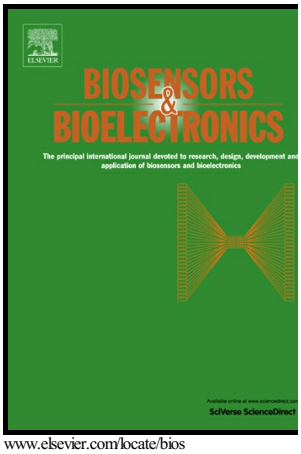

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www.elsevier.com/locate/bios

PII: S0956-5663(15)30340-7
DOI: http://dx.doi.org/10.1016/j.bios.2015.08.013
Reference: BIOS7909

To appear in: *Biosensors and Bioelectronic*

Received date: 18 June 2015
Revised date: 4 August 2015
Accepted date: 8 August 2015

Cite this article as: Xiafei Zhang, Rui Cheng, Zhilu Shi and Yan Jin, A PCR-free fluorescence strategy for detecting telomerase activity via double amplification strategy, *Biosensors and Bioelectronic* http://dx.doi.org/10.1016/j.bios.2015.08.013

A PCR-free Fluorescence Strategy for Detecting Telomerase Activity via Double Amplification strategy

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ABSTRACT

As a universal tumor biomarker, research on the activity and inhibition of telomerase is of great importance for cancer diagnosis and therapy. Although the telomeric repeat amplification protocol (TRAP) has served as a powerful assay for detecting telomerase activity, its application has been significantly limited by amplification related errors and time-consuming procedure. To address the limitations of PCR-based protocol, a dual amplification fluorescence assay was developed for PCR-free detecting telomerase activity. Briefly, we designed an arch-structure DNA probe to specifically control strand displacement reaction and subsequent enzyme-aided amplification. Telomerase substrate (TS) primer was extended by telomerase to form long elongation products which contain several TTAGGG repeat units. So, one elongation product can release more than one trigger DNA (t-DNA) via strand displacement reaction to realize first amplification. Subsequently, t-DNA specifically opened molecular beacon (MB) to restore the fluorescence of MB. Meanwhile, t-DNA was recycled by the aid of nicking endonuclease to continuously open more and more MBs, leading to a second amplification. Owing to the double amplification strategy, the proposed method allowed the measurement of telomerase activity in crude cell extracts equivalent to 5 HeLa cells and 10 CCRF-CEM cells without PCR amplification. Besides, the influence of telomere-binding ligands on the telomerase activity demonstrated that the proposed method holds the potential to evaluate the inhibition efficiency of telomerase inhibitors.

Keywords: Telomerase; PCR-free; Fluorescence; Dual amplification;

1. Introduction

Telomerase activity is unregulated in greater than 85% of human tumors, such as breast, colon, lung, prostate, ovary, stomach, and skin, whereas it is absence or low expression in most healthy tissues (Morin et al., 1989; Moyzis et al., 1988; Shay et al., 1997). This renders this enzyme as an important therapeutic target and a promising biomarker in cancer diagnosis and therapy (Shay et al., 2002). Sensitivity is still a significant challenge for reliable detection of telomerase activity due to the extreme low amount of telomerase in cancer cells. Therefore, it is of significant theoretical and practical importance to realize sensitive and reliable detection of telomerase activity. Up to date, many approaches have been developed to measure telomerase activity (Counter et al., 1992; Gelmini et al., 1998; Hou et al., 2001; Nemos et al., 2003; Dalla et al., 2002). Among them, the telomeric repeat amplification protocol (TRAP) is the most commonly applied method. This PCR-based method enabled exponential amplification of the primer-telomeric repeats generated in the telomerase reaction, and the resulting improvements dramatically increased the efficiency and sensitivity of telomerase activity detection (Patel et al., 2004). This PCR-based amplification, although highly sensitive, is susceptible to amplification related errors and time-consuming procedure (Krupp et al., 1997), expensive equipment and reagents. In recent years, a number of alternative, PCR-free approaches to detect telomerase activity have been developed, including fluorescence (Schmidt et al., 2002; Ding et al., 2010; Wang et al., 2012; Zuo et al., 2011; Sharon et al., 2014; Quach et al., 2013; Tian et al., 2013; Alves et al., 2008; Qian et al., 2014； Wang et al. 2013), colorimetry

(Wang et al., 2012), surface plasmon resonance (SPR) (Sharon et al., 2010; Maesawa et al., 2003), chemiluminescence (Zhou et al., 2009; Zhang et al., 2014; Ma et al., 2012; Sato et al., 2005), electrochemical detection (Eskiocak et al., 2007; Shao et al., 2008; Yang et al., 2011; Liu et al., 2013; Yi et al., 2014), and so on (Zheng et al., 2008). These strategies avoid the PCR-derived problems and requirement for gel analysis in TRAP assay, permitting the measurement of telomerase activity from cell extracts in a simple and isothermal way. Willner and co-workers (Xiao et al., 2004a; Pavlov et al., 2004; Xiao et al., 2004b) reported several methods for telomerase assay by using the catalytic DNAzyme, which consists of hemin and single-stranded guanine-rich nucleic acids and possesses peroxidase-like activities. Li (Li et al., 2011) reported a method for telomerase activity detection based on horseradish peroxidase (HRP)-mimicking DNAzyme formed by intercalating hemin into the G-quadruplex of the human telomere. Mirkin and co-workers (Weizmann et al., 2004) devised a bio-barcode assay based on polyvalent oligonucleotide-functionalized gold nanoparticles conjugates with silver staining technique to amplified telomerase detection. However, most of them have the disadvantages of lacking simplicity or the time-consuming protocol. Therefore, the development of facile and highly sensitive method for telomerase detection is still urgent and in great demand.

Herein, we report a facile strategy for highly sensitively and specifically detecting telomerase activity based on the double amplification technique. An arch-structure DNA probe is formed by partial hybridization between complementary DNA of elongation product of telomerase substrate (TS) primer and trigger DNA (t-DNA) of

strand displacement reaction. In the presence of telomerase, the TTAGGG repeat units were added to the 3′-end of the TS primer in the presence of the nucleotide mixture (dNTPs) to form a long elongation strand of telomerase products which can release t-DNA via strand displacement reaction. One elongation product was able to hybridize with more than one complementary DNA to release numerous t-DNA. Subsequently, with the aid of Nt.BbvCI, the second amplification of NEase-aided DNA recycling amplification could be performed to amplify the detectable fluorescent signal of molecular beacons. By elaborately combining elongation characteristic of telomerase with two isothermal signal amplification strategies, a facile and reliable fluorescence strategy was offered for PCR-free detecting telomerase activity.

2. Experimental section

2.1 Chemicals and instruments.

3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS), β-Mercaptoethanol, glycerol, phenylmethylsulfonyl fluoride (PMSF), ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), Tween 20 and Tris-(hydroxymethyl) aminomethane (Tris) were purchased from Sigma-Aldrich. Nicking endonuclease Nt.BbvCI and NEB buffer were purchased from New England Biolabs (Ipswich, MA, USA). Diethyl pyrocarbonate (DEPC) was purchased from Sangon Biotechnology Co. (Shanghai, China). All other reagents were of analytical reagent grade. All the water used in the work was RNase-free. The oligonucleotides used in this work were synthesized by Sangon Biotechnology Inc (Shanghai, P. R. China). Sequences of the oligonucleotides are listed in Table S1 in supporting

information. They were all purified by reverse-phase high-performance liquid chromatography (HPLC).

All fluorescence measurements were performed on Hitachi F-7000 fluorescence spectrophotometer (Kyoto, Japan). The vertical electrophoresis system was purchased from Bio-Rad Laboratories, Inc. The Molecular Imager system was purchased from Shanghai Peiqing science & Technology. Co., Ltd (Shanghai, China).

2.2 Fluorescence measurements.

All fluorescence measurements were carried out at an F-7000 fluorometer with excitation at 480 nm and emission at 520 nm for the hairpin fluorescence probes labeled by 5′- FAM as a fluorophore. When the samples were excited at 480 nm, the emission was scanned from 500 to 600 nm in steps of 1 nm; the scanning speed was 1200 nm/min.

2.3 Cell culture.

HeLa cells and CCRF-CEM cells were separately cultured in DMEM medium and RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 U mL^{-1} of penicillin-streptomycin. The cells were maintained at 37 °C in a humidified atmosphere (95% air and 5% CO_2). The cancer cell densities were determined using a hemocytometer.

***2.4** Extraction of telomerase.*

The telomerase was extracted by the CHAPS method. Cells were collected in the exponential phase of growth. Cells were counted, and an aliquot containing 1.0×10^6 cells was pelleted (850 rpm in a 1.5 mL EP tube for 5 min) in culture medium. The

pellet was washed twice with ice-cold PBS, and then, the pellet was resuspended in 200 μL of ice cold lysis buffer (0.5% CHAPS, 10 mM Tris-HC1, pH 7.5, 1 mM $MgC1_2$, 1 mM EGTA, 5 mM β-mercaptoethanol, 0.1 mM PMSF, 10% glycerol) by retropipeting at least three times and kept on ice for 30 min. The mixture was centrifuged at 12000 rpm for 20 min at 4 °C, and the supernatant was collected carefully. The resulting extract was used immediately or was flash frozen in liquid nitrogen at -80 °C.

2.5 Telomerase extension.

Telomerase extracts were diluted in lysis buffer with respective number of cells, the extracts (2 μL) were added to 20 μL of RNA secure pretreated extension solution containing 1×TRAP buffer, (20 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween 20, 1 mM EGTA, BSA 0.1 mg /mL) 0.2 mM dATP, dTTP, dCTP and dGTP, and 200 nM TS primer. For inhibition of telomerase, different volumes of catechinic acid or ginseno-side was mixed with telomerase extract from 1000 HeLa cells. Then the solution was incubated at 30 ℃ for 60 min. For negative control experiments, telomerase extracts were heat-treated at 95 ℃ for 10 min.

2.6 Detection of telomerase activity

After the TS elongation reaction, MB, arch-structure DNA probe and DEPC water were added to the preceding reaction solution. Then, the reaction mixture was incubated at 37 °C for 10 min. Finally, the above mixture incubated with 15 U/ml Nt.BbvCI at 37 °C for 30min, and then the fluorescence intensities were recorded immediately. The total volume of the reaction mixture was 100 μL, and the final

concentrations of hairpin fluorescence probe, TS primer, and arch-structure DNA probe were 200 nM, 20 nM, and 100 nM, respectively.

2.7 Gel electrophoresis analysis.

A 12.5% polyacrylamide gel was prepared using 1×TBE (100 mM Tris–HCl, 83 mM boric acid, 1 mM EDTA, pH 8.0). 20 μL of the reaction product along with 2 μL loading buffer was loaded onto a 12.5% polyacrylamide gel and then run in 1×TBE buffer at 200 V for 20 min. The gels were stained with silver for 20 min. The visualization and photography were performed using a molecular imager with a Gel Doc system.

2.8 Conventional TRAP assays

5μL of telomerization products were added into 45μL of solution which contains 1×PCR buffer, 200 μM dNTPs, 2.5 U of Taq DNA polymerase, 400 nM of TS primer and 400 nM of ACX primer. PCR was carried out using a Bio-Rad T100 TM thermal cycler with the following program: 94 ℃ for 4 min; 30 cycles at 94℃ for 30 s, 58℃ for 30 s, and 72 ℃ for 30 s; 72 ℃ for 5 min; 4℃ hold. PCR products were analyzed using a Bio-Rad (Bio-Rad Laboratories, U.S.A.) slab electrophoresis system. The 10 μL samples were loaded onto a 12% native polyacrylamide gel (29:1 acryl/bisacryl) in 0.5×Tris borate EDTA (TBE). Gels were run at room temperature for 40 min at 200 V. The gel was confirmed by silver staining.

3. Results and discussion

3.1 Proof of principle.

The principle of the PCR-free fluorescence assay for the detection of telomerase activity based on double amplification strategy was schematically shown in Scheme 1. An arch-structure DNA probe is formed by partial hybridization between complementary DNA (cTSDNA) of elongation product of telomerase substrate (TS) primer and trigger DNA (t-DNA) of strand displacement reaction. Molecular beacon (MB) was designed as fluorescence reporter which can be opened by free t-DNA. In the absence of telomerase, the stable arch-structure DNA probe cannot open the hairpin structure of MB, leading to a very weak fluorescence. In the presence of telomerase, the TTAGGG repeat units were added to the 3′-end of the TS primer in the presence of the nucleotide mixture (dNTPs) to form a long elongation strand (E-TSDNA). The E-TSDNA could displace t-DNA from the arch-structure DNA to form a more stable double-stranded DNA with cTSDNA. Then, t-DNA hybridized with MB to restore the fluorescence of MB. E-TSDNA contains several TTAGGG repeat units. So, one E-TSDNA was able to hybridize with more than one cTSDNA to release more t-DNA for realizing first step amplification. Furthermore, the MB strand in MB/t-DNA hybrid was cleaved at the recognition site (red region) of Nt.BbvCI to release t-DNA again for initiating the next cycle of hybridization and cleavage reaction. More and more MBs were opened to achieve fluorescence accumulation for sensitively detecting telomerase activity based on the double amplification.

Scheme.1

To prove the feasibility, firstly, a chemical synthesized telomerase elongation product (CST) was used to verify the sensing mechanism. Through recognition and

hybridization with the chemical synthesized telomerase product, an obvious fluorescence enhancement of MB was obtained (Figure S1). In addition, with the proceeding of the Nt.BbvCI-aided DNA recycling amplification, the fluorescence intensity further dramatically enhanced, demonstrating that it is feasible to detect telomerase activity via the double amplification strategy. Then, the fluorescence response of telomerase elongation products was studied. The fluorescence enhancement in the presence of the telomerase equivalent to 1000 HeLa cells was shown in Figure 1A. The mixture of MB and arch-structure DNA shows low fluorescence (a). In the presence of telomerase elongation products, an obvious fluorescence enhancement was observed (d). This result suggested that t-DNA was released from the arched structure DNA to open MB. Furthermore, a significant fluorescence enhancement was obtained by introducing NEase (e). The preliminary results revealed the feasibility of this amplification strategy.

To further verify the reliability, more control experiments were performed. First, the influence of lysis buffer was studied. The fluorescence intensity of the mixture of MB and arched structure DNA remained unchanged upon the addition of lysis buffer in Figure 1A (b). That is, lysis buffer has no influence on the fluorescence intensity of MB. Then, the influence of NEase was studied. It is clear from Fig.1B that NEase alone cannot cause the fluorescence restoration of MB. That is, the fluorescence change is not due to the non-specific cleavage of NEase. Finally, to further confirm the telomerase-dependent fluorescence enhancement, telomerase was replaced by heat-treated telomerase. It is well known that heat will destroy the essential RNA

template and reverse transcriptase protein of telomerase. Telomerase activity depends on its RNA and reverse transcriptase protein. As shown in Figure 1A, the fluorescence intensity remained unchanged in the presence of inactive telomerase (c). Once again, it demonstrated that the fluorescence enhancement dependent on the telomerase activity. All the results described above implied that the present design has the capability to detect the telomerase activity in cancer cells.

Fig.1

3.2 Optimization of experimental conditions.

To improve the sensitivity, the concentration of TS and molecular beacon were optimized. As shown in Figure 2A, the fluorescence intensities increased rapidly with the increase of the TS concentration at first and reached an equilibration step at the concentration of 20 nM. Thus, 20 nM TS was used throughout the experiment. To investigate the effect of MB concentration, TS (20 nM), arched structure DNA probe (100 nM) and the telomerase extracts (2μL) were mixed with different concentrations of MB ranged from 100 nM to 500 nM. As shown in Figure 2B, both the fluorescence enhancement in the presence of telomerase extras (F) and the background fluorescence (F_0) increased as the MB concentration increased. The best signal-to-noise was achieved when the concentration of MB was 200 nM.

The amount of NEase was optimized. At the fixed reaction time, the fluorescence intensity increased significantly with the increasing concentration of NEase and reached an optimal value at 15 U mL^{-1} (Figure 2C). Therefore, the optimum

concentration of NEase was 15 U mL^{-1}. The NEase reaction time, which determined the circle times, was another influence condition for the fluorescence assay. The influence of the NEase reaction time was investigated, as shown in Figure 2D. The fluorescence intensities increased with the increase of reaction time, and a plateau effect was reached after 30 min. Therefore, reaction time of NEase-aided assistant DNA recycling was 30 min**.**

Fig.3

3.3 Detection of Telomerase Activity.

To validate the sensitivity of the newly developed telomerase assay, telomerase extracted from 1.0×10^6 HeLa cells were serially diluted with lysis buffer before reacting with TS primers. As the elongation of telomere primer was controlled by the content of telomerase in the cell lysate samples, the amount of released t-DNA and fluorescence restoration all were related to the telomerase activity. Under the optimal conditions, the relationship between fluorescence intensity and cell numbers of HeLa cell was studied. It is clear from Figure 3 that the fluorescence intensities increased with the increasing of cancer cells. The telomerase activity in the HeLa extracts equivalent to 5-1000 cells could be detected by introducing NEase. That is, the telomerase activity equivalent to 5 cancer cells can be detected. The inset of Figure 3B depicts the linear relationship between fluorescence enhancement and the cells numbers from 0 to 100. A series of eleven repetitive measurements of 1000 target cells were used for estimating the precision, and the relative standard deviation was 2.3%. It showed that the amplification fluorescence detection had good reproducibility.

Compared with the previous methods (Table S2), the current protocol is one of the most sensitive assays for the determination of telomerase activity without PCR amplification.

Fig.3

In Figure S2A, the telomerase activity form CCRF-CEM cells was also measured via similar procedures as previous described. The fluorescence intensities enhanced when the cell number was raised from 10 to 1500. That is, the telomerase activity equivalent to 10 CCRF-CEM cells can be detected by the double amplification technique. Figure S2B shows the relationship between the fluorescence enhancement and the cell numbers. The inset of Figure S2B depicts the linear relationship between fluorescence enhancement and the cells numbers from 50 to 1000. Compared with the telomerase activity in the same number of HeLa cells, the telomerase activity in CCRF-CEM cells is lower (Figure S3).

3.4 Inhibition Assay of Telomerase Activity.

Telomerase is overexpressed in over 85% of all known human tumors, while telomerase activity is absent in human somatic cells (Reed et al., 2006; Zahler et al., 1991). Telomerase has been regarded as an important target for the development of new antiproliferative agents. Telomerase activity requires an unfolded single-stranded telomeric overhang. Telomeric DNA may form a G-quadruplex, which is an ineffective substrate for telomerase. Therefore, G-quadruplex-interacting agents that selectively bind and stabilize the G-quadruplex structure can inhibit telomerase activity (Perry et al., 1998). The inhibition of telomerase activity in the presence of

G-quadruplex binding ligand was studied to investigate the feasibility by using the developed method. In this work, the G-quadruplex binding ligand, aloe-emodin (AE) and its derivative (AED3) which was designed and synthesized by our group, were utilized as telomerase inhibitors. Telomerase extracted from 1000 HeLa cells was incubated with serial concentrations of the inhibitors for 30 min at 37℃ before performing the telomerase extension reaction. Then, the elongation products were detected by the proposed strategy. It has been found that the fluorescence enhancement induced by telomerase significantly decreased in a dose–response manner when telomerase incubated with inhibitors, revealing that our assay can effectively study the inhibition of telomerase activity (Figure 4). The IC50 value of aloe-emodin derivative (AED3) and aloe-emodin (AE) were 3.45 μM and 4.08 μM respectively.

Fig.4

To further confirm the reliability, the most commonly applied method, TRAP, has been performed to verify the telomerase activity and the inhibition ability of G-quadruplex-binding ligands. As shown in Figure 5, the TRAP amplification products were characterized by polyacrylamide gel electrophoresis (PAGE). Lanes 1 and 2 is corresponding to the negative controls. It is clear that PCR product did not produce when cell extract was absent or heat-inactivated. Lane3 represented amplification products in the presence of telomerase, which showed the multiple bands in ladder-like patterns. Lane 4 and Lane 5 represented the amplification products when telomerase incubated with 8 μM AE and 6 μM AED3 respectively.

Compared with lane 3, the bands of PCR products is less and shallower, indicating that the telomerase activity had been partially inhibited. AED3 achieves better inhibition effect, which is accordance with fluorescence results. Above research result indicates that the proposed strategy can be effectively and reliably applied to study inhibition of telomerase activity as well as detect telomerase activity.

Fig.5

4. Conclusion

In summary, we have developed a PCR-free double amplification fluorescence strategy for sensitively and reliably detecting telomerase activity. In comparison with the telomere repeat amplification protocol (TRAP), the proposed method is more simple, reliable, highly sensitive, and rapid with an isothermal condition but without employing the complicated denaturation-annealing cycles or gel analysis. Compared with the existing assays based on TS-primer conjugated to nanoparticles, electrode or other solid supports, TS elongation reaction is carried out in homogeneous solution, which will avoid the steric hindrance or other influences of these solid supports to achieve more real study of telomerase activity. Moreover, by ingeniously combining amplification induced by TS elongation with t-DNA recycling, telomerase activity has been highly sensitive detected. Without PCR amplification, telomerase activity equivalent to 5 HeLa cells and 10 CCRF-CEM cells were reliably determined, which is comparable with or even superior to most previously reported telomerase assays. Furthermore, arch-structure DNA and hairpin structure of MB highly ensured the specificity and reliablity. The influence of G-quadruplex-binding ligands on the

telomerase activity revealed that it holds great promise for screening potential telomerase inhibitors. Therefore, it holds great promise as a facile and sensitive method for studying telomerase inhibitors as well as detecting telomerase activity, which is of great importance for telomerase-related cancer diagnosis and therapy.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 21075079, 21375086), the Fundamental Research Funds for the Central Universities (GK261001097), the Program for Changjiang Scholars and Innovative Research Team in University (IRT_14R33), and the Program for Innovative Research Team in Shaanxi Province (No. 2014KCT-28).

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Figures captions

Scheme 1. Schematic illustration of PCR-free detection of telomerase activity based on the double amplification fluorescence strategy.

Figure 1. (A) The fluorescence spectra of MB under different conditions. (a) MB+arch-structure DNA probe, (b) a+lysis buffer, (c) a+heat-inactivated telomerase elongation product, (d) a+E-TSDNA, (e) d+Nt.BbvCI. (B) Fluorescence spectra of MB under different conditions. (a) MB, (b) MB+cTSDNA, (c) MB+arched structure DNA probe, (d) c+Nt.BbvCI. The concentrations of MB, cTSDNA, arched structure DNA probe and Nt.BbvCI were 200 nM, 100 nM, 100 nM and 15 U ml^{-1} respectively.

Figure 2. The effect of (A) TS， (B) molecular beacon (MB), (C) Nt.BbvCI concentration and (D) Nt.BbvCI cleavage time on the fluorescence response.

Figure 3. (A) Fluorescent spectra in response to telomerase extracts from different number of HeLa cells, the curves from a to m represent the extracts from 0, 5, 10, 20, 40, 60, 80, 100, 200, 400, 600, 800 and 1000 HeLa cells, respectively. The insert is the amplification of the lines a–d. (B) The relationship between the fluorescence intensity and telomerase (red line) and heat-inactivated telomerase (black line) from different numbers of HeLa cells. The concentrations of molecular beacon (MB), TS, arched structure DNA probe and Nt.BbvCI were 200 nM, 20 nM, 100 nM and 15 U/mL, respectively.

Figure 4. Inhibition of telomerase activity by aloe-emodin derivative (AED3) (A-1) and aloe-emodin (AE) (B-1) with the double amplification fluorescence assay. The

Relative activity of Telomerase incubated with AED3 (A-2) or AE (B-2) of different concentrations.

Figure 5. Gel image for TRAP assays of extracts from HeLa Cells. Lane M , the DNA ladder; Lane 1, negative control in the absence of telomerase; Lane2, negative control with heat-inactivated telomerase; Lane3, telomerase extracted from 1000 HeLa cells; Lane4, telomerase extracted from 1000 HeLa cells and 8 μM AE; Lane5, telomerase extracted from 1000 HeLa cells and 6 μM AED3.

Highlights

- A PCR-free fluorescence strategy was proposed for telomerase activity by combining self-amplification with two isothermal signal amplification techniques.
- Arch-structure DNA probe and hairpin structure of molecular beacon highly ensure the reliability and specificity of telomerase-induced signal transfer.
- Telomerase activity equivalent to 5 HeLa cells and 10 CCRF-CEM cells were reliably determined, which is comparable with or even superior to most previously reported telomerase assays.
- The influence of G-quadruplex-binding ligands on the telomerase activity revealed that it holds great promise for discovering potential telomerase inhibitors.

Scheme 1

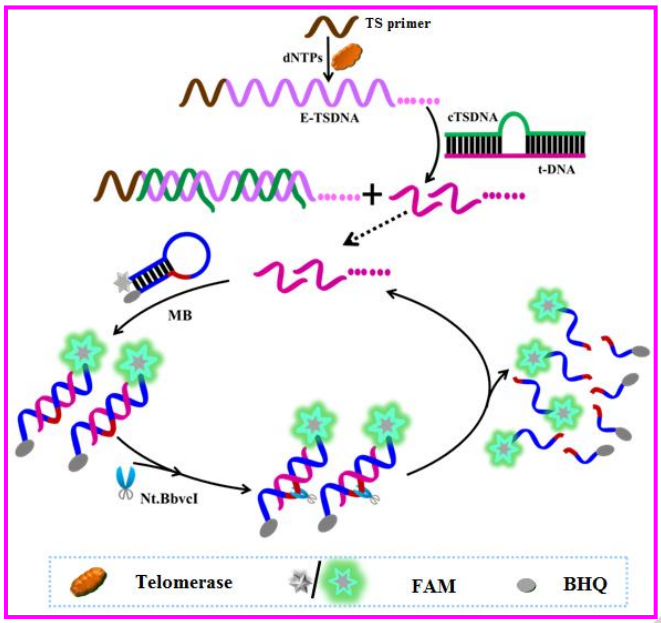

Figure 1

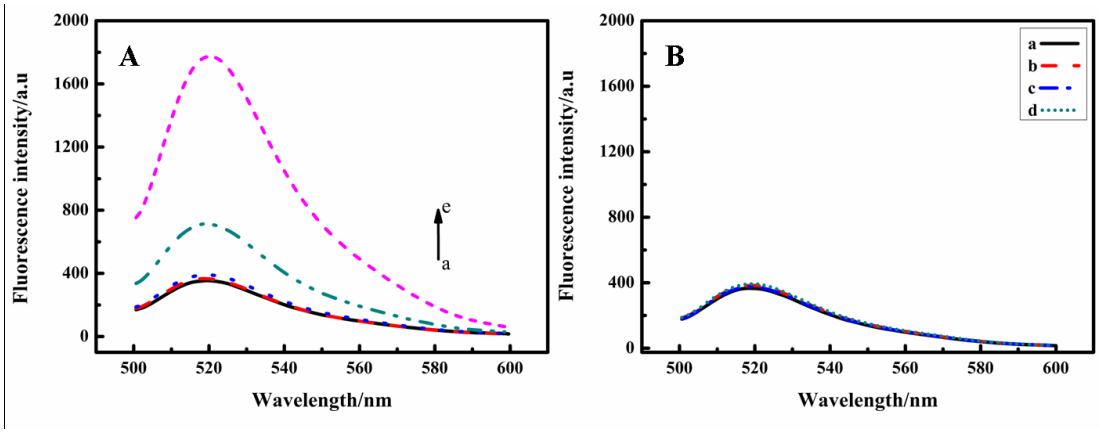

Figure 2

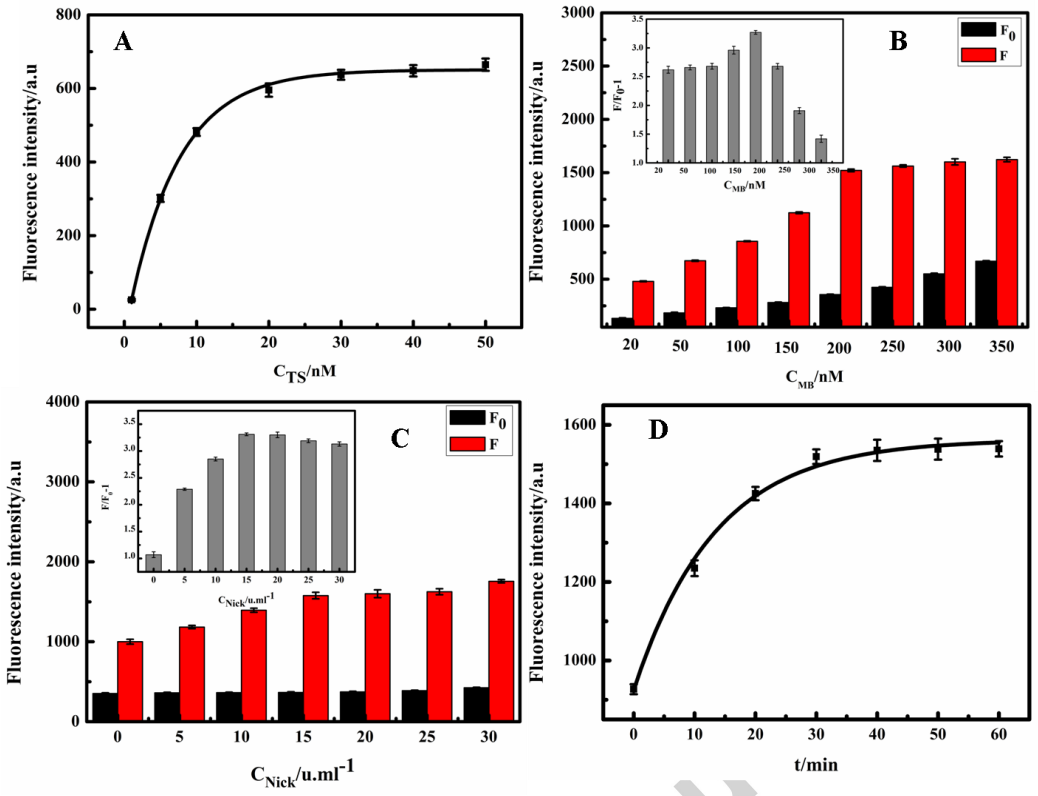

Figure 3

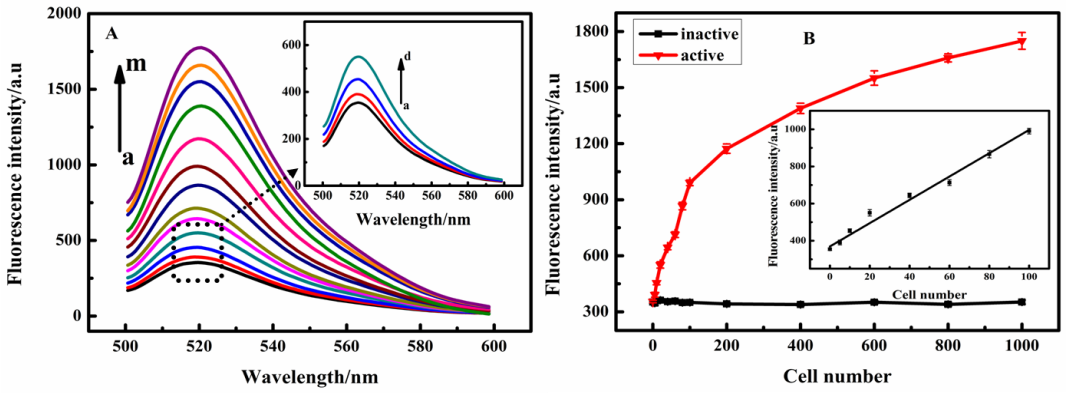

Figure 4

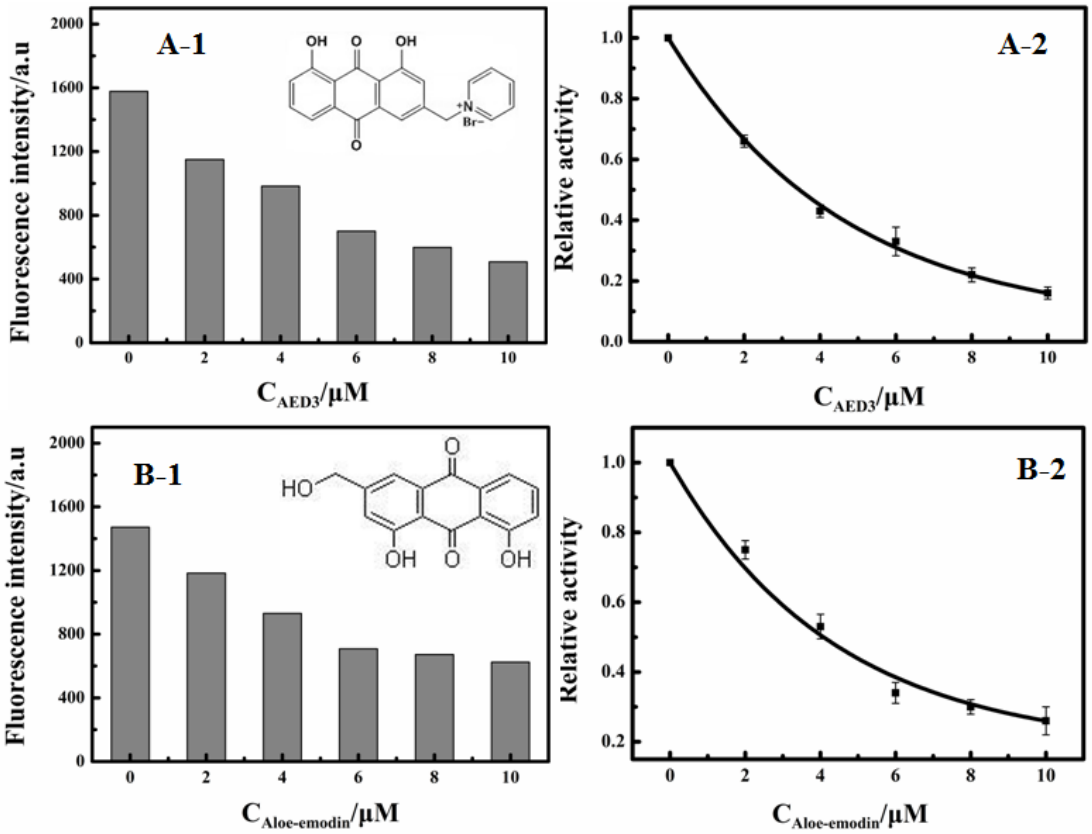

Figure 5