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# Graphical Abstract

## Simple, PCR-free telomerase activity detection using G-quadruplex-hemin DNAzyme

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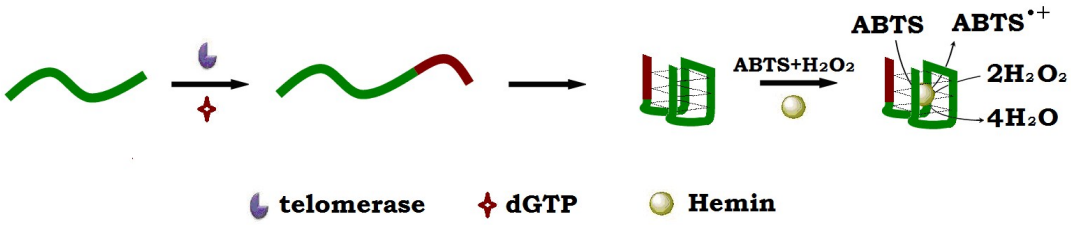


A G-quadruplex DNAzyme-based telomerase activity detection method is developed by utlizing telomerase-triggered generation of short G-rich extension products.

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# Simple, PCR-free telomerase activity detection using G-quadruplex-hemin DNAzyme

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A simple, cost-effective and polymerase chain reaction (PCR)-free telomerase activity detection method was developed on the basis of telomerase-triggered formation of G-quadruplex-hemin DNAzyme. In this method, a short, unlabelled telomerase primer was used. Because this primer contains only three GGG repeats, it cannot fold into stable G-quadruplex structure. In the presence of active telomerase and dGTP, a GGG repeat is added to the 3′-end of the primer. The extended primer can fold into G-quadruplex, which is able to bind hemin to form catalytically active G-quadruplex-hemin DNAzyme, catalyzing the oxidiation of 2,2′-azinobis (3-ethylbenzothiozoline)-6-sulfonic acid (ABTS) by $H_2O_2$ to green $ABTS^{\bullet+}$. Because the primer extension product is very short, telomerase might show high turnover rate, thus providing the method with improved sensitivity. Using this method, the telomerase activity originating from 200 HeLa cells can be detected.

## Introduction

Telomerase is a ribonucleoprotein enzyme that can add telomeric repeats (5′-GGGTTA-3′) onto the ends of human chromosomes and thus is responsible for the uncontrolled growth of cancer cells.[1] In contrast to healthy cells, in which telomerase activity is repressed, about 85% of malignant tumors show reactivated telomerase activity. The differential expression of telomerase between healthy somatic cells and tumor cells makes telomerase an attractive tumor marker and a potential target for chemotherapy.[2] In addition, telomerase is a potentially important biomarker for the early diagnosis and prognostic evaluation of cancer.[3]

Until now, some analytical methods were developed to assay telomerase activity. The most promising and frequently used one is the telomeric repeat amplification protocol (TRAP) assay.[4] Based on the TRAP, several modified versions have been developed.[5] These methods are sensitive and efficient since the telomerase products generated in the presence of active telomerase are exponentially amplified. However, TRAP is limited since it is based on polymerase chain reaction (PCR) amplification, which is time-consuming, prone to carry-over contamination, susceptible to inhibition by cell-extract, and requires expensive equipments and reagents. To circumvent this, several alternative PCR-free methods, including quantum dots-based fluorescence detection method, surface plasmon resonance (SPR) sensing platforms, chemiluminescence and electrochemical techniques, have been developed for telomerase activity analysis.[6] However, each of them still has its shortcomings such as low sensitivity, complicated manipulation, or the requirement of elaborate instruments and expensive fluorescent labels.

Constructing a simple, label-free, and cost-effective telomerase activity detection method has attracted more and more attention in these years. In 2004, Willner *et al* reported a simple, PCR-free telomerase activity detection method using G-quadruplex-hemin DNAzyme.[7] G-quadruplex-hemin DNAzyme is a peroxidase-like complex formed by hemin and G-quadruplex, an unordinary nucleic acid secondary structure formed by G-rich DNA or RNA sequence.[8] Compared to natural peroxidase, this artificial DNAzyme shows several important advantages and has been widely used in the detection of various targets, from proteins and DNAs to small molecules and metal ions.[9] In the method reported by Willner *et al*, G-rich sequence is embedded in a hairpin structural telomerase primer. The extension of the primer by telomerase can release the G-rich sequence, thus leading to the formation of G-quadruplex-hemin DNAzyme. This work opens up a new approach for simple telomerase activity analysis using G-quadruplex-hemin DNAzyme.[10]

In this report, we design another convenient, cost-effective and PCR-free method for telomerase activity detection based on G-quadruplex-hemin DNAzyme. This method adopts a different primer design and G-quadruplex formation mechanism from the reported work. In this method, only three guanine (G) bases are needed to be added to the 3′-end of the primer, telomerase might have high turnover rate, thus might provide the proposed method with improved detection sensitivity.

## Experimental section

### Materials and reagents

All oligonucleotides, ethylene glycol tetraacetic acid (EGTA), β-mercaptoethanol, 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid (CHAPS) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sangon Biotech. Co. Ltd. (Shanghai, China). The concentrations of the oligonucleotides were represented as single-stranded concentrations. Single-stranded concentrations were determined by measuring the absorbance at 260 nm. Molar extinction coefficients were determined using a nearest neighbour approximation. $H_2O_2$, 2,2′-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS), Triton X-100, and hemin were obtained from

Sigma. All chemical reagents were of reagent grade and used without further purification.

### Preparation of telomerase extract

HeLa (Human epithelial carcinoma) cells were kindly provided by Jiatong Chen (Common Lab of Cell Culture, College of Life Science, Nankai University, Tianjin, China). Cell extract containing telomerase was prepared as described previously.[4] Cells were collected in the exponential phase of growth, and $1 \times 10^7$ cells were dispensed in a 1.5 mL EP tube, washed twice with ice-cold phosphate buffered saline (PBS) solution, and re-suspended in 200 μL of ice-cold CHAPS lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM $MgCl_2$, 5 mM β-mercaptoethanol, 10% W/V glycerol, 0.5% CHAPS and 0.1 mM PMSF). The mixture was incubated for 30 min in ice, and then centrifuged at 14,000 rpm at 4 °C for 30 min to pellet insoluble material. Without disturbing the pellet, the cleared lysate was carefully transfered to a fresh 1.5 mL EP tube. The lysate was used immediately for telomerase assay or frozen at -70 °C. For the heat pretreatment control, cell extract was heated at 95 °C for 10 min.

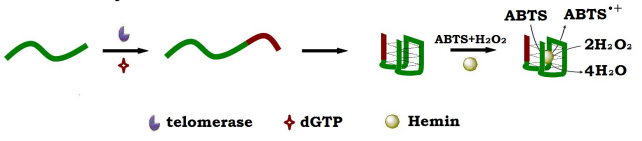
### Telomerase activity detection

Reaction mixture containing 20 mM Tris-HCl buffer (pH 8.2), 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween 20, 1 mM EGTA, 0.1 mg/mL BSA, 1 mM dGTP, 0.2 μM telomerase primer and 5 μL cell extract, was prepared. The mixture was incubated at 37 °C for 1.5 h for telomerase-triggered primer extension. After that, hemin (1 μM, final concentration) was added to the mixture. The mixture was held for 1 h at 20 °C. Then, 3.2 mM of ABTS and 2.2 mM of $H_2O_2$ were added. The final volume of the mixture was 100 μL. The absorption spectrum of the reaction product $ABTS^{\bullet+}$ was recorded by a TU-1901 UV-vis spectrophotometer after the reaction had run for 5 min. The absorbance at 419 nm was used for quantitative analysis. The color of the reaction mixture was recorded by a digital camera.

## Results and discussion

### Design of the telomerase activity detection system

The formation of a stable intramolecular G-quadruplex needs an oligonucleotide containing at least four G-tracts. In the proposed telomerase activity detection method (Scheme 1), a DNA oligonucleotide with only three G-tracts is used as the primer of telomerase (Fig. 1). Because this primer cannot form stable G-quadruplex, peroxidase-like G-quadruplex-hemin DNAzyme might not be formed in the presence of hemin. However, in the presence of active telomerase, telomerase-triggered primer extension reaction adds a GGG repeat to the 3′-end of the primer, thus making the extended product fulfill the requirement for the formation of stable intramolecular G-quadruplex, which binds hemin to form peroxidase-like G-quadruplex-hemin DNAzyme, catalyzing the oxidation of 2,2′-azinobis(3-ethylbenzothiozoline)-6-sulfonic acid (ABTS) by $H_2O_2$. Correspondingly, the absorption intensity of the reaction mixture at 419 nm increased greatly. According to the absorption signal change of the detection system, telomerase activity can be easily detected.




**Scheme 1** Schematic representation of G-quadruplex-hemin DNAzyme-based telomerase activity detection method.

### Design of telomerase primer

One key of the proposed telomerase activity detection method is the design of telomerase primer. A desired primer should meet two requirements: (1) It should be effectively extended by telomerase; (2) The designed primer should not form G-quadruplex-hemin DNAzyme in the absence of telomerase, but highly active G-quadruplex-hemin DNAzyme could be formed after extension by telomerase. Telomerase can add GGGTTA repeats to the 3′-end of its primer. To ensure that the designed primer can be effectively extended by telomerase, the six nucleotides at the 3′-end of telomerase primer were designed as telomeric sequence. That is, GGGTTA.

We and other groups have demonstrated that the activity of G-quadruplex-hemin DNAzyme is highly relevant with the structure of G-quadruplex.[11] The activity of DNAzyme formed by different structural G-quadruplexes follows the order: parallel G-quadruplex > parallel/antiparallel-mixed hybrid G-quadruplex > antiparallel G-quadruplex. It is reported that G-rich sequences with short linking loops, which connect adjacent two G-tracts, tend to fold into parallel G-quadruplexes, and the G-rich sequences containing long loops incline to fold into antiparallel ones.[12] Considering that the G-rich telomeric sequence, which has "TTA" loops, tends to fold into hybrid G-quadruplex with relatively low catalytic activity,[13] we did not select short telomeric sequence containing three GGG repeats as telomerase primer, but designed several G-rich sequences with short linking loops as the candidate telomerase primers.

| DNAs | Sequence |
|---|---|
| P1 | *ggg*T*ggg*T*ggg*TTA |
| T1 | *ggg*T*ggg*T*ggg*TTA***ggg*** |
| P2 | *ggg*T*ggg*C*ggg*TTA |
| T2 | *ggg*T*ggg*C*ggg*TTA***ggg*** |
| P3 | *ggg*C*ggg*C*ggg*TTA |
| T3 | *ggg*C*ggg*C*ggg*TTA***ggg*** |
| P4 | *ggg*TA*ggg*C*ggg*TTA |
| T4 | *ggg*TA*ggg*C*ggg*TTA***ggg*** |
| P5 | T*ggg*TA*ggg*C*ggg*TTA |
| T5 | T*ggg*TA*ggg*C*ggg*TTA***ggg*** |

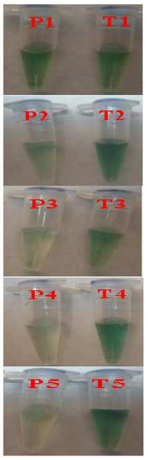



**Fig. 1** Potographed images of the ABTS-$H_2O_2$ reaction systems containing designed telomerase primers (P1~P5) or their individual mimicking extended products (T1~T5).

Five telomerase primers (P1~P5, Fig. 1) were designed, and a GGG repeat was added to the 3′-ends of these primers to mimic their extended products by telomerase (T1~T5). The peroxidase-like activities of these primers and their individual mimicking extended products were compared in the presence of hemin. As shown in Fig. 1, obvious green color was given by the reaction system containing P1, indicating that highly active G-quadruplex-hemin DNAzyme was formed in this system. The reason is that as a G-rich sequence with three GGG repeats, P1 might fold into intermolecular G-quadruplex, which would also bind with hemin to form active G-quadruplex-hemin DNAzyme, catalyzing the oxidation of ABTS by $H_2O_2$ to green product $ABTS^{\bullet+}$. When a linking loop was changed from thymidine (T) in P1 to cytosine (C) in P2, the catalytic activity of corresponding reaction system decreased greatly, but pale green color could also be observed. However, obvious color change could

be observed between the two systems containing P2 and T2, respectively. When the two linking loops were both changed to C bases (P3), the catalytic activity of corresponding reaction system further decreased. However, the green color of the reaction system containing its mimicking extended product T3 was weaker than those containing T1 or T2. That is, using P3 as the telomerase primer could decrease background signal, but its extended product could not give satisfactory catalytic activity. Above results suggested that G-rich sequences containing "T" linking loops tend to fold into intermolecular G-quadruplexes, which can also form G-quadruplex-hemin DNAzymes. P4 contains a "TA" loop and a "C" loop. The reaction system containing P4 could give very low background, and obvious signal change could be observed compared to its extended product T4. Adding a "T" base at the 5′-end of P4 can further enlarge the signal change between the resulted primer P5 and its mimicking product T5 to a little degree. This can be due to the positive effect of T base(s) tail located at 5′-end of G-rich sequence on catalytic activity as previously reported.[14] Therefore, P5 was selected as the telomerase primer in subsequent experiments.

**Optimization of telomerase detection conditions**

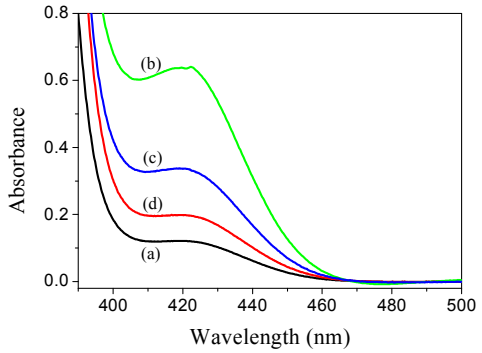



**Fig. 2** Absorption spectra of the detection mixtures containing (a) no cell tract, (b) HeLa cell extract + dGTP, (c) HeLa cell extract + dNTPs, and (d) heat-inactivated HeLa cell extract + dGTP. The number of HeLa cells was 10,000.

The designed telomerase primer P5 has three GGG repeats. To meet the requirement for intramolecular G-quadruplex formation, only one GGG repeat is needed to be added at the 3′-end of P5. In fact, telomerase-triggered primer extension reaction can add a long GGGTTA repeats to the 3′-end of P5. As a result, two reaction models were designed by us for telomerase activity detection. In the first model, dGTP was added in the detection system to assay the telomerase activity in HeLa cell extract. When a GGG repeat was added to the 3′-end of P5, this primer could not be further extended any longer because of the lack of dTTP. As a result, a short extended product TGGGTAGGGCGGGTTAGGG (T5) was produced. Such a reaction model gave a high absorption signal at 419 nm (Fig. 2, Line b), which is the maximum absorption wavelength of $ABTS^{\bullet+}$. In the second model, a dNTP mixture, containing dGTP, dTTP, dATP and dCTP, was added instead of dGTP. In this model, P5 could be extended by telomerase to form a long product $(TGGGTAGGGCGGGTTAGGGTTA(GGGTTA)_n)$, which might fold into multimeric G-quadruplex containing several G-quadruplex units.[15] However, corresponding detection system gave a much weaker absorption signal than the first model (Fig. 2, Line c). This is not surprise. Although the long extended product might fold into multimeric G-quadruplex, the G-quadruplex unit formed by $(GGGTTA)_4$ is hybrid G-quadruplex showing weak catalytic activity in the presence of hemin.[13] In the first model, because P5 cannot be further extended after addition of a GGG repeat, the telomerase reacted on it might then dissociate from this primer, and turn to react on other unextended P1. That is to say, in this model, telomerase has high turnover rate,[16] and more primers are extended to form highly active G-quadruplex-hemin DNAzyme. Therefore, the first reaction model was selected for subsequent experiments. That is, dGTP, but not dNTPs, is added in the detection system. To demonstrate that a short extension product is produced in this reaction model, polyacrylamide gel electrophoresis (PAGE) was used to separate the reaction systems without and with active telomerase. As shown in Fig. S1, the reaction systems containing active telomerase only gave two DNA bands. The faster-moving one corresponds to unextended primers. The other band with slightly lower migrating rate corresponds to the short extension products. To further demonstrate the color change of the detection system is really related with active telomerase, HeLa cell extract was heated at 95 °C for 10 min to inactivate the telomerase activity and added into the detection system. As expected, the absorption signal change caused by heat-inactivated cell extract was much lower than that caused by active cell extract (Fig. 2, Line d).

Incubation time and temperature of telomerase-triggered primer extension reaction were also optimized. As shown in Fig. 3, the absorption signal of the detection system obviously increased with incubation time, then gradually levelled off when the incubation time exceeded 1.5 h. Then, 1.5 h was selected as a compromise of detection signal intensity and detection time. 37 °C was selected as the incubation temperature for the primer extension reaction due to the highest telomerase activity at this temperature, which was reflected by the highest signal intensity given by the detection system.

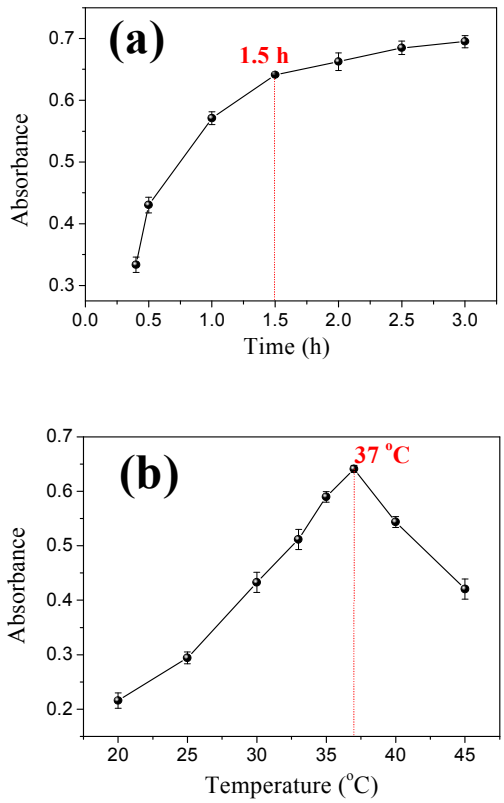



**Fig. 3** Absorption signal given by the detection systems performed for different incubation time (a) and under different incubation temperature (b). The number of HeLa cells was 10,000.

**Quantitation of telomerase activity in cell extract**

Under the optimized conditions, the telomerase activity originating from different numbers of HeLa cells was analyzed by the proposed method. As shown in Fig. 4, in the low cell number range, the detection signal increased with cell number, and nearly reached a plateau with 10,000 HeLa cells. A linear relationship ($R^2$ = 0.9967) was observed over a range of 100~1000 cells. If using heat-inactivated extract from 10,000 HeLa cells as the negative control, obvious detection signal change above this negative control was observed for 200 HeLa cells, indicating that the detection limit of the HeLa cells is ca. 200 cells. The obtained detection limit is lower than the reported G-quadruplex-based telomerase activity assay method.[7,17] This might be attributed to the high turnover rate of telomerase in our method. As mentioned above, in our detection method, telomerase might give high turnover rate because only a GGG repeat is needed to be added at the 3′-end of telomerase primer. On the contrary, in the reported method,[7] several GGGTTA repeats are needed to be added to the 3′-end of primer to release G-rich sequence to form a G-quadruplex. In addition, even if the primer was extended to produce a very long product, only a highly active G-quadruplex-hemin complex could be formed. In this method, colorimetric signal is used to analyse the activity of telomerase. If fluorescence or chemiluminescence assay is used,[18] the detection sensitivity might be further increased.

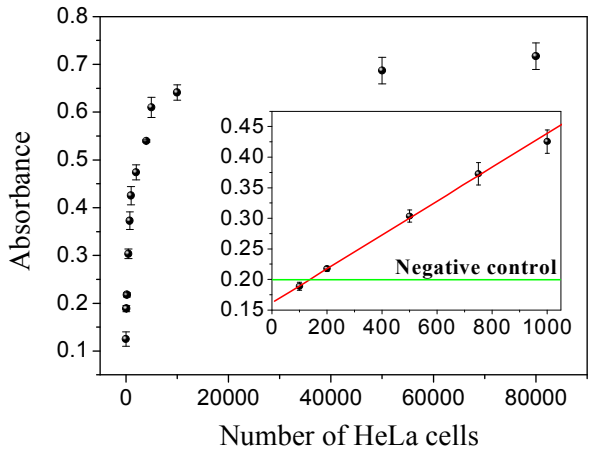



**Fig. 4** Telomerase activity-dependent change in the absorption signal at 419 nm. The inset shows the linear relationship between the absorbance and the number of cell in the range of 100~1000 HeLa cells. The green line represents the absorption signal of the negative control containing 10,000 heat- inactivated HeLa cells.

## Conclusions

In summary, a simple, cost-effective and rapid telomerase activity detection method was developed on the basis of telomerase-triggered formation of G-quadruplex-hemin DNAzyme. Compared with some reported telomerase activity detection methods, our method displays some important features: (1) Our method is PCR-free, thus greatly eliminating the risk of pseudo-positive results caused by cross-contamination of PCR products; (2) The operation is very simple and the whole telomerase detection operation (preparation of telomerase extracts is not included) can be finished in 3 h, thus making it suitable for high-throughput determination of telomerase activity; (3) Our method is very cost-effective. In this method, no fluorescent- or radioactive-labelled DNA probes are needed, and only commonly used UV-vis absorption sepectrophotometer is used, eliminating the needs for any expensive instruments; (4) Only three G bases are needed to be added at the 3′-end of the primer. Telomerase might have high turnover rate. As a result, more primers can be extended and fold into G-quadruplexes, thus providing the detection method with improved sensitivity. Using the proposed method, the telomerase activity originating from 200 HeLa cells can be detected.

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