Nonradioactive detection of telomerase activity using the telomeric repeat amplification protocol

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The telomeric repeat amplification protocol (TRAP) is a two-step process for analyzing telomerase activity in cell or tissue extracts. Recent modifications of this sensitive assay include elimination of radioactivity by using a fluorescently labeled primer instead of a radiolabeled primer. In addition, the TRAP assay has been modified for real-time, quantitative PCR analysis. Here, we describe cost-effective procedures for detection of telomerase activity using a fluorescent-based assay as well as by using real-time PCR. These modified TRAP assays can be accomplished within 4 h (from lysis of samples to analysis of telomerase products).

INTRODUCTION

The discovery of telomerase[1] as a ribonucleoprotein complex that synthesizes telomeric repeats onto the 3′ end of chromosomes led to the development of an assay for the detection and measurement of its activity in cells and tissues. TRAP was developed as a PCR-based method to assess the level of telomerase activity in a given sample[2–4]. Briefly, a cell or tissue sample is lysed with a buffer containing detergent, and an aliquot of the lysate is mixed with a reaction solution containing elements for the two-step process of telomerase product formation and amplification. In the first step, the telomerase substrate and dNTPs within the reaction solution are used for the addition of telomeric repeats by telomerase if it is present within the sample lysate (represented as a ladder on acrylamide gels). In the second step, forward and reverse primers for these products are used for amplification. In addition amplification of an internal standard PCR control using separate forward and reverse primers is performed. Mismatches in the reverse ACX primer reduce primer–dimer artifacts while still being able to amplify telomerase addition products[2,5]. Improvement of the TRAP assay and lysis methods have allowed increased linearity and sensitivity[4–6]. For example, the incorporation of an internal standard PCR control with a separate set of primers allows the identification of false negatives. In addition, normalizing the intensity of the telomerase ladder to that of an internal standard PCR control permits the TRAP assay to become linear and allows accurate comparison between samples[4,5]. The NP-40-based lysis buffer, in addition to using whole-cell lysates, allows maximal detection of TRAP activity in a given sample as compared with the CHAPS-based lysis buffer[6]. The TRAP assay has been adapted to detect or quantitate telomerase activity in a diverse set of samples: tissue, normal and cancerous cells, proliferating or nonproliferating cells, as well as cells that have been treated with agents that may affect telomerase activity[7–10].

A variety of detection methods have been developed for TRAP[10,11]. Recently, nonradioactive detection methods have been developed for the TRAP assay to eliminate the handling of radioisotopes when labeling the telomerase substrate (TS) primer. For example, the TS primer can be designed to contain a Cy5 fluorescent label at the 5′ end of the oligonucleotide, which allows a sensitive, specific and nonradioactive approach (Cy5 fluorescent gel-based whole-cell TRAP (Cy5-TRAP)) for detecting telomerase activity in whole-cell lysates when telomerase products are analyzed on an acrylamide gel.

Real-time quantitative PCR analysis of telomerase activity has also been recently described by different groups[7,10–16]. This method allows a more rapid, high-throughput, quantitative analysis of telomerase activity in cell or tissue samples; therefore, this assay is optimal for clinical use[7,14]. For the real-time, quantitative TRAP (Q-TRAP) protocol described here, we reviewed various published protocols and considered the following criteria: simplicity, cost-effectiveness, reliability and reproducibility. Our protocol uses the SYBR Green method of detection[13,16], which is more cost-effective and easier to set up than the commercial TRAPeze® XL kit from Chemicon described previously[7]. However, the commercial TRAPeze® XL kit allows an internal standard control to normalize TRAP activity in the presence of PCR inhibitors. Although the SYBR Green method does not permit this, as SYBR Green and the sequence detection system cannot distinguish internal standard control products from telomerase products, the presence of PCR inhibitors in real-time PCR is obvious from the slope of the curves during real-time analysis. The use of the SYBR Green PCR master mix kit specifically designed for the ABI sequence detection system, as described in our analyses, simplifies the set-up and optimization and reduces potential troubleshooting problems. The addition of EGTA (as described in refs. 13,16) to the reaction mixture improves amplification cycle threshold (C_t) values. Analysis of Q-TRAP described in this protocol is based on standard real-time PCR analysis, which uses a relative standard curve method. The C_t of an unknown sample is compared to a standard curve to quantify the relative amount of telomerase activity, which can then be normalized to the standard. Conventional TRAP assays are typically analyzed using cell equivalents from whole-cell lysates. For the Q-TRAP protocol, we added a precisely measured and consistent amount of cell protein lysate each time. We also analyzed samples with Q-TRAP using cell equivalents with similar results after normalizing for protein amounts (μg) per cell equivalents.

Finally, it is important to note that care must be taken not to contaminate reagents or samples with RNases or DNases during the TRAP assay. Furthermore, telomerase is heat sensitive; therefore, samples must be prepared and stored at cold temperatures (4 and

−80 °C), taking care not to heat the samples (as in tissue processing)[9]. When comparing the Cy5-TRAP and Q-TRAP methods, there are some quantitative and qualitative differences. The fraction of total telomerase liberated in a cell extract has been shown to be variable in different samples; hence, ideally, whole-cell lysates rather than cell extracts are used. However, tissues often contain "PCR inhibitors," much of which is removed by making extracts and spinning down the debris. Whole-cell lysates are preferred for Cy5-TRAP, whereas cell extracts are used for Q-TRAP to remove cellular debris for real-time SYBR Green PCR analysis; therefore, Q-TRAP may underestimate the amount of telomerase activity in samples with weak activity, as maximal activity has been shown to be in whole-cell lysates. On the other hand, the Q-TRAP method can eliminate human error during quantification because ABI software determines the C_t values. However, the lack of a firm identification of telomerase extension products in Q-TRAP can be a limitation in accurately ruling out false positives. Therefore, it is recommended that one performs the Cy5-TRAP, which includes an internal control, to precede or confirm the Q-TRAP results. The Q-TRAP protocol can then be optimal for high-throughput, quantitative analysis of telomerase activity. Although prices vary by country, the relative costs of the different assays in the USA are about $0.53 per CY5-TRAP reaction, $1.13 per Q-TRAP reaction using the ABI SYBR Green PCR master mix and $3.79 per TRAPeze®XL kit reaction.

MATERIALS

REAGENTS

- Cell pellet from 25,000 to 100,000 cells
- RNase/DNase-free H_2O (Ambion)
- BCA protein assay kit (Pierce)
- SYBR Green PCR master mix kit (Applied Biosystems, cat. no. 4309155; keep RNase-free)
- 10 mM EGTA (RNase/DNase-free)
- *Taq* DNA polymerase (New England Biologicals)
- Acrylamide (19:1 acrylamide:bis-acrylamide; Bio-Rad)
  ! CAUTION (acrylamide is a neurotoxin and should be handled with gloves).
- 10× TBE
- TEMED (N,N,N′,N′-Tetramethylethylenediamine) (Bio-Rad)
- 10% (wt/vol) ammonium persulfate solution
- Ultrapure BSA (Ambion, cat. no. 2616)
- RNA-Zap to help eliminate RNases
- 50× dNTP mix (2.5 mM each of dATP, dTTP, dGTP and dCTP in RNase-free H_2O)
- 100 ng μl^{-1} TS primer, HPLC-purified (5′-AATCCGTCGAGCAGAGTT)
- 100 ng μl^{-1} Cy5-TS primer, HPLC-purified (5′-Cy5-AATCCGTCGAGCAGAGTT)
- 1 $\mu g\ \mu l^{-1}$ ACX primer, HPLC-purified (5′-GCGCGGCTTACCCTTACCCTTACCCTAACC-3′)
- 1 $\mu g\ \mu l^{-1}$ NT primer, HPLC-purified (5′-ATCGCTTCTCGGCCTTTT-3′)
- NP-40 lysis buffer (RNase/DNase-free): 10 mM Tris-HCl, pH 8.0; 1 mM $MgCl_2$; 1 mM EDTA; 1% (vol/vol) NP-40; 0.25 mM sodium deoxycholate; 10% (vol/vol) glycerol; 150 mM NaCl; 5 mM β-mercaptoethanol; 0.1 mM AEBSF (4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride)
- 10× TRAP buffer (RNase/DNase-free): 200 mM Tris-HCl, pH 8.3; 15 mM $MgCl_2$; 630 mM KCl; 0.5% Tween 20; 10 mM EGTA
- Loading dye: 0.25% (wt/vol) bromophenol blue in 50% (vol/vol) glycerol/50 mM EDTA
- 50× TRAP primer mix recipe for Cy5-TRAP; see REAGENT SETUP

EQUIPMENT

- DNase-, RNase-free microfuge tubes
- DNase-, RNase-free filter pipette tips
- 96-well PCR plates with optical lids
- Liquid nitrogen or snap-freeze box
- Tabletop centrifuge (up to 16,000*g* capability) at 4 °C (Eppendorf 5415R or equivalent)
- Thermocycler
- Power supplies and vertical gel apparatus
- PhosphorImager capable of reading Cy5 fluorescence
- ABI Prism 7000, 7700 Sequence Detector (PE Applied Biosystems) or equivalent

REAGENT SETUP

50× TRAP primer mix recipe for Cy5-TRAP The primer mix includes the substrate for the 36-bp internal standard control (TSNT) and reverse primers (NT and ACX) for amplification of internal standard control (IC) and telomerase products. See **Box 1** for details on how to prepare this. The TSNT primer should be ordered either from a different company or at a different time from the NT and ACX primers to avoid any possibility of cross-contamination, as it functions in the reaction as a PCR substrate rather than a primer. ▲ CRITICAL The stock TSNT primer should be prepared in a separate room from the other TRAP steps and with separate pipetmen[9]. The reason for separating PCR setup from PCR analysis in different rooms is that the PCR product is so abundant that minor aerosols can contaminate primers, etc. and cause problems. The TSNT primer is essentially a PCR product that is spiked into the reaction to compare its relative amplification by the TS and ACX primers; therefore, it needs to be prepared in a different room for exactly the same reasons as using a different room for PCR products.

PROCEDURE

Preparation of cell lysates ● TIMING 30 min

1| Harvest 25,000–100,000 cells into a DNase-, RNase-free 1.5-ml microfuge tube. Pellet cells by centrifugation in a tabletop centrifuge at 3,000*g* for 5 min at room temperature (18–25 °C). Carefully discard the supernatant. It is not necessary to wash the pellet, but ensure that all residual liquid is removed. Proceed to Step 2 if samples are ready for lysis.

BOX 1 | PROTOCOL FOR MAKING UP THE 50× PRIMER MIX FOR CY5-TRAP

This recipe makes enough primer mix for 100 sample reactions.

1. In a separate room with separate pipetmen and tips from the other steps, dilute HPLC-purified TSNT oligonucleotide (5′-AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT-3′) to 100 μM with RNase-free H_2O. Perform serial dilutions (e.g., 1:100, 1:1,000 and 1:1,000) so that the final concentration of the TSNT stock is 1.0×10^{-18} mol μl^{-1}.
2. Mix 10 μl each of the ACX and NT primers and 79.0 μl of RNase-free H_2O together in an RNase-free tube (final concentration, 100 ng μl^{-1}).
3. Move to the area where the TSNT was prepared and add 1 μl of TSNT (final concentration, 0.01×10^{-18} mol μl^{-1}) to the mix. Clean the outside of the tube with diluted bleach.
4. Aliquot the primer mix in a separate room and store at −20 °C.

■ **PAUSE POINT** In case it is required to collect samples for use over a period of time, snap-freeze the cell pellet in liquid nitrogen and store at −80 °C until ready for lysis.

2| Resuspend the cell pellet in ice-cold NP-40 lysis buffer at a concentration of 500–1,250 cells μl^{-1} and incubate on ice for 30 min. We have observed that NP-40 lysis buffer is more efficient in extracting telomerase activity than the CHAPS-based lysis buffer[9].

▲ **CRITICAL STEP** Care must be taken not to contaminate any step in the TRAP assay with RNases.

3| Prepare lysates for the TRAP assay. This step can be performed using option A (Cy5 fluorescent gel-based TRAP on whole-cell lysates) or option B (real-time Q-TRAP on cell extracts).

(A) Cy5 fluorescent gel-based whole-cell TRAP ● **TIMING 3 h**

(i) Keep samples on ice.

■ **PAUSE POINT** Whole-cell lysates can be snap-frozen in liquid nitrogen and placed at −80 °C until ready for analysis.

(ii) Prepare samples so that the final volume is 2 µl. Keep all the samples on ice.

(iii) Choose a telomerase-positive sample for making a standard curve (e.g., this can be human MCF-7 breast carcinoma, H1299 lung carcinoma, HeLa cervical carcinoma or immortalized 293 human embryonic kidney cells). Prepare a dilution series of your standard sample (e.g., 1:10, 1:5 or 1:2 starting at 2,500 cell equivalents).

(iv) Prepare control samples: (a) 5 µl of lysate incubated with 1 µg of RNase at 37 °C for 20 min or heat-inactivated at 85 °C for 10 min before the telomerase assay; (b) a positive control lysate containing known telomerase activity as described above; and (c) a lysis buffer-only control should also be included to check for the presence of contamination in the lysis buffer. Keep all the samples on ice.

(v) Prepare the TRAP master mix and reaction mixture for the appropriate number of samples (n + 2). The final volume of sample, primers, buffers and enzyme is 50 µl per sample. Prepare a master mix containing 1× TRAP buffer, 1× dNTP mix, 1× TRAP primer mix, 100 ng of Cy5-TS primer per sample, 20 µg ml^{-1} BSA, RNase-free H_2O (to bring the final volume to 48 µl per sample) and 2 U of *Taq* DNA polymerase. For example, for ten samples:

Component	Volume
RNase-free H_2O	392 µl
10× TRAP buffer	50 µl
50× dNTP mix	10 µl
50× TRAP primer mix	10 µl
Cy-5 TS primer (100 ng μl^{-1})	10 µl
BSA (50 mg ml^{-1})	4 µl
Taq DNA polymerase (5 U μl^{-1})	4 µl

(vi) Add 48 µl of the master mix to each sample (total volume 50 µl). Incubate the samples at 22–30 °C for 30 min for the extension of the substrate by telomerase.

(vii) Amplify the extension products by PCR: 95 °C for 5 min to inactivate telomerase, and 24 cycles at 95 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s.

■ **PAUSE POINT** After PCR, samples can be kept at 4 °C for no more than 2 days or at −20 °C for a longer time (<1 month) until analysis on an acrylamide gel.

(viii) Following PCR, add 5 µl of loading dye to each TRAP reaction mixture and run 25 µl on a 10% nondenaturing acrylamide gel in 0.5× TBE (1.5 h, 17–20 V cm^{-1}). Fixing the gel is optional, but fixation prevents DNA diffusion and results in slightly sharper bands. Fix the gel by incubating in 0.5 M NaCl, 50% (vol/vol) ethanol and 40 mM sodium acetate (pH 4.2) for 15 min.

(ix) Visualize the gel using a PhosphorImager that is capable of reading Cy5 fluorescence, and determine the intensity of the telomerase products (6-bp ladder) and the 36-bp IC band with ImageQuant software (Molecular Dynamics). Calculate the relative telomerase activity (RTA) as the ratio of the intensity of the TRAP ladder to that of IC. Normalize the relative intensity of each sample to that of a positive control (i.e., percentage). For example, ((intensity of sample's TRAP ladder/intensity of sample's IC band)/(intensity of positive control's TRAP ladder/intensity of positive control's IC band))×100. **? TROUBLESHOOTING**

(B) Real-time Q-TRAP ● **TIMING 3 h**

(i) Centrifuge the sample lysates to remove cellular debris at 16,000*g* for 20 min at 4 °C using a tabletop microcentrifuge.

(ii) Keep the samples on ice. Collect 80% of the supernatant in a fresh DNase/RNase-free Eppendorf tube making sure that no traces of cell debris from the pellet are withdrawn.

■ **PAUSE POINT** Extracts can be snap-frozen in liquid nitrogen and placed at −80 °C until ready for analysis.

(iii) Quantify the total protein concentration of your samples using a BCA protein assay kit (Pierce) according to the manufacturer's protocols.

(iv) Prepare control samples: (a) 5 µl of extract incubated with 1 µg of RNase at 37 °C for 20 min or heat-inactivated at 85 °C for 10 min before the telomerase assay; (b) a positive control lysate containing known telomerase activity; and (c) a lysis buffer-only control to check for the presence of contamination. Keep all samples on ice.

(v) Choose a telomerase-positive sample for making the standard curve (e.g., this can be human MCF-7 breast carcinoma, H1299 lung carcinoma, HeLa cervical carcinoma or immortalized 293 human embryonic kidney cells).

(vi) Create a 1:10 or 1:5 dilution series of your standard to yield at least five separate curves that are spaced widely apart. Note that >1 µg protein lysate may interfere with the reaction and increase C_t values.

(vii) Choose how much protein of your unknown/test samples you will analyze so that the C_t values for these samples fall within the standard range. For example, 1–3 µg protein lysate is usually sufficient (however, the amount of protein from the unknown samples may need to be empirically determined as 1 µg protein lysate may contain only ~300–500 cell equivalents, which may be too low to detect TRAP products in samples with weak activity). Prepare each sample in triplicate as 2 µl final volume aliquots.

(viii) Prepare the TRAP master mix and reaction mixture for the appropriate number of samples (n + 2). The final volume of the reaction mixture (sample, primers, buffers and enzymes) is 25 µl per sample. Prepare a master mix containing 1× SYBR Green Master Mix (includes *Taq* polymerase and a passive reference dye, ROX), 100 ng TS primer per sample, 100 ng ACX primer per sample, 1 mM EGTA and enough water to bring the final volume to 25 µl. For example, for ten samples:

Component	Volume
RNase/DNase-free H_2O	60 µl
ABI SYBR Green PCR master mix	125 µl
EGTA (10 mM)	25 µl
ACX primer (100 ng μl^{-1})	10 µl
TS primer (100 ng μl^{-1})	10 µl

(ix) Add 23 µl of Q-TRAP master mix to each sample well of a 96-well PCR plate that will be analyzed (it is helpful to prepare a template of a 96-well PCR plate in order to plan the location of samples for analysis).

(x) Add your sample volume (2 µl) to each well to bring the total volume to 25 µl per well.

(xi) Incubate the 96-well plate for 30 min at 22–30 °C in the dark for extension of the substrate by telomerase.

(xii) Take the plate to the ABI Prism Sequence Detector and cycle (under SYBR Green setting) using the standard protocol that comes with the kit: 95 °C for 10 min (to activate the hot start *Taq* polymerase in the master mix); 40 cycles at 95 °C for 15 s and at 60 °C for 60 s.

(xiii) After PCR, collect real-time data according to the sequence detector manual and analysis software program. Set the baseline and threshold to be 10 standard deviations (s.d.) above the background (10 s.d. = 1 power of 10).

(xiv) You can also run the real-time PCR products on a 10% nondenaturing acrylamide gel to confirm the presence/absence of telomerase products.

(xv) Plot a standard curve according to the guidelines for the sequence detector using your telomerase-positive sample dilution series. Take the $\log_{10}$ of your protein concentration values for each sample. Plot your standard curve's average C_t values (± s.d.) on the y axis and the $\log_{10}$ protein concentration on the x axis. Add a linear trend line and record the equation on the graph. Ensure that R^2 is >0.90.

(xvi) Convert your unknown/test sample data into RTA units that are defined by your standard curve and linear equation. For each raw C_t value, perform the following calculation: converted RTA of sample=$10((C_t \text{ sample} - Y_{int})/\text{slope})$. Calculate the average RTA (± s.d.) for each test sample. Normalize the RTA to that of a positive control (for example, percentage telomerase activity compared to a positive control).

? TROUBLESHOOTING

● **TIMING**

Steps 1 and 2: 30 min

Step 3A: 3 h

Step 3B: 3 h

? TROUBLESHOOTING

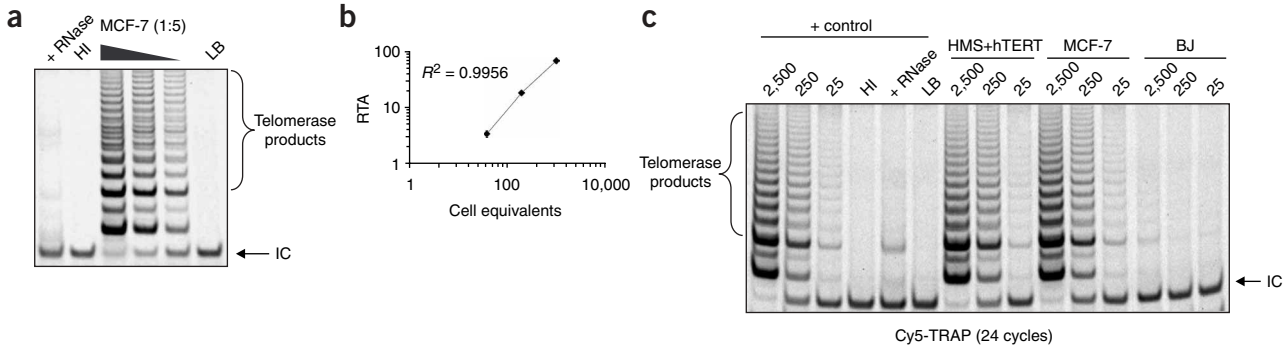
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Problem	Possible causes	Solution
No products/very high C_t for any sample including the positive control.	RNase/DNase contamination.	Practice sterile technique and use only RNase/DNase-free reagents.
	PCR amplification not initiated.	Check thermocycler for proper temperature and cycling conditions.
		Taq is a hot start enzyme and you must incubate samples at 95 °C for 10 min to activate it.
		Make sure you correctly added primers and that your lysates are still active (keep them at −80°C and avoid freeze–thaw).
		Check to see if the *Taq* is still active (keep all reagents on ice).
Products present/low C_t in negative controls.	Lysis buffer contamination.	Collect fresh lysates using freshly prepared lysis buffer.
	Kit, water or primer stocks contaminated.	Run quality control to see where the contamination is and then replace with fresh reagent.
	Background.	Inevitably 40 cycles may pick up background noise; hence do not consider it contamination unless the C_t is below 30–35 cycles.
	DNA-contaminated plates or tubes.	Use only RNase/DNase-free consumables.
Jagged amplification plots.	Unoptimized kit.	Optimize the protocol by adjusting [$MgCl_2$], [EGTA], [Primer], and/or add BSA or T4 protein.
		Adjust thermocycler conditions.
		Test another brand of SYBR Green Q-PCR kit; however, we have found that the ABI kit works best on ABI systems.
High background fluorescence.	Primer dimers and unoptimized thermocycler conditions.	Empirically adjust temperatures for annealing and cycle time.

ANTICIPATED RESULTS

As human telomerase is processive, varying numbers of hexameric repeats are added to it during the initial 30-min incubation in the presence of the TS primer. When subsequently amplified, a 6-bp DNA incremental ladder of telomerase products is visualized (**Fig. 1a,c**). This ladder is reflective of the amount of telomerase activity per cell equivalent or protein used in the assay. In addition, a 36-bp internal standard control band is seen on the acrylamide gel when using the Cy5-TRAP method. The RTA can then be expressed as the ratio of the intensity of the entire ladder to that of the IC band (**Fig. 1b,d**). When performing a dilution series on a sample, the RTA should behave in a linear manner (**Fig. 1b**; $R^2 > 0.90$). Dividing the intensity of the telomerase addition products by that of the internal standard permits the linear relationship to be maintained. Samples with high amounts of telomerase activity will appear to compete with the IC amplification as well as having extra bands between 36 and 50 bp (the first telomerase product) because the *Taq* polymerase becomes limiting (**Fig. 1c**). The amount of lysate should then be reduced so that it does not compete with the internal standard. The number of PCR cycles can also be adjusted to determine a more optimal end point of amplification. In addition, tissue samples often contain inhibitors of *Taq* polymerase, which will reduce the intensity of both the TRAP ladder and the



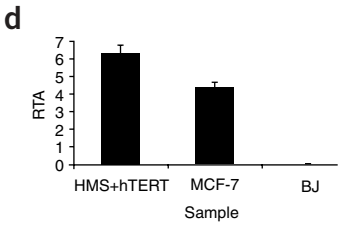


Figure 1 | Analysis of telomerase activity using Cy5-TRAP. (**a**) Human cancer cell lysates (MCF-7 breast carcinoma cells) are shown to be positive for telomerase activity, as evidenced by the 6-bp incremental TRAP ladder. Lysis buffer (LB) only, RNase-treated (+RNase) and heat-inactivated (HI) MCF-7 cell lysates served as negative controls. Each TRAP reaction includes a 36-bp internal standard control (IC). Serial dilutions (1:5) of MCF-7 whole-cell lysates (1,000, 200 and 40 cell equivalents) result in a decreased intensity of the telomerase products TRAP ladder and return of the internal standard control band (IC). (**b**) Quantification of telomerase activity using Cy5-TRAP. The total peak intensity of the telomerase products TRAP ladder for each sample was divided by the intensity of the IC band. The RTA ± s.d. for each cell equivalent was plotted using a logarithmic scale. The serial dilution of MCF-7 whole-cell lysate exhibited a linear relationship for RTA ($R^2 > 0.90$). Note that each fivefold dilution in (**a**) only decreases the intensity of the actual TRAP ladder two- to threefold, and it is only by dividing the intensity of the actual TRAP ladder by the increasing intensity of the IC that one gains the linearity of response shown in (**b**). (**c**) Cy5-TRAP analysis of telomerase activity using 25, 250 and 2,500 cell equivalents from normal fibroblasts (BJ), a cancer cell line with endogenous telomerase activity (MCF-7) and human stromal cells with exogenous hTERT (HMS+hTERT). Positive telomerase activity is evidenced as the 6-bp incremental TRAP ladder of telomerase products. A serial dilution of whole-cell lysates from a positive control (MCF-7 cells) served as a positive control (+control). Lysis buffer (LB) only, RNase-treated (+RNase) and heat-inactivated (HI) MCF-7 cell lysates served as negative controls. Each TRAP reaction includes a 36-bp internal standard control (IC). (**d**) Relative amounts of telomerase activity (RTA±s.d.) for 250 cell equivalents from HMS + hTERT, MCF-7 and BJ whole-cell lysates analyzed by Cy5-TRAP. The ratio of the intensity of the sample's TRAP ladder (telomerase products, TP) to that of the internal control (IC) band was calculated after compensating for the background in the LB sample (i.e., $(TP - TP_{LB})/IC$).

internal control; therefore, these samples should be diluted with lysis buffer to achieve detectable telomerase products. Cy5-TRAP allows a relatively inexpensive method to detect reliably the presence or absence of a ladder that can be documented for figures as authentic telomerase addition products without major artifacts.

Q-TRAP follows traditional quantitative, real-time PCR experimental design, where the threshold of amplification can be determined (C_t). In this protocol, we describe the analysis of samples using known protein concentrations so that we can be confident about the amount of starting material for real-time PCR analysis with SYBR Green. We also performed this protocol using cell equivalent with similar results after normalizing for protein amounts (μg) per cell equivalents. Repeating the serial dilution series for a cell line as illustrated in **Figure 1**, the data screen that appears during real-time PCR (here with the ABI Prism 7000 sequence detector software) shows separate curves with increasing threshold cycles (C_t, *x* axis) for each sample (**Fig. 2a**). The green line represents the threshold that can be manipulated within the file so that it is ten s.d.s. above the baseline signal or background (the background lines of the curves can also be omitted as in these figures). Following the relative standard curve method for real-time data analysis (which should be included in the procedure handbook associated with the sequence detector), the C_t values of the samples were plotted against log[protein] to calculate the linear equation (**Fig. 2b**). The R^2 should be >0.90. The *Y*-intercept and the slope values from the equation are used to quantify the RTA of unknown samples (RTA of unknown sample$= 10^{[(C_{t\,sample} - Y_{int})/slope]}$). Using the relative standard curve method, as described above, allows one to compare the RTA of one sample with thatof another sample performed in the same experiment, which is standard for analyzing real-time PCR products.

Analysis of telomerase activity of various samples should also reveal individual curves as shown in **Figure 2c** (the standard curves were omitted in this figure). The relative standard curve method as described above is used to quantify the RTA of different samples performed in the same experiment (**Fig. 2d**). Confirmation of real-time PCR analysis can be performed using an acrylamide gel, which is then stained with SYBR Green; however, this step is optional and can also reveal artifacts and spurious PCR products from the interaction of the ACX primer with the telomerase substrate, genomic DNA or PCR product contamination of reagents as the samples were collected after 40 cycles. These artifact signals are easily distinguishable from true telomerase products. An additional consideration is the functional linear range of the two assays. The single band in

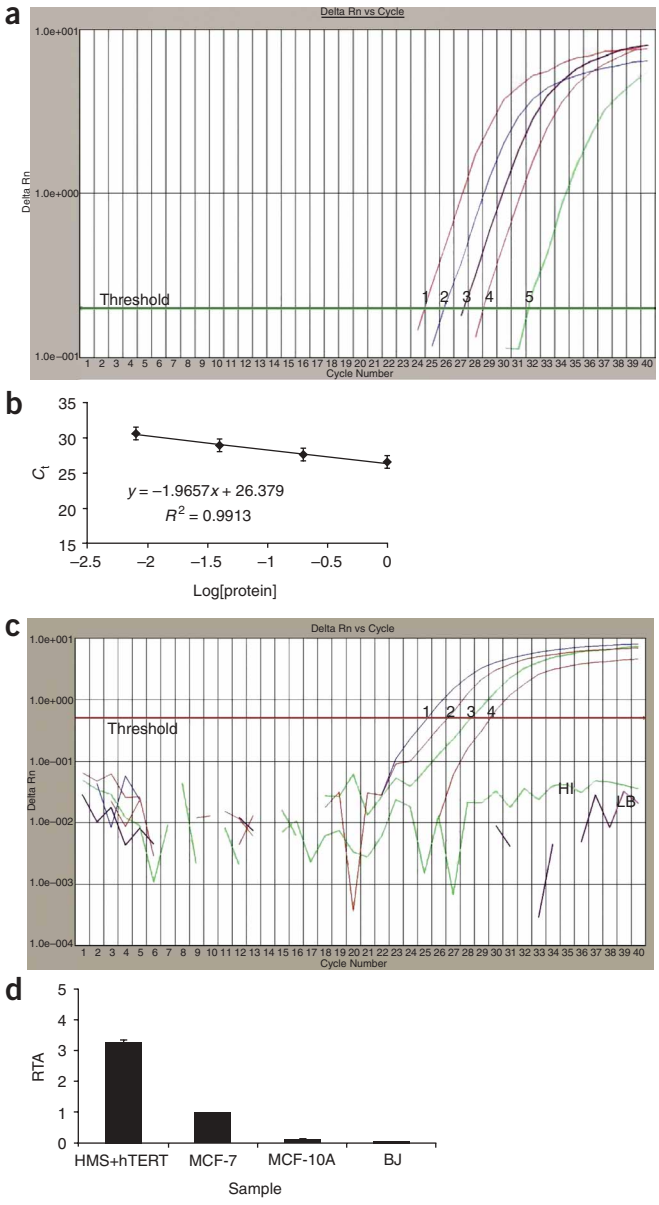


Figure 2 | Real-time Q-TRAP analysis of telomerase activity: preparation of the standard curve. (**a**) The amount of cellular protein in MCF-7 breast carcinoma cell lysates was determined and then analyzed for telomerase activity using Q-TRAP and an ABI Prism 7000 Sequence Detector. Real-time amplification plots of serially diluted (1:5) MCF-7 breast carcinoma cell lysates (curves 1–5: 1.0, 0.2, 0.04, 0.008 and 0.0016 μg, respectively). The change in fluorescence of SYBR Green (Delta Rxn) was plotted against cycle number. C_t represents the cycle at which fluorescence is first detected above the threshold (baseline signal plus 10 s.d.). Serial dilutions of protein extract result in increased C_t values. (**b**) Determination of the standard curve and linear relationship for Q-TRAP. The C_t values (±s.d.) of the standard control were plotted against log[protein] to calculate the linear equation. The *Y*-intercept and the slope values from the equation are used to quantify the RTA of unknown samples ($=10^{[(C_{t\ sample}-Y_{int})/slope]}$). (**c**) Real-time Q-TRAP analysis of telomerase activity in cell extracts. Various samples representing normal fibroblasts (BJ), a cancer cell line with high telomerase activity (MCF-7), an immortal cell line with low telomerase activity (MCF-10A) and human cells with exogenous hTERT (HMS + hTERT) were analyzed for telomerase activity using both the Cy5-TRAP and Q-TRAP protocols. Amplification plots of 1-μg protein extracts from human cells with exogenous hTERT (curve 1), a cancer cell line with high endogenous telomerase activity (curve 2), an immortal cell line with low telomerase activity (curve 3) and normal fibroblasts with no detectable hTERT mRNA (curve 4). HI, heat inactivated; LB, lysis buffer. (**d**) Samples were quantified as described in the protocol and plotted as RTA ± s.d. For Q-TRAP quantification, the RTA for an unknown sample was calculated based on the following standard curve and equation obtained for the same Q-TRAP assay: $y=-1.9657x + 26.379$; RTA$= 10^{[(C_{t\ sample}-Y_{int})/slope]}$.

the 2,500 BJ cell lane of **Figure 1c** is often seen in the lysis buffer control and thus represents an artifact; therefore, there is at least a 100-fold difference in telomerase activity between BJ and MCF-7 cells. Because cell extracts (rather than whole-cell lysates) and only 370 cell equivalents were analyzed by Q-PCR, BJ fibroblasts appear to only have an approximately tenfold rather than a 100-fold lower activity than MCF-7 cells. Q-PCR thus tends to overestimate the activity of very low-activity samples.

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