- Dispase: from *Bacillus polymyxa*

2.3.3 System Components

- Liver Tissue Dissociation System, OptiTDS™, (2 x 1.0 ml)
- Liver OptiTDS™ Digestion Buffer, (4 x 100 ml)

2.3.4 Procedures For Tissue Preparation and Dissociation

1. Warm the 200 ml Liver Tissue Washing Medium and 400 ml Liver OptiTDS™ Digestion Buffer in a water bath (usually approximately 38-39℃ to achieve 37℃ in the liver) for 10 min. Oxygenation is not necessary. Combine 300 ml Liver OptiTDS™ Digestion Buffer, and 1 ml Liver Tissue Dissociation System, OptiTDS™, mix well and incubate in the water bath until use. The remaining 100 ml Liver OptiTDS™ Digestion Buffer will be used to flush the portal vein described in step 5 below.
2. Set the pump flow rate at 30 ml/min.
3. After the tissue has been prepared, place a loosely tied ligature around the portal vein (approximately 5 mm from the liver), insert the cannula up to the liver, and ligate.
5. Rapidly incise the sub-hepatic vessels to avoid excess pressure, and start the perfusion using 100 ml Liver Tissue Washing Medium followed by 100 ml of Liver OptiTDS™ Digestion Buffer (without tissue dissociation enzymes) at a flow rate of 30 ml/min; verify that the liver whitens within a few seconds.
6. Next, perfuse the liver with 300 ml of Liver Tissue Dissociation System, (OptiTDS™ working solution prepared in step 1) at a flow rate of 15 ml/min for 30 min. The liver should swell.
7. Remove the liver and wash it with 100 ml Liver Tissue Washing Medium. Disrupt the Glisson capsule and disperse the cells in 100 ml of complete Human Hepatocytes PrimaCell™ Medium (see preparation below in 2.4.1).

2.3.5 Storage:

The tissue dissociation solutions should be reconstituted just before use and can only be stored for 2-4 days at 4 ^{o}C. For long-term use, solutions should be aliquoted before reconstitution and stored at -20 ^{o}C. Avoid repeated freeze-thaw cycles.

2.4 Culture of primary Human Liver cells

2.4.1 Medium Preparation

Thaw out the Hepatocytes PrimaCell™ Medium Supplements with Serum and Liver Fibroblast Growth Inhibitors, Liver FibrOut™ on ice. To every 100 ml Human Hepatocytes PrimaCell™ Basal Culture Medium, add 1 ml of Human Hepatocytes PrimaCell™ Medium Supplements with Serum; and 1 ml of Liver Fibroblast Growth Inhibitors, Liver FibrOut™, mix thoroughly and warm the complete medium in a 37^{o} C water bath for 10 min prior use. (**Important:** *Long term use of FibrOut™ in the culture medium may result in loss of hepatocytes, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or until an acceptable level of fibroblasts is reached*).

2.6.1 Treatment of Culture Dishes

To enhance cell attachment to the culture dishes, collagen I-coated plates (Corning, NY) MUST be pre-treated with the complete Human Hepatocytes PrimaCell™ Culture Medium (enough to cover the whole cell-growth area) and incubated at 37^{o} C for 5 min. Aspirate the medium and allow the dishes to air-dry in a ventilated cell culture hood for 5-10 min.

2.4.3 Standard primary culture conditions

8. Filter the suspension through two-layers of gauze or 60-80-μm nylon mesh, allow the viable cells to sediment for 20 min (usually at room temperature), and discard the supernatant (60 ml) containing debris and dead cells.
9. Wash the cells twice with 1 x PBS and once with complete Human Hepatocytes PrimaCell™ Medium by slow centrifugations (50 g for 40 s) to remove tissue dissociation enzymes, damaged cells, and non-parenchymal cells.
10. Collect the hepatocytes in 10 ml complete Human Hepatocytes PrimaCell™ Medium.
11. Count the cells and seed 5-7 x 10^5 cells/100-mm dishes. Isolated Hepatocytes can grow for 4-6 days and undergo 1-3 rounds of division on collagen I-coated culture dishes without obvious differentiation. Our experience has shown that freshly isolated human hepatocytes seeded at a density of 2×10^5 to 3×10^5 cells/ml medium on collagen-coated plates reach a confluence (after plating) of approximately 80–90%, with hepatocyte viability greater than 90% as assessed by Trypan blue exclusion. After one round of plating, hepatocytes were maintained in the complete culture medium without addition of Liver FibrOut™ and Medium was changed daily.

2.4.4 Alternative Primary Culture conditions

The attachment of hepatocytes during primary culture and subculture is more reproducible and efficient when cells are inoculated onto collagen-coated flasks. When short term (4-6 hours) growth of cell is acceptable for the experiment, plain culture dish without biomatrix can be used.

2.4.5 Subculture and Propagation

Most primary cultures cannot be passaged presently using routine trypsin / EDTA procedures. Disaggregation to single cells of the cultured liver cells with 0.1% trypsin in 0.25 mM (0.1%) EDTA will result in extremely poor or no growth. To avoid these consequences Dispase is used. An advantage with this procedure is that dispase can only efficiently detach epithelial cell but not fibroblast, thereby, increasing the purity of the target cell population.

12. Add 0.5% Dispase (Sigma, w/v) to the cell monolayer, just enough to cover the cells (~2.5 ml/25-cm^2 flask), and leave the solution to stand for 40-60 min for primary cultures and 20-40 min for cell lines.
13. Once the epithelial layers begin to detach (they do so as sheets rather than single cells), pipette to help detachment and dis-aggregation into smaller clumps.
14. Wash and replate the cells under standard culture conditions. It may take several days for clumps to attach, so replace the medium carefully when feeding.

III Fibroblast Contamination

There are several techniques have been published in the literature to deal with fibroblast contamination during primary cell culture. These include: (1) Physically remove a well-isolated

fibroblast Livery by scraping it with a sterile blunt instrument (e.g., a cell scraper). Care has to be taken to wash the culture up to six times to remove any fibroblasts that have detached in order to prevent them from reseeding and reattaching to the flask. (2) Differential trypsinization can be attempted with the carcinomas. (3) Dispase preferentially (but not exclusively) removes the epithelium during passaging and leaves behind most of the fibroblastic cells attached to the culture vessel. During subculture, cells that have been removed with dispase can be preincubated in plastic Petri dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the epithelium. Clumps of epithelial cells still floating can be transferred to new flasks under standard culture conditions. This technique takes advantage of the fact that fibroblasts in general attach more quickly to plastic than do clumps of epithelial cells, so that a partial purification step is possible. (4) Reduce the concentration of serum to about 2.5-5%, if there are heavy concentrations of fibroblasts. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and using any or all of the techniques mentioned above will eventually push the cells through so many divisions that the fibroblasts will eventually senesce.

Human Liver PrimaCell™ includes a fibroblast elimination system, the Human Liver Fibroblast Growth Inhibitors, Liver FibrOut™. It contains a mixture of D-valine, collagenase, and formulated serum substitutes. The FibrOut™ kills contaminating fibroblasts, but shows no signs of toxicity toward the target cells, whether derived from an adenoma, a carcinoma or normal liver tissues.

IV References:

1. Guillouzo A, Le Bigot JF, Guguen-Guillouzo C, Kiechel JR. *Presence of phase I and phase II drug metabolizing enzymes in cultured human foetal hepatocytes.* Biochem Pharmacol. 1982 Jul 15;31(14):2427-30.
2. Guguen-Guillouzo C, Bourel M, Guillouzo A. *Human hepatocyte cultures.* Prog Liver Dis. 1986;8:33-50. Review.
3. Chen HL, Wu HL, Fon CC, Chen PJ, Lai MY, Chen DS. *Long-term culture of hepatocytes from human adults.* J Biomed Sci. 1998 Nov-Dec;5(6):435-40.
4. Kono Y, Yang S, Roberts EA. *Extended primary culture of human hepatocytes in a collagen gel sandwich system.* In Vitro Cell Dev Biol Anim. 1997 Jun;33(6):467-72.
5. Koebe HG, Pahernik S, Eyer P, Schildberg FW. *Collagen gel immobilization: a useful cell culture technique for long-term metabolic studies on human hepatocytes.* Xenobiotica. 1994 Feb;24(2):95-107.
6. Gibson-D'Ambrosio RE, Crowe DL, Shuler CE, D'Ambrosio SM. *The establishment and continuous subculturing of normal human adult hepatocytes: expression of differentiated liver functions.* Cell Biol Toxicol. 1993 Oct-Dec;9(4):385-403.
7. Strom SC, Jirtle RL, Jones RS, Novicki DL, Rosenberg MR, Novotny A, Irons G, McLain JR, Michalopoulos G. *Isolation, culture, and transplantation of human hepatocytes.* J Natl Cancer Inst. 1982 May;68(5):771-8.

Human Liver PrimaCell™: Hepatocytes

Human Liver Primary Cell Culture

Cat No.	Description	Qt.	Price
2-96131	**Human Liver PrimaCell™ system**	**kit**	**$550**
4-26132	Human Liver Dissociation System, Muscle OptiTDS™ (for 500 ml medium)	1 ml	$146
9-46013	Human Hepatocytes PrimaCell™ Basal Culture Medium	500 ml	$73
9-36132	Human Hepatocytes PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$160
7-66132	Human Liver Fibroblast Growth Inhibitors, Liver FibrOut™	ea	$195
9-86013	Human Liver Tissue Preparation Buffer Set	ea	$90

Human Primary Hepatocytes Epithelium Characterization

6-31511	**Human Hepatocyte Primarker™ Kit**	**kit**	**$220**
6-31512	Human Hepatocyte Primarker™ antibody set	set	$180
6-31513	Human Hepatocyte Primarker™ buffer system	set	$90

Human Muscle PrimaCell™: Skeletal Muscle Cells
(Cat No. 2-96141)

I. General Description

Of all the different tissues and organs in the body, skeletal muscles are the most frequent organ. A unique characteristic of skeletal muscle is that it is not made up of individual cells, but consists of large multinucleate syncytia, the myofibers, each of which can be several centimeters long. During embryogenesis, mononuclear precursor cells, or myoblasts, fuse to form these huge muscle fibers. Later life during regenerative process subsequent to muscle damage, quiescent mononuclear stem cells (satellite cells) lacated between the sarcolemma and basal lamina of the muscle fiber are activated. These cells begin to proliferate and either fuse with each other into novel myotubes, or they fuse with damaged muscle fibers. Both myoblasts and satellite cells can be isolated from the body and grown in tissue culture, and in optimal culture media they will fuse and differentiate into mature, spontaneously-contracting myotubes. Culture derived from normal and diseased human skeletal muscle cells therefore provide an excellent model to study several aspects of early muscle development under normal and pathological conditions. While these cells are extremely useful in the laboratory they are notoriously difficult to isolate and culture.

The Human Muscle PrimaCell™ kit (Cat No. 2-96141) is designed for the successful isolation and culture of skeletal muscle cells from human muscle allowing reproducible and dependable results.

1.1 Components of the Human Muscle PrimaCell™ System

- **Human Muscle Tissue Dissociation System , Muscle OptiTDS™** (2 × 1 ml) --- *A mixture of collagenase, collagenase I, Elastase, Pronase, trypsin and Muscle OptiTDS™ Reconstitution Buffer*.
- **Muscle Tissue Washing Medium**, (2 × 1 m)
- **Human Muscle OptiTDS™ Digestion Buffer,** (1 × 100ml) --- *A modified Ham's F12 without NaHCO3, with 20 mM HEPES, 200 U/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Human Muscle Fibroblast Growth Inhibitors, Muscle FibrOut™** ---Muscle FibrOut™ (5 × 200 μl) --- *A mixture of D-valine, collagenase, gentamycin.*
- **Human Skeletal Muscle Cells PrimaCell™ Basal Culture Medium** (5 × 100 ml) --- *A modified Ham's F12*.
- **Human Skeletal Muscle Cells PrimaCell™ Medium Supplements with Serum** (10 × 10 ml): *Highly purified and special-treated fetal bovine serum, penicillin, streptomycin.*

1.2 Required materials but NOT included:

- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or human fibroblast (optional)

- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, fresh gelatin-coated plate or culture dishes are recommended (see below for treatment of culture dishes).

2.2 Principle

It is possible to culture myogenic cells from adult skeletal muscle of several species under conditions in which the cells continue to express at least some of their differentiated traits. A cell tuped called *satellite cells*, partially mimic the first steps of skeletal muscle differentiation. They proliferate and migrate randomly on the substratum and then align and finally undergo a fusion process to form multinucleated myotubes. Although three to four passages can be performed by means of trypsinization, subculture is no longer possible once differentiation (i.e., fusion) has taken place. For the same reason, proliferation is very difficult to estimate once some cells have started to fuse. The procedure described here is an enzymatic method of digesting a muscle biopsy. Primary cultures from human healthy muscle biopsies are highly enriched in myogenic cells, as evidenced by at least 80% positively to desmin by immunostaining at day 10 after seeding. Primary cultures can be grown easily in **Human Complete Skeletal Muscle PrimaCell™ Culture Medium**. Without modifying the culture conditions, these cells proliferate and differentiate by fusing to form multinucleated myotubes, confirming the myogenicity of the cultivated cells.

2.3 Human Muscle Tissue Preparation

Human Muscle: Fresh non-fixed and non-frozen muscle specimens are usually obtained during corrective surgery or from biopsies. Biopsies are usually taken under local anesthesia using scissors or a muscle biopsy clamp, or by percutaneous needle biopsies. Muscle specimen can be stored for a conveniently long period.

1. The muscle specimens for culture were immediately placed in a 100-mm tissue culture dish containing 5-10 ml of **Human Muscle Tissue Washing Medium**.
2. Trim off nonmuscle tissue from the specimens with a scalpel, and rinse in **Human Muscle Tissue Washing Medium**.
3. Cut the muscle tissue into fragments parallel to the fibers and wash in **Human Muscle Tissue Washing Medium** prior to weighing the biopsy.
4. Place the fragments parallel to each other in the lid of a Petri dish, cut the fragments into thinner cylinders and then, finally into 1-mm^3 pieces, without crushing the tissue. The final cutting can be done in a tube with long scissors, again avoiding crushing.
5. Discard the **Human Muscle Tissue Washing Medium** and incubate tissue specimens with 5-10 ml 70% ethanol for 1 min, followed by inoculating with 5-10 ml fresh **Human Muscle Tissue Washing Medium** for 5 min.

2.4 Skeletal Muscle Cell Separation and Tissue Dissociation

2.4.1 Human Muscle OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Human Muscle Tissue Dissociation System is suited for optimal dissociation of normal newborn human muscle tissues to yield maximum number of single skeletal muscle cell.

2.4.2 Enzyme Compositions

- Trypsin: from *Bovine Pancreas*
- Collagenase: from *Clostridium Histolyticum*
- Collagenase I: from *Clostridium Histolyticum*
- Elastase: from *Bovine Pancreas*
- Pronase: from *Bovine Pancreas*

2.4.3 System Components

- Human Muscle Tissue Dissociation System, Muscle OptiTDS™, (2 × 1 ml).
- Human Muscle OptiTDS™ Digestion Buffer, (2 × 9 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

6. Prepare fresh enzyme working solution: Add 1.0 ml of **Human Muscle Tissue Dissociation System, Bone OptiTDS™** to one vial of **Human Muscle OptiTDS™ Digestion Buffer** (9 ml). Warm the diluted enzyme working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 mg tissue samples per 10 ml diluted enzyme working solutions.
7. Rinse muscle tissue with **Human Muscle Tissue Washing Medium** and let the pieces settles; discard the supernatant.
8. Dissociation can be achieved in either of the following two methods

   (a) Rapid dissociation: Float muscle on Human Muscle Tissue Dissociation System working solution for 2-3 hrs at 37 ℃. This works particularly well with full-thickness muscle. Monitor the separation of the muscle carefully when using the rapid dissociation method.

   (b) Slow dissociation: Float the samples on ice-cold Human Muscle Tissue Dissociation System working solution at 4℃ for 15-24 h. This is particularly convenient for flexible scheduling of skeletal muscle cells preps.

2.7 Primary skeletal muscle cells Culture

2.5.1 Medium Preparation.

Thaw out **Human Skeletal Muscle PrimaCell™ Basal Culture Medium**, **Human Skeletal Muscle PrimaCell™ Medium Supplements With Serum** on ice.

Human Complete Skeletal Muscle PrimaCell™ Culture Medium: To every 100 ml **Human Skeletal Muscle PrimaCell™ Basal Culture Medium**, add 10 ml **Human Skeletal Muscle**

PrimaCell™ Medium Supplements With Serum, mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use.

Human Complete Skeletal Muscle PrimaCell™ Culture Medium/FibrOut: To every 100 ml **Human Skeletal Muscle PrimaCell™ Basal Culture Medium**, add 10 ml **Human Skeletal Muscle PrimaCell™ Medium Supplements With Serum**, one vial of **Human Muscle Fibroblast Growth Inhibitors, Bone FibrOut™**, mix thoroughly and warm the complete medium at a 37°C water bath for 10 min prior use. (**Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to an acceptable level of fibroblast cell contamination*)

2.5.2 Primary Cell Culture

9. Triturate the culture with a pipette after incubation. The medium should become increasingly opaque as more and more cells are released.
10. Let the fragments settle to the bottom by gravity, forming pellet P1 and supernatant S1.
11. Filter S1 through a 100-µm nylon mesh and into a 20-ml centrifuge tube. Shake or pipette the supernatant gently to resuspend the cells.
12. Centrifuge the tube 8-10 min at 350 g. Discard the supernatant by aspiration.
13. Resuspend the pellet very, very gently by means of a rubber-bulb pipette in precisely 10 ml of **Human Complete Skeletal Muscle PrimaCell™ Culture Medium/FibrOut**, and count the cells with a hemocytometer.
14. Dilute the suspension in growth medium to seed culture flasks with about 1.5×10^4 cells/ml. About $1 – 2 \times 10^5$ cells/g are obtained from healthy donor biopsies.
15. Add 15 ml of digestion medium to P1, and incubate the fragments for 30 min in a water bath at 37 ˚C, with periodic shaking.
16. Pipette the suspension to disaggregate the cells and then filter the suspension through nylon mesh. Rinse the filter with 20 ml of **Human Complete Skeletal Muscle PrimaCell™ Culture Medium/FibrOut**.
17. Centrifuge the suspension for 8 – 10 min at 350 g, count the cells, and seed as before.
18. Transfer the flasks to a 37 ˚C humidified incubator with 5% CO_2.
19. Maintenance of Cultures: Change the **Human Complete Skeletal Muscle PrimaCell™ Culture Medium/FibrOut** very gently 24 h after seeding and then every 3-4 d. The development of these cultures is mainly towards differentiation. The timing of the three phases for human muscle cells is about 4-6 d for peak proliferation; then the cells align at about day 8, and around day 10 to 12 an increase in cell fusion and the formation of myotubes are observed. Nevertheless, one must keep in mind that some cells may differentiate earlier and that others will still proliferate when the majority of the culture is undergoing differentiation. Change culture medium to **Human Complete Skeletal Muscle PrimaCell™ Culture Medium** after 3-5 cycles or an acceptable level of fibroblast cell contamination is observed.

Δ ***Safety Note.*** The rest of the biopsy and all tubes, pipettes, plates, etc., used in the procedure should be treated with hypochlorite before disposal.

2.7 Subculture

20. Add a small volume of EDTA gently to the cells and remove it immediately.
21. Add sufficient trypsin solution (0.25%) to form a thin layer over the cells.
22. When cells detach, add 5 to 10 ml of **Human Complete Skeletal Muscle PrimaCell™ Culture Medium**, pass the culture very gently in and out of a pipette, and then centrifuge the

cells for 10 min at 350 g.

23. Count an aliquot and seed the cells at the chosen concentration.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Skeletal muscle cells cultures derived from preconfluent layers.

23. Trypsinize cells as above, and centrifuge at 100 g for 10 min.
24. Resuspend cells in complete **Human Complete Skeletal Muscle PrimaCell™ Culture Medium** and count cells.
25. Prepare aliquots of 2×10^6 cells/ml in **Human Complete Skeletal Muscle PrimaCell™ Culture Medium** and 10% glycerol into cryovials.
26. Equilibrate at 4℃ for 1-2 h.
27. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
28. To recover cells:
    a. Thaw cryovials quickly in a 37℃ water bath.
    b. Dilute cells tenfold with appropriate **Human Complete Skeletal Muscle PrimaCell™ Culture Medium**.
    c. Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in **Human Complete Skeletal Muscle PrimaCell™ Culture Medium**.

IV Characterization

The growth curves of human myogenic cell cultures obtained in Human Complete Skeletal Muscle PrimaCell™ Culture Medium show the three traditional phases: the lag phase, the exponential phase, and the plateau, which corresponds to the onset of fusion. The last, evaluated in terms of the number of nuclei incorporated into myobubes or in terms of a fusion index (the percentage of nuclei incorporated into myotubes relative to the total number of nuclei), commences usually around day 8 after plating and rises dramatically around day 10. According to the sample, this chronology can gain or lose one day. Hence, differentiation, expressed as the number of nuclei per myotube/cm^2, may be observed morphologically. But the differentiation process can also be monitored by the use of biochemical markers (such as the sarcomeric proteins), enzymes involved in differentiation (e.g., creatine phosphokinase and its time-dependent muscle-specific isoform shift), or the appearance of α-actin. A family of genes, the best known of which is MyoD, was shown to activate muscle-specific gene expression in myogenic progenitors. Myogenic cell differentiation involves either the activation of a variety of other genes with concurrent changes in cell surface adhesive properties or the recently shown requirement of cell surface plasminogen activator urokinase and its receptor.

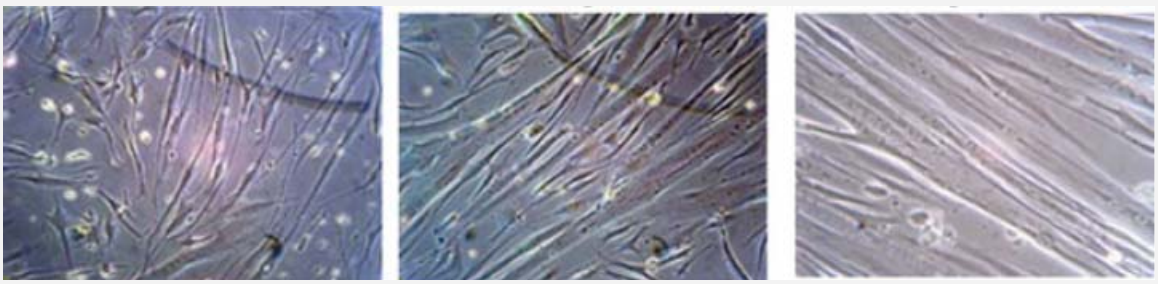


Figure. Light microscopy picture of undifferentiated myoblasts (left), after 4 days of differentiation (middle), and fused multinucleated myotubes after 8 days of differentiation (right) (40 × magnification).[2]

V References

1. Koller, M.R.P., B. O.; Masters, J. R. W., *Human Cell Culture: Primary Mesenchymal Cells* Vol. 5. 2001: Springer.
2. Solberg, R., et al., *Leptin expression in human primary skeletal muscle cells is reduced during differentiation.* J Cell Biochem, 2005. **96**(1): p. 89-96.
3. Askanas, V., A. Bornemann, and W.K. Engel, *Immunocytochemical localization of desmin at human neuromuscular junctions.* Neurology, 1990. **40**(6): p. 949-53.
4. Buckingham, M., *Making muscle in mammals.* Trends Genet, 1992. **8**(4): p. 144-8.
5. Dodson, M.V., B.A. Mathison, and B.D. Mathison, *Effects of medium and substratum on ovine satellite cell attachment, proliferation and differentiation in vitro.* Cell Differ Dev, 1990. **29**(1): p. 59-66.
6. Pegolo, G., V. Askanas, and W.K. Engel, *Expression of muscle-specific isozymes of phosphorylase and creatine kinase in human muscle fibers cultured aneurally in serum-free, hormonally/chemically enriched medium.* Int J Dev Neurosci, 1990. **8**(3): p. 299-308.
7. Quax, P.H., et al., *Modulation of activities and RNA level of the components of the plasminogen activation system during fusion of human myogenic satellite cells in vitro.* Dev Biol, 1992. **151**(1): p. 166-75.

Human Muscle PrimaCell™: Skeletal Muscle Cells

Human Muscle Primary Cell Culture

Cat No.	Description	Qt.	Price
2-96141	**Human Muscle PrimaCell™ system**	**kit**	**$550**
4-26142	Human Muscle Tissue Dissociation System, Muscle OptiTDS™ (for 500 ml medium)	1 ml	$146
9-46014	Human Skeletal Muscle Cells PrimaCell™ Basal Culture Medium	500 ml	$73
9-36142	Human Skeletal Muscle Cells PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$160
7-66142	Human Muscle Fibroblast Growth Inhibitors, Muscle FibrOut™	ea	$195
9-86014	Human Muscle Tissue Preparation Buffer Set	ea	$90

Human Primary Skeletal Muscle Cells Characterization

6-31611	**Human Skeletal Muscle Cell Primarker™ Kit**	**kit**	**$220**
6-31612	Human Skeletal Muscle Cell Primarker™ antibody set	set	$180
6-31613	Human Skeletal Muscle Cell Primarker™ buffer system	set	$90

Human Pancreas PrimaCell™ I: Pancreatic Epithelium
(Cat No. 2-96161)

I. General Description:

This protocol is developed for attachment and growth of normal Human Pancreatic epithelial cells from 1-3 mm^3 biopsies with Human Pancreatic PrimaCell™ system (Cat No. 2-96161). This system provides an optimal condition of tissue dissociation system, Pancreatic OptiTDS™ that yields 4-7 times of single cells more than most of the tissue dissociation protocols published in the literature. In addition, this system ensures a high viability of the target cells with improved gradient contained in the culture medium. With CHI's proprietary fibroblast inhibitory system, Pancreatic FibrOut™, cells are growing with contamination of minimized amount of the non-epithelial cells. The preparation of tissue specimens for cell culture is usually started within 1-2 h of removal from the patient. If this is impossible, fine cutting of the tissue into small pieces (1-2 mm) with scalpels and storage overnight at 4℃ in washing medium (see below) can also prove successful.

Human Pancreatic PrimaCell™ system applies to all types of normal adult Human biopsies samples. Biopsies samples contain pathological organism (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Human Pancreatic PrimaCell™ System

- **Human Pancreatic Tissue Dissociation System, Pancreatic OptiTDS™,** (2 aliquots) --- *A mixture of collagenase I, collagenase III, collagenase IV, collagenase, and trypsin*.
- **Human Pancreatic OptiTDS™ Reconstitution Buffer**, (2 x 1 ml).
- **Human Pancreatic OptiTDS™ Digestion Buffer**, (2 x 10 ml).
- **Human Pancreatic Tissue Washing Medium**, (5 x 10 ml) --- *Basal Pancreatic PrimaCell™ Culture Medium with 5% FBS, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Human Pancreatic Fibroblast Growth Inhibitors, Pancreatic FibrOut™** (5 x 200 μl) --- *A mixture of* anti-Thy-1 *monoclonal antibody, toxin ricin, and formulated serum substitutes*.
- **Human Pancreatic PrimaCell™ Basal Culture Medium**, (5 x100 ml) --- *Modified formulation based on NCTC 168 and Weymouth medium*.
- **Human Pancreatic PrimaCell™ Medium Supplements**, (5 x 1.0 ml) --- A mixture of *ethanolamine, phosphoethanolamine, hydrocortisone, ascorbic acid, transferring, insulin, epidermal growth factor, pentagastrin, and deoxycholic acid*.
- **Human Pancreatic PrimaCell™ Serum**, (1 x 50 ml) --- H*eat-inactivated and special-treated Fetal-bovine serum.*
- **Coating Solution**, (5 x 10 ml) --- *Basal growth medium containing 10 μg/ml BSA.*

1.2 Required Materials but not provided

- Hank's balanced salt solution (HBSS)
- Dispase (Sigma)

- Pasteur pipettes
- Collagen I-coated Culture dishes
- Scalpels, scissors, and forceps
- Pasteur pipettes and 10-ml pipettes
- Test tubes, 12 and 50 ml

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, collagen I-coated plate (Corning, NY) MUST be pre-treated with the provided BSA by adding 5 ml 10 μg/ml BSA in growth medium and incubate for 5 min. Aspirate the BSA solution; let the dishes be air-dry in the ventilated cell culture hood for 5-10 min.

2.2 Surgical specimens

Biopsies of about 1-3 mm3 are taken with biopsy forceps to sample only the mucosal layer and not the muscle layer. Surgical specimens from involved segments of the large intestine should be immediately placed in **Human Pancreatic Tissue Washing Medium** and transported on ice to the laboratory within 1 h and worked up immediately. With autoclaved scalpels, scissors, and forceps, carefully remove muscle and fat from specimens followed by washing procedures. Place specimens in a 10 ml falcon tube contain 5 ml Pancreatic Tissue Washing Medium followed by inoculating for 10 min at the room temperature. For large tissue specimens, 50 ml falcon tube and more wash medium is needed to ensure thoroughly washing. Aspirate the washing medium and repeat the washing procedures with fresh washing medium two more time. Washing tissue specimens sequentially in 70% ethanol for 1 min at the room temperature, in PBS for 5 min, and in fresh Pancreatic Tissue Washing Medium for 5 min. Collecting tissue specimen by centrifugation prior to tissue dissociation procedures (see below).

2.3 Tissue Preparation and Dissociation

2.3.1 Human Pancreatic OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Pancreatic Tissue Dissociation System, OptiTDS™, is suited for optimal dissociation of normal adult Human biopsies samples to yield maximum number of single primary cells of Pancreatic tissues.

2.3.2 Enzyme Compositions

- Collagenase I: from *Clostridium Histolyticum*
- Collagenase III: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*
- Collagenase: from *Clostridium Histolyticum*
- Trypsin: from *Bovine Pancreas*

2.3.3 System Components

- Pancreatic Tissue Dissociation System, OptiTDS™, 2 vials.
- Pancreatic OptiTDS™ Reconstitution Buffer, (2 x 1 ml).
- Pancreatic OptiTDS™ Digestion Buffer, (2 x 9 ml).

2.3.4 Procedures For Tissue Preparation and Dissociation

1. Prepare fresh enzyme working solutions: to each vial of Pancreatic Tissue Dissociation System, OptiTDS™, add 1.0 ml of the Pancreatic OptiTDS™ Reconstitution Buffer, Mix well.
2. Add 1.0 ml of the fresh enzyme working solution to one vial of Pancreatic OptiTDS™ Digestion Buffer (9.0 ml). Warm the diluted enzyme working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 4-5 mg tissue samples per 10 ml diluted enzyme working solutions.
3. Mince pre-washed tissue into pieces approximately 0.2-0.5 mm^2 in diameter with two scalpel and forceps.
4. Incubate minced tissues with the diluted enzyme working solutions by incubating minced tissue samples (up to 5 mg) in 10 ml diluted enzyme working solutions with slow magnetic stirring for 30 min at 37 ℃.
5. At the end of tissue dissociation period, gentle triturating tissue with a 10 ml pipette, constitutes filling the empty the barrel at a rate of 2-3 ml per second. Repeat this procedure for 5-6 times.
6. Collecting cells by filtration the mixture through a cell strainer followed by centrifugation at 1 x 100 g. Carefully remove the medium and resuspend the cell pellet with 1.0 ml complete culture medium.
7. Count the cells and seed cells in 3-4 T-25 collagen I-coated flasks (**Important:** pre-treat the flask with the provided BSA containing growth medium, see below) upon the density of viable cells (2.5-5 x 10^5 Cells/Dish).

2.3.5 Storage:

Reconstituted tissue dissociation systems should occur before use and can only be stored for 2-4 days at 4 ^oC. For long-term use, it should be aliquoted and stored at -20 ^oC. Avoid repeated freeze-thaw cycles.

2.4 Culture of primary Human Pancreatic cells

2.4.1 Medium Preparation.

Thaw out the Human Pancreatic PrimaCell™ Medium Supplements and Human Pancreatic PrimaCell™ Serum on ice. To every 100 ml Human Pancreatic PrimaCell™ Basal Culture Medium, add one vial of Human Pancreatic PrimaCell™ Medium Supplements; 10 ml Human Pancreatic PrimaCell™ Serum; and one vial of Human Pancreatic Fibroblast Growth Inhibitors, Pancreatic FibrOut™, mix thoroughly and warm the complete medium at a 37^oC water bath for 10 min prior use.

2.4.2 Treatment of Culture Dishes.

To enhance cell attachment to the culture dishes, collagen I-coated plate (Corning, NY) MUST be pre-treated with the provided Coating Solution (Basal growth medium containing 10 µg/ml BSA)

by adding appropriate volume of the Coating Solution (enough to cover the whole cell-growth area) and incubate for 5 min. Aspirate the BSA solution; let the dishes be air-dry in the ventilated cell culture hood for 5-10 min.

.

2.4.3 Standard primary culture conditions.

Inoculate epithelial tubules and clumps of cells derived from tissue specimens into T-25 flasks coated with collagen type I with pre-treatment of coating solution at 37℃ in a 5%-CO_2 incubator with 4 ml of complete culture medium. Change the culture medium twice weekly. The tubules and cells start to attach to the substratum, and epithelial cells migrate out within 1-2 d. Most of the tubules and small clumps of epithelium attach within 7 d, but the larger organoids can take up to 6 weeks to attach, although they will remain viable all that time.

2.4.4 Alternative Primary Culture conditions.

The attachment of epithelium during primary culture and subculture is more reproducible and efficient when cells are inoculated onto collagen-coated flasks, and significantly better growth is obtained with 3T3 feeders than without. When the epithelial Breasties expand to several hundred cells per Breasty, they become less dependent on 3T3 feeders, and no further addition of feeders is necessary. All medium and solutions described in the standard culture condition are applicable in this culture method.

2.4.5 Subculture and Propagation

Most pancreatic primary cultures cannot at present be passaged by routine trypsin / EDTA procedures. Disaggregation to single cells of the cultured pancreatic cells with 0.1% trypsin in 0.25 mM (0.1%) EDTA will result in extremely poor or even zero growth, so Dispase is used instead. One of the advantage using dispase is that dispase can only detach epithelial cell but not the fibroblast, thus increase the purity of epithelium.

8. Add 0.5% Dispase (Sigma, w/v) to the cell monolayer, just enough to cover the cells (~2.5 ml/25-cm^2 flask), and leave the solution to stand for 40-60 min for primary cultures and 20-40 min for cell lines.
9. Once the epithelial layers begin to detach (they do so as sheets rather than single cells), pipette to help detachment and disaggregation into smaller clumps.
10. Wash and replate the cells under standard culture conditions. It may take several days for clumps to attach, so replace the medium carefully when feeding.

III Fibroblast Contamination

There are several techniques have been published in the literature to deal with fibroblast contamination during pancreatic primary cell culture. These include: (1) Physically remove a well-isolated fibroblast Breasty by scraping it with a sterile blunt instrument (e.g., a cell scraper). Care has to be taken to wash the culture up to six times to remove any fibroblasts that have detached in order to prevent them from reseeding and reattaching to the flask. (2) Differential trypsinization can be attempted with the carcinomas. (3) Dispase preferentially (but not exclusively) removes the epithelium during passaging and leaves behind most of the fibroblastic cells attached to the culture vessel. During subculture, cells that have been removed with dispase can be preincubated in plastic Petri dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the epithelium. Clumps of epithelial cells

still floating can be transferred to new flasks under standard culture conditions. This technique takes advantage of the fact that fibroblasts in general attach much more quickly to plastic than do clumps of epithelial cells, so that a partial purification step is possible. (4) Reduce the concentration of serum to about2.5-5% if there are heavy concentrations of fibroblastic cells. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and that using any or all of the preceding techniques will eventually push the cells through so many divisions that any fibroblasts will senesce.

Human Pancreatic PrimaCell™ includes a fibroblast elimination system, the Human Pancreatic Fibroblast Growth Inhibitors, Pancreatic FibrOut™. It contains a mixture of anti-Thy-1 monoclonal antibody, toxin ricin and formulated serum substitutes. Thy-1 antigen is present on pancreatic fibroblasts, but not pancreatic epithelial cells; therefore, the conjugate kills contaminating fibroblasts, but shows no signs of toxicity toward the epithelium, whether derived from an adenoma, a carcinoma or normal Pancreatic tissues.

IV References:

1. Youngman KR, Simon PL, West GA, Cominelli F, Rachmilewitz D, Klein JS, Fiocchi C: Localisation of intestinal interleukin 1 activity and protein and gene expression to lamina propria cells. Gastroenterology 1993, 104:749-758.
2. Gibson PR, van de Pol E, Maxwell LE, Gabriel A, Doe WF: Isolation of Pancreatic crypts that maintain structural and metabolic viability in vitro. Gastroenterology 1989, 96:283-291.
3. Whitehead RH, Brown A, Bhathel PS: A method for the isolation and culture of Human Pancreatic crypts in collagen gels. In Vitro 1986, 23:436-442.
4. Knoll N, Weise A, Claussen U, Sendt W, Marian B, Glei M, Pool-Zobel BL. 2-Dodecylcyclobutanone, a radiolytic product of palmitic acid, is genotoxic in primary Human Pancreatic cells and in cells from preneoplastic lesions.
5. Buset M, Winawer S, Friedman E. Defining conditions to promote the attachment of adult Human Pancreatic epithelial cells.

Human Pancreas PrimaCell™: Pancreatic Epithelium

Human Pancreas Primary Cell Culture

Cat No.	Description	Qt.	Price
2-96161	**Human Pancreas PrimaCell™ system**	**kit**	**$550**
4-26162	Human Pancreas Tissue Dissociation System, Pancreas OptiTDS™ (for 500 ml medium)	1 ml	$146
9-46016	Human Pancreatic Epithelium PrimaCell™ Basal Culture Medium	500 ml	$73
9-36162	Human Pancreatic Epithelium PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$160
7-66162	Human Pancreas Fibroblast Growth Inhibitors, Pancreas FibrOut™	ea	$195
9-86016	Human Pancreas Tissue Preparation Buffer Set	ea	$90

Human Primary Pancreatic Epithelium Characterization

6-31711	**Human Pancreatic Epithelium Primarker™ Kit**	**kit**	**$220**
6-31712	Human Pancreatic Epithelium Primarker™ antibody set	set	$180
6-31713	Human Pancreatic Epithelium Primarker™ buffer system	set	$90

Human Prostate PrimaCell™: Prostate Epithelium
(Cat No. 2-96171)

I. General Description:

This protocol is developed for attachment and growth of normal Human Prostate epithelial cells from newborn or adult Human Prostate with Human Prostate PrimaCell™ system (Cat No. 2-96171). This system provides an optimal condition of tissue dissociation system, Prostate OptiTDS™ that yields 4-7 times of single cells more than most of the tissue dissociation protocols published in the literature. In addition, this system ensures a high viability of the target cells with improved gradient contained in the culture medium. With CHI Scientific's proprietary fibroblast inhibitory system, FibrOut™, cells are growing with contamination of minimized amount of the non-epithelial cells. This procedure involves explanting fragments of large Prostate tissue in a serum-free medium (LHC-9) in order to initiate and subsequently propagate fibroblast-free outgrowths of prostate epithelial cells; four subculturings and 30 population doublings are routine.

Human Prostate PrimaCell™ system applies to all type tissue samples from human at all age though younger tissue samples are recommended for yielding maximum amount of viable target cells. However, tissue samples contain pathological organism (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Human Prostate PrimaCell™ System

- **Prostate Tissue Dissociation System, Prostate OptiTDS™,** (2 aliquots) --- *A mixture of collagenase I, collagenase II, collagenase IV, dispase and trypsin*.
- **Prostate OptiTDS™ Reconstitution Buffer**, (2 x 1 ml).
- **Prostate OptiTDS™ Digestion Buffer**, (2 x 10 ml).
- **Coating Solution:** *A Mixure of Human fibronectin, collagen, and crystallized bovine serum albumin (BSA), in basal culture medium.*
- **Prostate Tissue Washing Medium**, (1 x 100 ml) --- *Basal Prostate PrimaCell™ Culture Medium with 5% FBS, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Prostate Tissue Healing Medium,** (1 x 100 ml) --- *A mixture of basal culture medium containing insulin; hydrocortisone; β-retinyl acetate; glutamine; penicillin; stretomycin (50 ug/ml); gentamucin; fungison; FBS.*
- **Human Prostate Fibroblast Growth Inhibitors, Prostate FibrOut™** (5 x 200 μl) --- *A mixture of cis-OH-proline, collagenase, D-valine, and formulated serum substitutes*.
- **Human Prostate PrimaCell™ Basal Culture Medium**, (5 x100 ml) --- *Modified formulation based on medium 199 and Weymouth medium*.
- **Human Prostate PrimaCell™ Medium Supplements**, (5 x 1.0 ml) --- A mixture of *of Human fibronectin, collagen, and crystallized bovine serum albumin (BSA)*.
- **Human Prostate PrimaCell™ Serum**, (1 x 50 ml) --- *Highly purified special-treated Fetal-bovine serum.*

1.2 Required Materials but not provided

- Plastic tissue culture dishes (60 and 100 mm)
- Scalpels No. 1621 (Becton Dickinson)
- Surgical scissors
- Half-curved microdissecting forceps
- Pipettes (10 and 25 ml)
- Trypsin (Cooper Biomedical), 0.02%, EGTA (Sigma), 0.5 mM, and polyvinylpyrrolidine (USB), 1% solution
- High-O2 gas mixture (50% O2, 45% N2, 5% CO2)
- Gloves sterilized with autoclave (Human tissue can be contaminated with biologically hazardous agents)
- Controlled atmosphere chamber
- Rocker platform
- Phase-contrast inverted microscope

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, culture plate (Corning, NY) must be coated with the provided coating solution.

2.2 Surgical specimens

1. Prostate tissue from autopsy of noncancerous donors is recommended for yielding a large number of cells with a 30-40% plating efficiency.
2. Incubate Prostate tissue biopsies for up to 10 min in Prostate Tissue Washing Medium to prevent infection. (This procedure should not affect cell viability.)
3. Incubate Prostate biopsies in 10 ml 70% sterile ethanol for 2 min.
4. Rinse tissue twice in Prostate Tissue Washing Medium for 10 min each and kept on ice.

2.3 Tissue Preparation and Dissociation

2.3.1 Human Prostate OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Human Prostate Tissue Dissociation System, OptiTDS™, is suited for optimal dissociation of normal adult and newborn Prostate biopsies samples to yield maximum number of single primary cells of colonic tissues.

2.3.2 Enzyme Compositions

- Trypsin: from *Bovine Pancreas*
- Dispase: from *Bacillus polymyxa*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase II: from *Clostridium Histolyticum*

- Collagenase IV: from *Clostridium Histolyticum*

2.3.3 System Components

- Prostate Tissue Dissociation System, OptiTDS™, 2 vials.
- Prostate OptiTDS™ Digestion Buffer, (2 x 9 ml).

2.3.4 Procedures For Tissue Preparation and Dissociation

5. Prepare fresh enzyme working solution: Add 1ml of the reconstituted tissue dissociation solution to one vial of Human Prostate OptiTDS™ Digestion Buffer (9 ml). Warm the diluted Human Prostate OptiTDS™ working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 5 ml Human Prostate OptiTDS™ working solutions.
6. Discard the **Human Prostate Washing Medium,** and float samples on 5 ml of Human Prostate Tissue Dissociation System working solution (prewarmed to 37℃) and transfer the minced tissue to a sterile centrifuge tube (10–12 pancreases each).
7. The tissue was digested in a shaking water bath at 37 C for 10-30 min.

2.5 Prostate Epithelial cells Isolation

Note: Please read section 2.5 for specific information on Cervical Epithelium culture and platting before preceding this section.

8. The resulting digests were washed three times with cold **Human Prostate Washing Medium.**
9. The pellets were resuspended in **Human Complete Prostate PrimaCell™ Culture Medium/FibrOut™** and distributed in 50 mm plastic culture dishes.
10. The Prostates were cultured for 5 days in 5 ml **Human Complete Prostate PrimaCell™ Culture Medium/FibrOut™** at 37 ^{o}C in a humidified atmosphere of 5% CO2.

2.6 Primary Prostate Epithelial Cells Culture

2.6.1 Medium Preparation.

Thaw out **Human Prostate PrimaCell™ Basal Culture Medium**, **Human Prostate cell PrimaCell™ Medium Supplements**, and **Human Prostate PrimaCell™ Serum** on ice.

Human Complete Prostate PrimaCell™ Culture Medium: To every 100 ml **Human Prostate PrimaCell™ Basal Culture Medium**, add 10 ml **Human Prostate PrimaCell™ Medium Supplements** and 1 ml **Human Prostate PrimaCell™ Serum** mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use.

Human Complete Prostate PrimaCell™ Culture Medium/FibrOut: To every 100 ml **Human Prostate PrimaCell™ Basal Culture Medium**, add 10 ml **Human Prostate PrimaCell™ Medium Supplements,** 1 ml **Human Prostate PrimaCell™ Serum**, and 1 ml **Human Prostate Fibroblast Growth Inhibitors, Prostate FibrOut™**,mix thoroughly and warm the complete medium at a 37^{o}C water bath for 10 min prior use. (**Important:** *Long term use of FibrOut™ in the culture medium may result loss of targeted primary cells, therefore, we recommend using FibrOut™ with 3-5 cycles of changing culture medium or to*

an acceptable level of fibroblast cell contamination)

2.6.2 Primary Cell Culture

11. The medium was changed every day. At the end of the preculture period, the Prostates attached to the bottom of the culture dishes were gently blown free using a sterilized Pasteur pipette under a stereomicroscope. The fibroblast layer remaining on the bottom of the culture dishes
12. The detached Prostates were cultured free-floating in 50 mm Petri dishes in **Human Complete Prostate PrimaCell™ Culture Medium/FibrOut™** changed every other day.

Δ ***Safety Note.*** The rest of the biopsy and all tubes, pipettes, plates, etc., used in the procedure should be treated with hypochlorite before disposal.

2.5.3 Subculture and Propagation

13. Gently rinse the culture dish twice with 0.02% (0.7 mM) EDTA.
14. Add 3 ml of 0.25% trypsin/0.1% (2.5 mM) EDTA, and incubate at 37℃. Examine the dish under phase microscopy every 5 min to detect cell detachment.
15. When most cells have detached, add 12 ml complete melanocyte growth medium to inactive the trypsin activity.
16. Pipette the contents of the dish to ensure complete melanocyte melanocyte detachment.
17. Aspirate and centrifuge the cells for 5 min at 350 g.
18. Aspirate the supernatant, resuspend the cells in a complete growth medium, and replate at 2-4 $\times$ 10^4 cells per 100-mm dish.
19. Refeed the culture twice a week with complete melanocyte growth medium.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample; the best results are reported when cells from preconfluent primary cultures are used.

20. Detach cells as for the subculture, and centrifuge at 100 g for 10 min.
21. Resuspend cells in complete culture medium with serum, and count.
22. Dispense aliquots of 2 x 10^6 cells/ml in complete growth medium with additional 10% FBS and 10% glycerol into cryopreservation tubes.
23. Equilibrate at 4℃ for 1-2 h.
24. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
25. To recover cells:
    (i) Thaw cryotubes quickly in a 37℃ water bath.
    (ii) Dilute cells tenfold with medium.
    (iii) Centrifuge cells and resuspend them at an appropriate concentHumanion in the desired culture medium, and seed culture vessel.

Human cells can be grown in all media for 4-7 weeks and can be subcultured only 4-5 times.

IV Fibroblast Contamination

There are several techniques have been published in the literature to deal with fibroblast contamination during pancreatic primary cell culture. These include: (1) Physically remove a well-isolated fibroblast colony by scraping it with a sterile blunt instrument (e.g., a cell scraper). Care has to be taken to wash the culture up to six times to remove any fibroblasts that have detached in order to prevent them from reseeding and reattaching to the flask. (2) Differential trypsinization can be attempted with the carcinomas. (3) Dispase preferentially (but not exclusively) removes the epithelium during passaging and leaves behind most of the fibroblastic cells attached to the culture vessel. During subculture, cells that have been removed with dispase can be preincubated in plastic petri dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the epithelium. This technique takes advantage of the fact that fibroblasts in general attach much more quickly to plastic than do clumps of epithelial cells, so that a partial purification step is possible. (4) Reduce the concentration of serum to about 2.5-5% if there are heavy concentrations of fibroblastic cells. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and that using any or all of the preceding techniques will eventually push the cells through so many divisions that any fibroblasts will senesce.

Human Prostate PrimaCell™ I system includes a fibroblast elimination system, the Human Prostate Fibroblast Growth Inhibitors, Prostate FibrOut™. It contains a mixture of of cis-OH-proline, collagenase, D-valine, and formulated serum substitutes. This system can effectively eliminate Prostate fibroblast contamination while has not affect on the behavior of targeted cells.

VI References:

1. Yim HW, Slebos RJ, Randell SH, Umbach DM, Parsons AM, Rivera MP, Detterbeck FC, Taylor JA. *Smoking is associated with increased telomerase activity in short-term cultures of Human Prostate epithelial cells.* Cancer Lett. 2006 Mar 3;
2. Doherty GM, Christie SN, Skibinski G, Puddicombe SM, Warke TJ, de Courcey F, Cross AL, Lyons JD, Ennis M, Shields MD, Heaney LG. *Non-bronchoscopic sampling and culture of Prostate epithelial cells in children.* Clin Exp Allergy. 2003 Sep;33(9):1221-5.
3. Mattinger C, Nyugen T, Schafer D, Hormann K. *Evaluation of serum-free culture conditions for primary Human nasal epithelial cells.* Int J Hyg Environ Health. 2002 Apr;205(3):235-8.
4. de Jong PM, van Sterkenburg MA, Kempenaar JA, Dijkman JH, Ponec M. *Serial culturing of Human Prostate epithelial cells derived from biopsies.* In Vitro Cell Dev Biol Anim. 1993 May;29A(5):379-87.
5. Robbins RA, Koyama S, Spurzem JR, Rickard KA, Nelson KJ, Gossman GL, Thiele GM, Rennard SI. *Modulation of neutrophil and mononuclear cell adherence to Prostate epithelial cells.* Am J Respir Cell Mol Biol. 1992 Jul;7(1):19-29.
6. Lechner JF, Wang Y, Siddiq F, Fugaro JM, Wali A, Lonardo F, Willey JC, Harris CC, Pass HI. *Human lung cancer cells and tissues partially recapitulate the homeobox gene expression profile of embryonic lung.* Lung Cancer. 2002 Jul;37(1):41-7.
7. Lechnewr JF, & LeVeck MA. *A serum free method for culturing normal Human Prostate epithelial cells at clonal density.* J. Tissue Cult. Methods 9: 43-48.

Human Prostate PrimaCell™: Prostate Epithelium

Human Prostate Primary Cell Culture

Cat No.	Description	Qt.	Price
2-96171	**Human Prostate PrimaCell™ system**	**kit**	**$550**
4-26172	Human Prostate Tissue Dissociation System, Prostate OptiTDS™ (for 500 ml medium)	1 ml	$146
9-46017	Human Prostate Epithelium PrimaCell™ Basal Culture Medium	500 ml	$73
9-36172	Human Prostate Epithelium PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$160
7-66172	Human Prostate Fibroblast Growth Inhibitors, Prostate FibrOut™	ea	$195
9-86017	Human Prostate Tissue Preparation Buffer Set	ea	$90

Human Primary Prostate Epithelium Characterization

6-31911	Human Prostate Epithelium Primarker™ Kit	kit	$220
6-31912	Human Prostate Epithelium Primarker™ antibody set	set	$180
6-31913	Human Prostate Epithelium Primarker™ buffer system	set	$90

Human Skin PrimaCell™ I: Melanocytes
(Cat No. 2-96181)

I. General Description:

This protocol is developed for attachment and growth of normal human skin melanocytes from newborn or adult human skin with Human Skin PrimaCell™ I system (Cat No. 2-96181). This system provides an optimal condition of tissue dissociation system, Skin OptiTDS™ that yields 4-7 times of single cells more than most of the tissue dissociation protocols published in the literature. In addition, this system ensures a high viability of the target cells with improved gradient contained in the culture medium. With CHI's proprietary fibroblast inhibitory system, FibrOut™, cells are growing with contamination of minimized amount of the non-epithelial cells. The preparation of tissue specimens for cell culture is usually started within 1-2 h of removal from mice. If this is impossible, fine cutting of the tissue into small pieces (2 x 2 mm) with scalpels and storage overnight at 4℃ in washing medium (see below) can also prove successful.

Human SkinPrimaCell™ I system applies to all types skin samples from mice at all age though newborn mice are recommended for yielding maximum amount of viable target cells. Skin samples contain pathological organism (virus, parasites, etc.) or tumor may not suitable for this system.

1.1 Components of Human Skin PrimaCell™ I System

- **Skin Tissue Dissociation System, Skin OptiTDS™,** (2 aliquots) --- *A mixture of collagenase I, collagenase II, collagenase IV, dispase and trypsin*.
- **Skin OptiTDS™ Reconstitution Buffer**, (2 x 1 ml).
- **Skin OptiTDS™ Digestion Buffer**, (2 x 10 ml).
- **Skin Tissue Washing Medium**, (5 x 10 ml) --- *Basal Skin PrimaCell™ I Culture Medium with 5% FBS, 200 u/ml of penicillin, 200 μg/ml of streptomycin, and 50 μg/ml of gentamycin*.
- **Human Skin Fibroblast Growth Inhibitors, Skin FibrOut™** (5 x 200 μl) --- *A mixture of cis-OH-proline, collagenase, D-valine, and formulated serum substitutes*.
- **Human Skin PrimaCell™ I Basal Culture Medium**, (5 x100 ml) --- *Modified formulation based on medium 199 and Weymouth medium*.
- **Human Skin PrimaCell™ I Medium Supplements**, (5 x 1.0 ml) --- A mixture of *basic fibroblast growth factor, cholera toxin, hydrocortisone, Insulin, transferring, insulin, and epidermal growth factor*.
- **Human Skin PrimaCell™ I Serum**, (1 x 50 ml) --- *Highly purified special-treated Fetal-bovine serum.*

1.2 Required Materials but not provided

- Pasteur pipettes
- Collagen I-coated Culture dishes
- Scalpels, scissors, and forceps
- Pasteur pipettes and 10-ml pipettes

- Test tubes, 12 and 50 ml
- Nylon gauze cell strainer

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, collagen I-coated plate (Corning, NY) is recommended.

2.2 Surgical specimens

1. Newborn (prefer 1-2 days) mice epidermis is recommended for yielding a large number of cells (5-10 x 10^6/epidermis), with a 30-40% plating efficiency. Mice are sacrificed by CO_2 narcosis 1-4 days postpartum (prior to the appearance of hair). Using an aseptic technique, limbs and tails are amputated, a longitudinal incision is made from tail to snout, and skin is peeled off the carcass using forceps.
2. Incubate skin biopsies for up to 10 min in Skin Tissue Washing Medium to prevent infection. (This procedure should not affect Melanocytes viability.)
3. Incubate skin biopsies in 10 ml 70% sterile ethanol for 2 min.
4. Rinse tissue twice in Skin Tissue Washing Medium for 10 min each and kept on ice.

2.3 Tissue Preparation and Dissociation

2.3.1 Human Skin OptiTDS™

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Human Skin Tissue Dissociation System, OptiTDS™, is suited for optimal dissociation of normal adult and newborn skin biopsies samples to yield maximum number of single primary cells of colonic tissues.

2.3.2 Enzyme Compositions

- Trypsin: from *Bovine Pancreas*
- Dispase: from *Bacillus polymyxa*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase II: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*

2.3.3 System Components

- Skin Tissue Dissociation System, OptiTDS™, 2 vials.
- Skin OptiTDS™ Reconstitution Buffer, (2 x 1 ml).
- Skin OptiTDS™ Digestion Buffer, (2 x 9 ml).

2.3.4 Procedures For Tissue Preparation and Dissociation

5. Prepare fresh enzyme working solutions: to each vial of Skin Tissue Dissociation

System, OptiTDS™, add 1.0 ml of the Skin OptiTDS™ Reconstitution Buffer, Mix well.

6. Add 1.0 ml of the fresh enzyme working solution to one vial of Skin OptiTDS™ Digestion Buffer (9.0 ml). Warm the diluted Skin OptiTDS™ working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 10 ml diluted Skin OptiTDS™ working solutions.
7. Mince pre-washed tissue into pieces approximately 0.2-0.5 mm^2 in diameter with two scalpel and forceps.
8. Incubate tissue with Skin Dissociation System by one of the following steps:
   - Rapid dissociation: Float skin samples in Skin Dissociation System solution for 2-3 h min at 37 ℃. This works particularly well also with full-thickness skin.
   - Slow dissociation: Float the samples on ice-cold Skin Dissociation System at 4℃ for 15-24 h.
9. Monitor the separation of the epidermis carefully. When the first detachment of the epidermis is visible at the cut edges of skin samples, carefully separate epidermis from dermis. Place the epidermis (dermis side down) in 100-mm plastic Petri dishes and irrigate with 10 ml fresh Skin OptiTDS™ solution.
10. Once all pelts have been processed, use scissors to cut epidermis and disrupt beta pleated sheets in the epidermis. Transfer all solution containing skin samples into a sterile beaker or container.
11. Rinse sterile stir bar in PBS and place in cell mixture and stir gently for 20-30 min.
12. Rinse woven cloth 2X in PBS and place into sterile funnel on top of sterile beaker and strain the cell mixture to remove debris (alternatively, a Nylon gauze cell strainer can be used).
13. Passing the cell mixture with sterile pipette several times to facilitate a single cell mixture, pass through the Nylon gauze cell strainer.
14. Collect cells by centrifugation at 100 g, washing cells with 10 ml PBS or washing medium twice. At the end of washing process, collect cells and dilute cell in 0.5-1.0 ml complete culture medium.
15. Count viable cells and plate 1.0 x 10^6/ 100mm dish.
16. Seed cells at 37℃ in Complete Human Skin PrimaCell™ I Culture Medium at desired densities:

2.3.5 Storage:

Reconstituted tissue dissociation systems should occur before use and can only be stored for 2-4 days at 4 ^oC. For long-term use, it should be aliquoted and stored at -20 ^oC. Avoid repeated freeze-thaw cycles.

2.4 Culture of Primary Human Melanocytes

2.4.1 Medium Preparation.

Thaw out the Human Skin PrimaCell™ Medium Supplements and Human Skin PrimaCell™ I Serum on ice. To every 100 ml Human Skin PrimaCell™ I Basal Culture Medium, add one vial of Human Skin PrimaCell™ I Medium Supplements; 10 ml Human Skin PrimaCell™ I Serum; and one vial of Human Skin Fibroblast Growth Inhibitors, Skin FibrOut™, mix thoroughly and warm the complete medium at a 37^oC water bath for 10 min prior use.

2.6.1 Primary Culture Conditions.

Inoculate epithelial cells derived from tissue specimens contains both Melanocytes and Melanocytes. Seed cells into T-25 flasks coated with collagen type I in a 5%-CO_2 incubator with 4 ml of complete culture medium. Change the culture medium twice weekly. The cultures will contain primary Melanocytes with scattered melanocytes. Melanocytes proliferation should cease within several days, and colonies should begin to detach during the second week. By the end of the third week, only melanocytes should remain. In most cases, cultures attain near confluence and are ready to passage within 2-4 weeks.

2.4.3 Subculture and Propagation

17. Gently rinse the culture dish twice with 0.02% (0.7 mM) EDTA.
18. Add 3 ml of 0.25% trypsin/0.1% (2.5 mM) EDTA, and incubate at 37℃. Examine the dish under phase microscopy every 5 min to detect cell detachment.
19. When most cells have detached, add 12 ml complete melanocyte growth medium to inactive the trypsin activity.
20. Pipette the contents of the dish to ensure complete melanocyte melanocyte detachment.
21. Aspirate and centrifuge the cells for 5 min at 350 g.
22. Aspirate the supernatant, resuspend the cells in a complete growth medium, and replate at 2-4 $\times$ 10^4 cells per 100-mm dish.
23. Refeed the culture twice a week with complete melanocyte growth medium.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample; the best results are reported when cells from preconfluent primary cultures are used.

24. Detach cells as for the subculture, and centrifuge at 100 g for 10 min.
25. Resuspend cells in complete culture medium with serum, and count.
26. Dispense aliquots of 2 x 10^6 cells/ml in complete growth medium with additional 10% FBS and 10% glycerol into cryopreservation tubes.
27. Equilibrate at 4℃ for 1-2 h.
28. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
29. To recover cells:
    (i) Thaw cryotubes quickly in a 37℃ water bath.
    (ii) Dilute cells tenfold with medium.
    (iii) Centrifuge cells and resuspend them at an appropriate concentration in the desired culture medium, and seed culture vessel.

Human cells can be grown in all media for 4-7 weeks and can be subcultured only 4-5 times.

IV Fibroblast Contamination

There are several techniques have been published in the literature to deal with fibroblast contamination during pancreatic primary cell culture. These include: (1) Physically remove a well-isolated fibroblast colony by scraping it with a sterile blunt instrument (e.g., a cell scraper). Care has to be taken to wash the culture up to six times to remove any fibroblasts that have detached in order to prevent them from reseeding and reattaching to the flask. (2) Differential trypsinization can be attempted with the carcinomas. (3) Dispase

preferentially (but not exclusively) removes the epithelium during passaging and leaves behind most of the fibroblastic cells attached to the culture vessel. During subculture, cells that have been removed with dispase can be preincubated in plastic petri dishes for 2-6 h to allow the preferential attachment of any fibroblasts that may have been removed together with the epithelium. This technique takes advantage of the fact that fibroblasts in general attach much more quickly to plastic than do clumps of melanocytes, so that a partial purification step is possible. (4) Reduce the concentration of serum to about 2.5-5% if there are heavy concentrations of fibroblastic cells. It is worth remembering that normal fibroblasts have a finite growth span *in vitro* and that using any or all of the preceding techniques will eventually push the cells through so many divisions that any fibroblasts will senesce.

Human Skin PrimaCell™ I system includes a fibroblast elimination system, the Human Skin Fibroblast Growth Inhibitors, Skin FibrOut™. It contains a mixture of of cis-OH-proline, collagenase, D-valine, and formulated serum substitutes. This system can effectively eliminate skin fibroblast contamination while has not affect on the behavior of melanocytes.

V Confirmation of Melanocytic Identity

Melanocyte cultures may be contaminated initially with melanocytes and at any time by dermal fibroblasts. Both forms of contamination are rare in cultures established and maintained by an experienced technician or investigator, but are common problems for the novice. The cultured cells can be confirmed to be melanocytes with moderate certainty by frequent examination of the culture under phase microscopy, assuming that the examiner is familiar with the respective cell morphologies. More definitive identification is provided by electron microscopic examination, DOPA staining, or immunofluorescent staining with Mel 5 antibody, directed against tyrosinase-related protein-1.

VI References:

1. Naeyaert JM, Eller M, Gordon PR, Park HY, Gilchrest BA. *Pigment content of cultured human melanocytes does not correlate with tyrosinase message level.* Br J Dermatol. 1991 Oct;125(4):297-303.
2. Gilchrest BA, Vrabel MA, Flynn E, Szabo G. *Selective cultivation of human melanocytes from newborn and adult epidermis.* J Invest Dermatol. 1984 Nov;83(5):370-6.
3. Wilkins L, Gilchrest BA, Szabo G, Weinstein R, Maciag T. *The stimulation of normal human melanocyte proliferation in vitro by melanocyte growth factor from bovine brain.* J Cell Physiol. 1985 Mar;122(3):350-61.
4. Naeyaert JM, Eller M, Gordon PR, Park HY, Gilchrest BA. *Pigment content of cultured human melanocytes does not correlate with tyrosinase message level.* Br J Dermatol. 1991 Oct;125(4):297-303.
5. Park HY, Gilchrest BA. *Protein kinase C: biochemical characteristics and role in melanocyte biology.* J Dermatol Sci. 1993 Dec;6(3):185-93. Review.
6. Guyonneau L, Murisier F, Rossier A, Moulin A, Beermann F. *Melanocytes and pigmentation are affected in dopachrome tautomerase knockout mice.* Mol Cell Biol. 2004 Apr;24(8):3396-403.
7. Hirobe T, Furuya R, Ifuku O, Osawa M, Nishikawa S. *Granulocyte-macrophage colony-stimulating factor is a keratinocyte-derived factor involved in regulating the proliferation and differentiation of neonatal human epidermal melanocytes in culture.* Exp Cell Res. 2004 Jul 15;297(2):593-606.

8. Hirobe T, Osawa M, Nishikawa S. *Hepatocyte growth factor controls the proliferation of cultured epidermal melanoblasts and melanocytes from newborn mice.* Pigment Cell Res. 2004 Feb;17(1):51-61.
9. Hirobe T. *Endothelins are involved in regulating the proliferation and differentiation of human epidermal melanocytes in serum-free primary culture.* J Investig Dermatol Symp Proc. 2001 Nov;6(1):25-31.
10. Morgan AC Jr, McIntyre RF. *Monoclonal antibody defined-human melanoma-associated antigens: molecular and phylogenetic studies in normal serum.* Mol Immunol. 1983 Dec;20(12):1293-9.

Human Skin PrimaCell™ I: Melanocytes

Human Skin Primary Cell Culture

Cat No.	Description	Qt.	Price
2-96181	**Human Skin PrimaCell™ I system**	**kit**	**$ 550**
4-20182	Human Skin Tissue Dissociation System, Skin OptiTDS™ (for 500 ml medium)	1 ml	$146
9-46018	Human Melanocytes PrimaCell™ Basal Culture Medium	500 ml	$73
9-36182	Human Melanocytes PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$ 160
7-66182	Human Skin Fibroblast Growth Inhibitors, Skin FibrOut™	ea	$ 195
9-86018	Human Skin I Tissue Preparation Buffer Set	ea	$90

Human Primary Melanocytes Characterization

6-32011	**Human Melanocytes Primarker™ Kit**	**kit**	**$220**
6-32012	Human Melanocytes Primarker™ antibody set	set	$180
6-32013	Human Melanocytes Primarker™ buffer system	set	$90

Human Skin PrimaCell™ II: Epidermal Keratinocytes
(Cat No. 2-96191)

I. General Description

Keratinocytes have a unique biology and thus are widely used for experiments to study the activity of oncogenes in epithelial neoplasias, and the molecular mechanisms implicated in warts and other skin associated disorders. In addition, several *in vitro* skin models have been developed that accurately mimic the epidermis making it possible to study the skin in a physiologically relevant context. While these cells are extremely useful in the laboratory they are notoriously difficult to isolate and culture.

The Human Skin PrimaCell™ II kit (Cat No. 2-96191) is designed for the successful isolation and culture of epidermal keratinocytes from human skin allowing reproducible and dependable results.

1.1 Components of the Human Skin PrimaCell™ II System

- **Human Skin Tissue Dissociation System II, Skin OptiTDS™ II** (2 aliquots) --- *A mixture of collagenase I, collagenase III, collagenase IV, collagenase, and trypsin*.
- **Human Skin OptiTDS™ II Reconstitution Buffer,** (2 x 1 ml)
- **Human Skin OptiTDS™ II Digestion Buffer,** (4 x 4.5 ml)
- **Human Skin Fibroblast Growth Inhibitors** ---Skin FibrOut™ (5 x 200 µl) --- *A mixture of toxin ricin, and formulated serum.*
- **Human Skin Keratinocytes Basic Culture Medium** (5 x 95 ml) --- *Modified formulation based on NCTC 168 and Weymouth medium*.
- **Human Skin Keratinocytes Culture Medium Supplements with Serum** (5 x 5 ml): *insulin, hydrocortisone, EGF, transferring, highly purified serum substitute, and L-ascorbic acid, $CaCl_2$, Bovine Pituitary Extract*, *antibiotics (penicillin, 100 U/ml), and streptomycin.*
- **Buffer Systems for Human Skin Preparation** (1 x 100 ml): Basal Culture medium containing Betadine solution.

1.2 Required materials but NOT included:

- Sterile Centrifuge tube, 50 ml
- Sterile nylon gauze
- Cell Strainer (BD Bioscience)
- Petri dishes, bacteriological grade, 100 mm
- Scalpels, curved forceps
- 3T3 cells or human fibroblast (optional)
- Collagen solution (Vitrogen 100)
- PBSA (PBS containing 10% BSA), sterile.

II. Procedures

2.1 Material Preparation

All materials used in this experiment must be sterile or autoclaved to prevent contamination. To enhance cell attachment to the culture dishes, fresh gelatin-coated plate or culture dishes are recommended (see below for treatment of culture dishes).

2.2 Principle

Separation of the epidermis from the dermis is accomplished by enzymatic digestion using the Skin Tissue Dissociation System II supplied in this kit. The Skin Tissue Dissociation System II contains a mixture of trypsin, dispase and type I, III, and IV collagenases at the optimal concentrations to gently detach the fragile epidermal layer from the dermis. The isolated epidermis is then further disrupted to release individual keratinocytes by enzymatic and mechanical agitation. The mixture is then filtered through Cell Strainers and seeded on specially coated tissue culture plates. The Keratinocytes are propagated in serum-free, low-calcium media or growth arrested feeder cells and the corresponding media. Sub-populations of keratinocyte stem cells can then be isolated based on their selective attachment to specific basement matrix substrates.

2.3 Human Skin Tissue Preparation

Human Skin: Skin tissues from newborn pups 1-4 days after birth can be used, however, pups 1-2 days are preferred since they proliferate well and have no hair. Each human epidermis yield large number of cells (5-10 x 10^6/epidermis), with a 30-40% plating efficiency.

1. Euthanize pups using halothane or other approved method. Using an aseptic technique, amputate limbs and tail at the base, make a longitudinal incision from tail to snout, and peel off the skin from the carcass using forceps.
2. Incubate skins in betadine solution for up to 5 min to prevent infection. (This procedure should not affect keratinocytes cell viability.)
3. Rinse skins twice in cold PBSA for 5 min while keeping on ice.

2.4 Epidermal Separation and Tissue Dissociation

2.4.1 Human Skin OptiTDS™ II

In the primary cell culture, there are several important factors can affect the yield and viability of cells, including type of tissues, origin of species, age of the animal used, enzymes, culture mediums and growth supplements. The Human Skin Tissue Dissociation System II is suited for optimal dissociation of normal newborn human skin tissues to yield maximum number of single keratinocyte cells.

2.4.2 Enzyme Compositions

- Trypsin: from *Bovine Pancreas*
- Dispase: from *Bacillus polymyxa*
- Collagenase I: from *Clostridium Histolyticum*
- Collagenase III: from *Clostridium Histolyticum*
- Collagenase IV: from *Clostridium Histolyticum*

2.4.3 System Components

- Human Skin Tissue Dissociation System II, Skin OptiTDS™ II, 2 vials.
- Human Skin OptiTDS™ II Reconstitution Buffer, (2 x 1 ml).
- Human Skin OptiTDS™ II Digestion Buffer, (4 x 4.5 ml).

2.4.4 Procedures For Tissue Preparation and Dissociation

4. Reconstitute tissue dissociation solution: to each vial of Human Skin Tissue Dissociation System II, Skin OptiTDS™ II, add 1.0 ml of the Human Skin OptiTDS™ II Reconstitution Buffer, Mix well.
5. Prepare fresh enzyme working solution: Add 500 µl of the reconstituted tissue dissociation solution to one vial of Human Skin OptiTDS™ II Digestion Buffer (4.5 ml). Warm the diluted Human Skin OptiTDS™ II working solution at 37 ℃ for 10 min prior to use. For optimal results of performance, we recommend use 2-3 g tissue samples per 5 ml Human Skin OptiTDS™ II working solutions.
6. Dissociation can be achieved in either of the following two methods

(a) Rapid dissociation: Float skins on Human Skin Tissue Dissociation System II working solution for 2-3 hrs at 37 ℃. This works particularly well with full-thickness skin.

(b) Slow dissociation: Float the samples on ice-cold Human Skin Tissue Dissociation System II working solution at 4℃ for 15-24 h. This is particularly convenient for flexible scheduling of keratinocyte preps.

7. Monitor the separation of the epidermis carefully when using the rapid dissociation method. When the first detachment of the epidermis is visible at the edge of each skin carefully separate the epidermis from dermis. Place the entire skin on a clean and dry tissue culture plate epidermis side up. Starting at the edge of the skin tease out the epidermis from the dermis using two forceps. Once you have the epidermis, gently start pulling it away from the dermis, while using one of the forceps to stabilize the dermis on the plate. Place the epidermis in 10 ml DMEM containing 10% FBS, antibiotics, and 500 µl reconstituted Human Skin Tissue Dissociation System II (not the working solution).

2.6 Keratinocytes Isolation

Note: Please read section 2.6 for specific information on Keratinocytes culture and platting before preceding this section.

8. Once all epidermis have been separated, use scissors to mince the epidermi in order to disrupt the beta pleated sheets that hold the keratinocytes together. Particular attention should be given to this step to ensure that the epidermi are completely minced to a fine pulp. Transfer all medium containing the epidermal pulp into a sterile beaker or plastic container.
9. Rinse a sterile stir bar in PBSA and then place the stir bar into the pulp mixture. Stir gently for 30 min.
10. Strain the cell mixture through a sterile cell strainer (70-100µm) to remove debris. Cell strainers fit perfectly in 50 ml conical and are very convenient for this procedure. If cell strainers are not available, nylon gauze can be used after rinsed twice in PBS and placed at the opening of a 50 ml conical.
11. Centrifuge the strained mixture at 1500 rmp for 10 min at 4°C.
12. Carefully pour off the supernatant and discard.
13. Add 10 ml of fresh DMEM containing 10% FBS and 100µl Human Skin FibrOut™ to the

Keratinocytes pellet and pipette with a sterile pipette several times to ensure that the keratinocytes are in a single cell suspension.

14. Count viable cells and plate 1.0 x 10^6/ 100mm dish.
15. Check cells after 2-3 hrs for attachment. Please be sure cells are attached before preceding the following.
16. At which time most cells have attached, carefully aspirate off DMEM, and add 10ml of complete Human Skin Keratinocytes Culture Medium (see below).

2.6 Primary Keratinocytes Culture

17. Primary keratinocytes can be cultured alone or on a layer of feeder cells depending on the nature of the experiments.

(a) Keratinocyte Culture with feeder layer cells:

- Prepare feeder layers by culturing 3T3 cells or human fibroblasts for 3 days. When the cells have reached confluence irradiate the 3T3 at 30 Gy or the human fibroblasts at 70 Gy.
- Prepare keratinocytes as usual (see procedure above) in complete DMEM containing 10% FBS and seed on dishes that contain the already irradiated feeder layers. Seed keratinocytes at 2-5 x 10^4 cells/cm^2.
- Change to complete Human Skin Keratinocytes Culture Medium after 2-3 hrs or visible attachment, however, the time should not exceed 5 hours.

(b) Keratinocyte Culture without feeder layer:

- Prepare complete DMEM containing 10% FBS, add 100µl Human Skin FibrOut™
- Prepare complete Human Skin Keratinocytes Culture Medium (95 ml Human Skin Keratinocytes Basic Culture Medium + 5 ml Human Skin Keratinocytes Culture Medium Supplements with Serum +100 µl Skin FibrOut™).
- Pre-coat tissue culture dishes with collagen freshly. Dilute collagen solution (Vitrogen 100, Palo Alto, CA) in sterile PBS and completely coat tissue culture dishes. Place in the incubator for at least 1 hr before platting the keratinocytes. When ready to plate the cells, aspirate off the collagen solution and seed the keratinocytes. (We have directly used collagen-coated plates from Vitrogen 100 but resulted lower efficiency).
- Place the isolate Keratinocytes from step 13 (1-5 x 10^4 cells/cm^2) and seed in the collagen coated plates with 10 ml complete DMEM for 2-3 hr. After cells have attached aspirate off DMEM and add complete Human Skin Keratinocytes Culture Medium.

Note: Keratinocytes do well when maintained at high densities on tissue culture dishes

18. Keratinocytes will form stable layers for 1-3 days. To maintain viability in culture rinse cells several times with Human Skin Keratinocytes Basic Culture Medium to eliminate nonattached dead and differentiated cells. If cells are to be cultured further then it is necessary to change culture medium every 2 days. Differentiation and growth arrest can be achieved by increasing the Ca^{2+} concentration to 1.2 mM by adding $CaCl_2$ to complete Human Skin Keratinocytes Culture Medium which contains 0.05 mM $CaCl_2$.

2.7 Subculture

19. Propagating keratinocytes in culture can be somewhat challenging, especially when keratinocytes are cultured alone on plastic, however the following methods have worked consistently in many laboratories.

(a) Keratinocytes grown on a feeder layer:
   (i) Incubate in 0.05-0.1% EDTA for 5-15 min to initiate cell detachment. Cells start to detach when the areas around cells become enlarged.
   (ii) Incubate in 0.1% trypsin and 1.3 mM (0.05%) EDTA at 37 ℃ for 5-10 min, followed by gentle pipetting, to completely detach the cells.

(b) Cultures in complete Human Skin Keratinocytes Culture Medium:
   (i) EDTA should NOT be used when keratinocytes are cultured without feeder cells.
   (ii) Remove complete Human Skin Keratinocytes Culture Medium and wash keratinocytes in 0.1% trypsin two times. Place just enough trypsin (0.1 % trypsin without EDTA) to moisten keratinocyte layer (200-500 μl depending on size of the dish). Incubate for 1-5 min at 37°C.
   (iii) Gently pipette keratinocytes and resuspend in complete Human Skin Keratinocytes Culture Medium (FibrOut™ is not necessarily needed at this step) for counting and replatting on collagen coated tissue culture plates.

III Cryopreservation

Cryopreservation is often necessary to maintain large quantities of cells derived from the same tissue sample. The best results have been reported from Keratinocytes cultures derived from preconfluent layers.

20. Trypsinize cells as above, and centrifuge at 100 g for 10 min.
21. Resuspend cells in complete complete Human Skin Keratinocytes Culture Medium without FibrOut™ and count cells.
22. Prepare aliquots of 2 x 10^6 cells/ml in complete DMEM medium with additional normal 10% FBS (total 20-25% FBS) and 10% glycerol into cryovials.
23. Equilibrate at 4℃ for 1-2 h.
24. Freeze cells with a programmed freezing apparatus (Planer) at a cooling rate of 1℃ per min.
25. To recover cells:
   (i) Thaw cryovials quickly in a 37℃ water bath.
   (ii) Dilute cells tenfold with appropriate culture media.
   (iii) Centrifuge cells get rid of the glycerol and resuspend at an appropriate concentration in Media A or B depending on whether feeder layers are used.

IV Characterization

Keratinocytes can be characterized based on their specific for their epidermal (epithelial) phenotype to exclude contamination by mesenchymal cells. This is best achieved using cytokeratin-specific antibodies for the epithelial cells. Contaminating endothelial cells can be identified by antibodies against CD31 or factor VIII-related antigen. Identifying fibroblasts unequivocally is difficult, because the use of antibodies against vimentin (the mesenchymal cytoskeletal element) is not specific; Keratinocytes *in vitro* may initiate vimentin synthesis at frequencies that depend on culture conditions. As a practical assessment for mesenchymal cell contamination, cells should be plated at clonal densities (1-5 x 10^2 cells/cm^2) on feeder cells, and clone morphology should be identified at low magnification following fixation and haematoxylin

and eosin (H&E) staining of 10- to 14-d cultures. A more specific and highly sensitive method to identify contaminating fibroblasts is the analysis of expression of Keratinocytes growth factor (KGF) by RT-PCR. Since this factor is produced in fibroblasts and not in Keratinocytes, it represents a selective marker. Moreover, KGF expression is enhanced by co-cultured Keratinocytes so that a minority of contaminating fibroblasts will be detected by this assay.

V References

1. Bickenbach, J. R., and Chism, E. 1998. Selection and extended growth of murine epidermal stem cells in culture. *Exp. Cell Res.* **244**:184-195.
2. Boyce, S. T., and Ham, R. G. 1983. Calcium-regulated differentiation of normal human epidermal kaeratinocytes in chemically difined clonal cultue and serum-free serial culture. *J. Invest. Dermatol.* **81**:33-40s.
3. Dlugosz, A. A., Glick, A. B., Tennenbaum, T., Weinberg, W. C., and Yuspa, S. H. 1995. Isolation and utlization of epidermal keratinocytes for oncogene research. *Methods in Enzymol.* **254**:3-20.
4. German, L., Rouabhia, M., Guignard, R., Carrier, L., Bouvard, V., and Auger, F. A. 1993. Improvement of human keratinocyte isolation and culture using thermolysin. *Burns* **19**:99-104.
5. Smola, H., Thiekotter, G., and Fusenig, N. E. 1993. Mutual induction of growth factor gene expression by epidermal-dermal cell interaction. *J. Cell Biol.* **122**:417-429.
6. Stark, H.-J., Baur, M., Breitkreutz, D., Mirancea, N. and Fusenig, N. E. 1999. Organotype keratinocyte cocultures in defined medium with regular epidermal morphogenesis and differentiation. *J. Invest. Derm.* **112**:681-691.

Human Skin PrimaCell™ II: Epidermal Keratinocytes

Human Skin Primary Cell II Culture

Cat No.	Description	Qt.	Price
2-96191	**Human Skin PrimaCell™ II system**	**kit**	**$550**
4-26182	Human Skin Tissue Dissociation System, Skin OptiTDS™ (for 500 ml medium)	1 ml	$146
9-46019	Human Keratinocytes PrimaCell™ Basal Culture Medium	500 ml	$73
9-36192	Human Keratinocytes PrimaCell™ Medium Supplements with Serum (for 500 ml medium)	set	$160
7-69182	Human Skin Fibroblast Growth Inhibitors, Skin FibrOut™	ea	$195
9-86019	Human Skin II Tissue Preparation Buffer Set	ea	$90

Human Primary Keratinocytes Characterization

6-32711	**Human Epidermal Keratinocyte Primarker™ Kit**	**kit**	**$220**
6-32712	Human Epidermal Keratinocyte Primarker™ antibody set	set	$180
6-32713	Human Epidermal Keratinocyte Primarker™ buffer system	set	$90

For rat and human primary cell culture protocols, please dowonload and view the Part 2 – Rat PrimaCell™; and Part 3 – Human PrimaCell™.

For information about primary cell culture and related products, please send email to:
primarycells@chiscientific.com
For technique support about primary cell culture and research, please send email to:
primatech@chiscientific.com
For general inqury about CHI Scientific's products and services, please send email to:
info@chiscientific.com

Additional Contact information:

U. S. and Canada Customers

Phone:
Toll Free: 800.986.6008
978.897.5460

Facsimile:
978.897.5462

Internet:
Web www.chiscientific.com
E-mail: help@chiscientific.com

Mail:
CHI Scientific, Inc.
Customer Service Department
63 Great Road
Maynard, MA 01754
USA

International Customers

Phone:
001.978.897.5461
001.978.897.5460

Facsimile:
978.897.5462

Internet:
Web www.chiscientific.com
E-mail: help@chiscientific.com

Mail:
CHI Scientific, Inc.
Customer Service Department
63 Great Road
Maynard, MA 01754
USA