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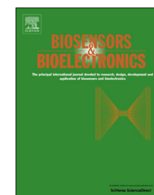

# Fluorescence detection of telomerase activity in cancer cell extracts based on autonomous exonuclease III-assisted isothermal cycling signal amplification

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ABSTRACT

Based on the extension reaction of a telomerase substrate (TS) primer in the presence of the telomerase, strand-displacement process to perform more stable longer duplex chain, and stepwise hydrolysis of mononucleotides from the blunt or the recessed 3′-hydroxyl termini of duplex DNA in the presence of Exonuclease III (Exo III), an amplified fluorescence detection of telomerase activity in the cancer cells was described in this manuscript. A fluorescence probe DNA, a quencher DNA, and a TS primer were mixed to construct a three-chain DNA structure and a two-chain DNA structure because the amount of the TS primer was less than the other two DNA. In the presence of the telomerase, the quencher DNA was replaced from the probe DNA and the telomerase activity could be determined with the fluorescence enhancement. The telomerase activity in HeLa extracts equivalent to 6-2000 cells was detected by this method. Moreover, the strategy was further proved by using telomerase extracted from Romas cells. With the multiple rounds of isothermal strand displacement and the hydrolysis process, constituted consecutive of signal amplification for the novel detection paradigm that allowed measuring of telomerase activity in crude cancer cell extracts confirmed the reliability and practicality of the protocol, which reveal this platform holds great promise in the biochemical assay for the telomerase activity in early diagnosis for cancers.

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## 1. Introduction

Human telomerase is a ribonucleoprotein responsible for the maintenance of chromosome integrity by adding tandem repeats (TTAGGG)n to the ends of telomere through its RNA template, which is one of the most common cancer markers due to its strong association with cellular immortality and carcinogenesis (Blackburn 1991, 2001; Harley 2008). In most normal somatic cells, as the telomerase expression is highly repressed or absent, telomeres are shortened after each replication cycle, which leads to cell senescence and finally cell apoptosis (Blasco, 2005; Rodier and Campisi, 2011; Shay and Wright, 2005). However, in most types of cancer cells, the length of telomeres is maintained because of the upregulation or activation of telomerase, which makes cancer cells divide indefinitely (Petel et al., 2004; Masutomi et al., 2003).

Therefore, telomerase and telomere structures play key roles in the studies of many diseases, such as cancer, gene regulation, cell/organism aging, and the cloning of mammals (Artandi and DePinho, 2010; Cong and Shay, 2008). Thus, a variety of strategies have been proposed for the detection of telomerase activity since its discovery in 1985 (Greider and Blackburn, 1985). Telomere repeat amplification protocol (TRAP) is a conventional assay for detecting telomerase activity based on classic polymerase chain reaction (PCR) technique (Liu et al., 2010; Herbert et al., 2006; Wu et al., 2000). However, TRAP is time-consuming and could inherently generate false positive/negative artifacts. So it is not suitable for quantitative analysis and screening compounds for telomerase inhibition (Xiao et al., 2010a, 2010b; Zuo et al., 2011). Recently, a variety of strategies, such as isothermal DNA amplification, DNAzyme and nanomaterial-based biosensing protocoles has been proposed to improve the quantification of telomerase activity and simplify the PCR steps in TRAP (Tian et al., 2013; Wang et al., 2013a, 2013b, 2012; Zhao et al., 2013; Li et al., 2011; Patolsky et al., 2003). Plaxco and coworkers detected telomerase activity using telomere strand (TS) primer modified gold nanoparticles by a PCR-based method (Xiao et al., 2010a). On the basis of the combined use of aggregation-induced emission (AIE) fluorogens and quencher, Zhang et al. developed a high specificity strategy for detection of telomerase activity from cell extracts and cancer

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patients' urine specimens (Yuan et al., 2015). Despite these works establish detection assay of telomerase activity in cell extract, it can not realize in situ detection and not provided telomerase information in single-cell level. Subsequently, they designed a novel telomerase activity probe by using gold nanoparticles functionalized with Cy5-tagged molecular beacon, which can be conveniently used for intracellular in situ detection and imaging of telomerase activity through a one-step incubation procedure (Ruo et al., 2014a, 2014b).

Recently, Li's group reported a highly sensitive fluorescence biosensing platform for hOGG1 activity detection based on autonomous exonuclease III (Exo III)-assisted signal amplification (Xiu et al., 2014). According to this novel and high sensitive method and a former work of our group for the fluorescence detection of telomerase activity (Cai and Xiang, 2010; Piatyszek et al., 1995), a telomerase activity assay with high selectivity and sensitivity was designed in this work. The fluorescence of the probe DNA was quenched by part hybridization with the quencher DNA at first and the fluorescence was restored after the quencher DNA was displaced by a longer DNA elongated from TS primer with the help of telomerase. By measurement of the intensity of fluorescence spectra, the telomerase activity in the extracts extracted from 6 to 2000 HeLa cells was detected in this strategy. After the probe DNA was successive digestion by Exo III, the telomerase reaction products could replace the quencher DNA, which in turn triggered the cycling cleavage of fluorescence probe DNA. Therefore, vast fluorophore fragments were released, leading to a significantly amplified fluorescence signal toward telomerase activity detection. The high performance of this assay is related to the determination of telomerase activity from cell extracts equivalent down to 6 HeLa cells. The results show that the present fluorescence strategy holds great promise in the biochemical assay for the early diagnosis of cancers.

## 2. Experimental section

### 2.1. Reagents

All oligonucleotides used in this work were synthesized and purified by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). Their base sequences are as below: Probe DNA sequence, 5′-FAM-CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CTC TGC TCG ACG GAT T-3′; TS primer sequence, 5′-AAT CCG TCG AGC AGA GTT −3′; Quencher DNA sequence, 5′-TTA GGG TTA GGG TTA GGG TTT T-BHQ1-3′.

[Tris (hydroxymethyl) aminomethane] (Tris), Exonuclease III (Exo III) and ethylenediaminetetraacetic acid (EDTA) were purchased from Sangon Biotech Co.,Ltd. (Shanghai, China). Deoxynucleotide solution mixture (dNTPs) was obtained from SaiBaiSheng Gene Technology Co., Ltd. (Beijing China). Telomerase was purchased from shanghai Fu life Industry Co., Ltd. (Shanghai, China). All other chemical reagents were of analytical grade.

HeLa and Ramos cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 100 IU $mL^{-1}$ penicillin-streptomycin. The cells were maintained at 37 °C in a humidified atmosphere (95% air and 5% $CO_2$).

### 2.2. Apparatus

Fluorescence measurements were performed by using a Hitachi F-4600 spectrofluorimeter (Tokyo, Japan) with a scan rate of 1200 nm/min. The excitation wavelength was set to 490 nm, and the 24 photomultiplier tube voltage was set to 700 V. The slits for excitation and emission were set at 10 nm/10 nm. ELIAS assays was performed on a microplate reader (ELx800, BioTek, USA).

### 2.3. Detection of the telomerase

In this assay, 10 μL of 500 nM Probe DNA, 15 μL of 500 nM Quencher DNA and 3 μL of 500 nM TS primer were added into the reaction buffer (10 mM Tris–HCl, 50 mM NaCl, 10 mM $MgCl_2$, pH 7.4) to give a total volume of 100 μL. The above solutions were incubated at 37 °C for 90 min. The 10 μL of telomerase at different concentrations and 5 μL dNTPs were added in the solutions and incubated at 37 °C for 2 h. The 4 μL of Exo III (30 IU) was added the above solution was incubated at 37 °C for 1 h before fluorescence measurement.

### 2.4. .Telomerase extract preparation from HeLa cells grown in culture

The telomerase was extracted by the CHAPS method as described in the reference (Piatyszek et al., 1995). The cancer cells were first suspended in 1 × CHAPS lysis buffer (lysis buffer, Millipore) and incubated on ice for 30 min. Then, the mixture was centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was transferred, aliquoted, and stored at −80 °C. Telomerase extracts were prepared as follows with the conventional method described in Ref. (Shippenlentz and Blackburn, 1990). HeLa cells were removed from the substrate by trypsinization, washed twice with phosphate buffer solution (pH 7.4) and centrifugated at 3500 rpm for 4 min at 4 °C. The cells were resuspended in a cold CHAPS lysis buffer at a concentration of $5.8 \times 10^5$ cells/mL, ultrasonicated for 30 min in ice and then centrifugated for 20 min (12,000 rpm, 4 C). The supernatant was stored at 20 °C for further experiment.

## 3. Results and discussion

### 3.1. Principle of fluorescent telomerase activity assay

The strategy for the sensitive telomerase activity detection was based on the autonomous exonuclease III-assisted isothermal cycling strand displacement and fluorescence signal amplification as shown in Scheme 1. A fluorescence probe DNA, a quencher DNA and a TS primer were used to construct the telomerase activity detection system. The probe DNA partially hybridized with both quencher DNA and TS primer (three-chain DNA), or hybridized only with quencher DNA (two-chain DNA) because the amount of the TS primer was less than the other two DNA. The TS primer was elongated in the presence of telomerase and nucleotide mixture dNTPs. With the telomerisation reaction, repeat units of "TTAGGG" were continuously added to the 3′-end of the TS primer in the three-chain DNA structure to form a longer single strand DNA (S1), which was complemented to the fluorescence probe DNA.

The quencher DNA was replaced by S1 and released from the probe DNA. Then a duplex strand DNA was formed and the fluorescence of probe DNA was restored. After that, the probe DNA in the duplex strand was hydrolyzed by Exo III in the direction from 3′ to 5′, releasing the S1 to replace the quencher DNA from the two-chain DNA structure to form a more stable new duplex DNA, which triggered another hydrolysis reaction. It is noteworthy that the telomerase played a key role during this process. It initiated the telomerization reaction to generate S1, displacement of quencher DNA and recovery of the fluorescence signal, and then the hydrolysis reaction. S1 extended from TS primer could replace the quencher DNA and trigger the hydrolysis reaction circularly, large amount of fluorophore fragments originally contained in fluorescence probe DNA could be released to achieve significant fluorescence enhancement even in the presence of trace amount of the telomerase. Therefore, the telomerase activity could be detected with high sensitivity by this facile biosensing platform.

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