JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

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Real-Time Detection of Telomerase Activity Using the Exponential Isothermal Amplification of Telomere Repeat Assay

Leilei Tian and Yossi Weizmann*

Department of Chemistry, The University of Chicago, 929 East 57th Street, Chicago, Illinois 60637, United States

Supporting Information

ABSTRACT: As crucial pieces in the puzzle of cancer and human aging, telomeres and telomerase are indispensable in modern biology. Here we describe a novel exponential isothermal amplification of telomere repeat (EXPIATR) assay—a sensitive, simple, and reliable *in vitro* method for measuring telomerase activity in cell extracts. Through a strategically designed path of nucleic acid isothermal amplifications, EXPIATR abandons the expensive thermal cycling protocol and achieves ultrafast detection: telomerase activity equivalent to a single HeLa cancer cell can be detected in ~25 min.

The discovery of telomeres and telomerase by Blackburn, Greider, and Szostak, who were awarded a Nobel Prize in 2009, added a new dimension to the understanding of cancer mechanisms and stimulated the development of potential new therapeutics for cancer.[1] Telomerase is a ribonucleoprotein responsible for telomere maintenance by adding tandem repeats $(TTAGGG)_n$ to the ends of chromosomes.[2] It is one of the most common cancer markers due to its strong association with cellular immortality and carcinogenesis. A vast majority of human tumors (~85−90%) express the up-regulation or reactivation of telomerase activity; however, in most normal somatic cells, telomerase activity is highly depressed.[3] Besides relating to cancer, telomerase and telomere structures also play crucial roles in the studies of other diseases, gene regulation, cell/organism aging, and the cloning of mammals.[4]

The real-time telomere repeat amplification protocol (RT-TRAP),[5] based on the classic polymerase chain reaction (PCR) technique, has served as a powerful assay for detecting telomerase activity. TRAP is a closed-tube protocol, avoiding the risk of carryover contamination and providing very high throughputs.[6] Exponential nucleic acid amplification in TRAP assay is driven by several thermal cycling steps, increasing the complexity of instrumentation and making the method time-consuming. Substantial attempts have been made to improve the RT-TRAP assay. For example, different detection means were used to assess the telomerase activity, such as optical fiber sensors,[7] surface plasmon resonance,[8] fluorescence,[9] electrochemistry,[10] electrochemiluminescence,[11] and magnetomechanical methods.[12] Recently, nanotechnologies have also been applied to telomerase detection. Plaxco and coworkers detected telomerase activity using telomerase-substrate-oligonucleotide-modified gold nanoparticles by a PCR-based method.[13] Mirkin and coworkers devised a bio-barcode assay based on polyvalent oligonucleotide-functionalized gold nanoparticles and reported a detection limit of 10 HeLa cells.[14] In addition, a silicon nanowire assay based on field-effect devices was developed by Lieber and coworkers that could realize multiplexed real-time detections of telomerase activity and achieved the same level of sensitivity as TRAP.[15] Although these methods were ingeniously designed and some of them showed great improvements in eliminating the thermal cycling protocol, none of them was as practical as TRAP. Most of these new methods were still not able to overcome shortcomings such as using complicated and time-consuming protocols and the need for expensive instrument.

Here we report an exponential isothermal amplification of telomere repeat (EXPIATR) assay, by which telomerase extracted from a single HeLa cancer cell can be detected in ~25 min under isothermal conditions. EXPIATR is based upon a strategically designed path of DNA amplification, inspired by strand-displacement amplification (SDA),[16] using the ability of a restriction enzyme to nick a recognition site and a polymerase to replicate and displace the target repeatedly. EXPIATR not only shares the superiorities of TRAP: (1) high sensitivity with a limit of detection (LOD) of telomerase from a single cancer cell or even less, (2) simple protocol allowing results to be read out by just mixing crude cell extracts with the reaction master mix, and (3) closed-tube assay making results reliable and reproducible. EXPIATR also makes several improvements: (4) it abandons the expensive thermal cycling protocol, making the method cost-efficient and more versatile for clinical applications, and (5) the detection time is greatly shortened to ~25 min (compared with ~1.5−2 h for RT-TRAP[6a]), resulting in a higher throughput.

The principle of the EXPIATR assay is illustrated in Figure 1. It is a method based on a system of two primers, the nicking telomerase substrate (NTS) primer and the nicking fluorescent reporter probe (NFRP) primer, and three enzymes, Bst 2.0 WarmStart DNA polymerase, the nicking endonuclease (NEase) Nt.BspQI, and telomerase extracted from crude cancer cells. The NTS primer contains a non-telomeric sequence (black color) that telomerase can recognize as a substrate,[5c] and the NFRP primer in a hairpin structure carries a single-stranded DNA tail (brown color) that can bind to the telomeric repeat sequence. By incorporating the Nt.BspQI recognition sequence (red color) in both primers, DNA amplification can be performed at a fixed temperature via the activity of the Nt.BspQI NEase to cut one strand of a double-stranded (ds) DNA at the recognition site and the ability of the Bst 2.0 polymerase to extend the 3′ end at the nick and displace the downstream strand. In the presence of telomerase, a minor number of NTS primers are converted to

Received: September 17, 2012
Published: November 27, 2012

   dx.doi.org/10.1021/ja309198j | J. Am. Chem. Soc. 2013, 135, 1661−1664

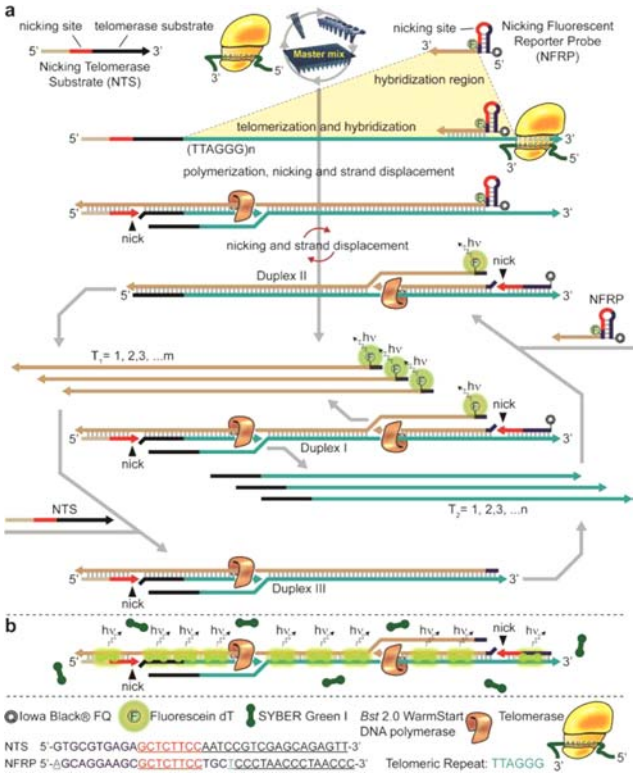

Figure 1. Schematic diagram of the EXPIATR assay (a) using the NFRP primer as a fluorescence probe and (b) using SYBR Green I dye probe.

telomerase extension products (telomeric repeats are synthesized onto the 3′ ends of NTS primers), which act as templates for the DNA amplification stage. Hybridization between the extended NTS telomeric template and the NFRP primer initiates polymerization, forming a nick on the extended NTS template, and driving polymerization from the nick in the opposite direction; likewise, the newly formed strand subsequently opens the hairpin structure of the NFRP primer, activates the nicking site, and initiates nicking and strand-extension/displacement downstream. Thus, there duplex I forms, in which the DNA reactions can be repeated in both directions, continuously releasing strands T_1 and T_2. As NTS and NFRP primers are present in excess, strands T_1 and T_2 can further hybridize with the free NTS and NFRP primers respectively, forming duplexes II and III that can perform the DNA reactions in a single direction. Cycling between II and III finally results in the exponential amplification of telomerase products, in which strands displaced from duplex II (same as T_1) serve as targets for the NTS primer (to form the new duplex III), while strands displaced from duplex III (same as T_2) serve as targets for the NFRP primer (to form the new duplex II).

For EXPIATR, two ways can be used to detect the amplification process in real-time: (a) In addition to a pure amplification primer, NFRP can serve as a fluorescence probe by modified with an internal fluorescein fluorophore at the stem region and an Iowa Black quencher at the 5′ terminus. The thermal denaturation profile of the NFRP primer (Figure S1) indicated that the NFRP primer itself could not open and generate fluorescent signals before the temperature reached 65 °C. As a result, only in the amplification process can fluorescent signals from the cleaved NFRP primers be liberated progressively. The absolute separation between fluorophore and quencher during the reaction reduces the possibility of any quenching effects and enhances the signal-to-noise ratio of the assay. Considering the enhancement in specificity and sensitivity of the assay, using NFRP as the fluorescence probe was mainly used in the following discussions. (b) Alternatively, the EXPIATR assay can also simply use SYBR Green I dye[17] as the fluorescence probe, with the unmodified NFRP primer.

Synthetic telomerase product TPC8, corresponding to primer NTS extended with eight telomeric repeats (TTAGGG), was used as a model for optimization and understanding of the DNA amplification process in EXPIATR (Figures S2−S5), and further was used as a positive control to estimate the telomerase activity from a given cell extract. The DNA amplification reaction showed an optimal response at 55 °C, much lower than the opening temperature of NFRP; as a result, any visible fluorescent signal before the signal of the negative control should ideally come from the reaction only. The NFRP-TPC8 hybrid showed a melting temperature (T_m) of 57.8 °C (Figure S6), and the NTS-T_1 hybrid was calculated to have $T_m \approx 60$ °C, ensuring that the primers can bind with targets in programmed ways when the EXPIATR assay is performed at 55 °C. Exponential amplification of telomerase product in EXPIATR is believed to depend on the presence of excess NTS and NFRP primers to drive the construction of duplexes I, II, and III and trigger the cycling reaction between II and III. By using TPC8 as the template, the reaction was performed under the condition that one of the primers was absent (Figure S7). There were no obvious signals observed in the absence of the NFRP primer, while when NTS was eliminated from the system, the amplification reaction proceeded in a slow and linear manner, as only duplexes I and II could be formed and the critical step of exponential growth was inhibited by the absence of duplex III (T_1·NTS). Primer concentration can also affect the thermodynamics of the DNA reaction, as it is highly related with the primer−target hybridization rate. It was found that the DNA amplification reactions reached optimal efficiency when the primer concentration was >50 nM (Figure S8).

Under the optimized conditions, the amplification reactions using NFRP as the fluorescence probe and TPC8 as the template (a series of concentrations 10^{-9}−10^{-18} M) were monitored by real-time fluorescence (Figure 2a). Triplicate measurements at each concentration were applied, showing a standard deviation (SD) ≤ 0.128, and the point of inflection (POI)—the time at which the fluorescence rises significantly above the back-

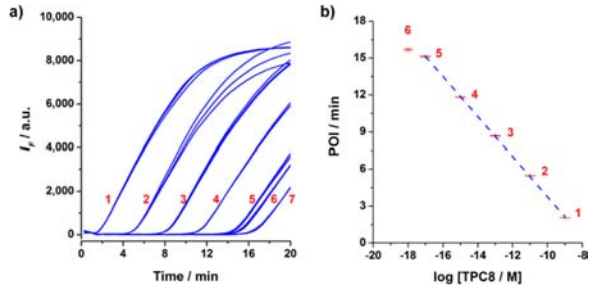

Figure 2. EXPIATR reactions monitored through real-time fluorescence by using TPC8 as a model. (a) Amplification curves of different concentrations of TPC8 (triplicate measurements of each concentration): 1×10^{-9} M (1), 1×10^{-11} M (2), 1×10^{-13} M (3), 1×10^{-15} M (4), 1×10^{-17} M (5), 1×10^{-18} M (6), and absence of TPC8 template (7). (b) Linear relationship between the POI values of the corresponding amplification curves and the logarithm of template concentrations. Error bars indicate standard error of triplicate tests.

dx.doi.org/10.1021/ja309198j | J. Am. Chem. Soc. 2013, 135, 1661−1664

ground—was used to quantitatively evaluate performances of the amplification reactions. The result indicated that EXPIATR could detect TPC8 at a concentration $<1 \times 10^{-18}$ M.[18] When the POI values were plotted against the logarithm of concentrations (Figure 2b), the resulting standard curve showed a linear relationship in the range of 10^{-9}–10^{-17} M for TPC8 with the correlation equation of POI = −12.68 − 1.64 log[TPC8] (R^2 = 0.9995).

Thereafter, the EXPIATR assay was tested on a series of diluted HeLa cells from telomerase-positive human cervical carcinoma (Figure 3a). A solution of master mix containing all

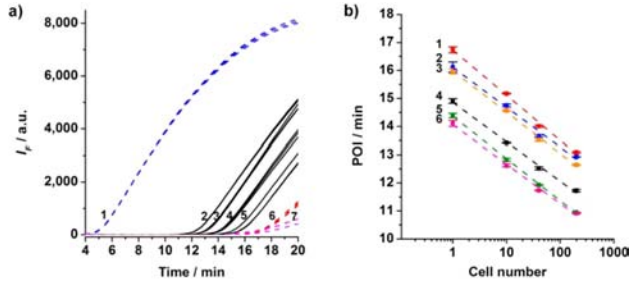

Figure 3. Detection of telomerase activity in human HeLa cancer cells by the EXPIATR assay. (a) The assay included a TPC8 positive control (1×10^{-11} M) (1), 200 cells, 40 cells, 10 cells, and 1 cell equivalent extracts (2–5), negative control in absence of cell extracts (6) and heat-inactivated extracts (1000 cells equivalence) (7). (b) Standard curves of the detections with 10 min incubations at different temperatures: 20 °C (1), 30 °C (2), and 37 °C (4); and standard curves of the detections with different incubation times at 37 °C: 5 min (3), 10 min (4), 20 min (5), and 30 min (6). Error bars indicate standard error of triplicate tests.

ingredients except the cell extract was prepared first, and the cell extract was added immediately before the onset of each test. In a typical EXPIATR analysis, each sample extract was tested in triplicate, accompanied by one positive control using 1×10^{-11} M TPC8 as template, and two negative controls including one using the same volume of CHAPS lysis buffer instead of the cell extract and the other performed with heat-inactivated extract (10 min at 85 °C). The assay was performed with a 10 min incubation time at 30 °C for telomerase extension (Figures S9 and S12) before initiating the DNA amplifications at 55 °C. The real-time result revealed that the LOD of the assay reached one HeLa cancer cell or less equivalent telomerase activity, comparable with the best result reported by RT-TRAP assay;[19] besides, the detection time was greatly shortened to ∼25 min (10 min for telomerase extension and 15 min for DNA amplification). EXPIATR is a method to detect telomerase activity that varies with incubation conditions.[20] Analyzing the assay under different conditions of incubation for telomerase extension, the resulting standard curves (Figures S10 and S11) showed distinct behaviors, except all of them were in linear relationships with the logarithm of cell numbers (Figure 3b). The assay generated faster signals when a longer incubation time was applied since more telomere templates were produced over time; furthermore, the amplification process also accelerated as the incubation temperature increased, consistent with the previous findings that the catalytic activity of human telomerase increases with temperature until 37 °C.[20] To make the EXPIATR assay a more versatile method, we also tested the possibility of using SYBR Green I dye as the fluorescence probe. A detection sensitivity of telomerase activity equivalent to 10 HeLa cancer cells was achieved (Figures S13 and S14). Although the SYBR Green I was found less sensitive than the NFRP probe due to its nonspecific binding, the assay still showed a great sensitivity.

As a further support for this new EXPIATR method, the amplification products were characterized by polyacrylamide gel electrophoresis (PAGE) (Figure 4). Lanes 2 and 3, correspond-

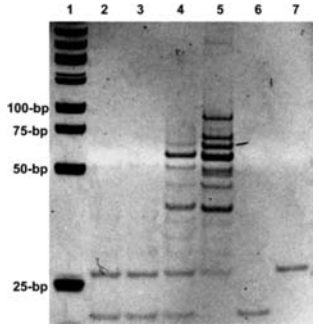

Figure 4. Non-denaturating PAGE analysis of EXPIATR assay: lane 1, DNA markers; lane 2, negative control in the absence of cell extract; lane 3, negative control with heat-inactivated cell extract; lane 4, cell extract from 1000 HeLa cancer cells; lane 5, positive control of TPC8 model (1×10^{-11} M); lane 6, NFRP primer (100 nM); and lane 7, NTS primer (100 nM).

ing to the negative controls, in which cell extract was absent or heat-inactivated, did not produce any observable bands. Meanwhile, the TPC8 positive control (lane 5) and HeLa cancer cell assay (lane 4) showed multiple bands in ladder-like patterns. As discussed in Figure 1, for EXPIATR assay, dsDNA products $T_1 \cdot T_2$ can be expected when the NTS and NFRP primers become limiting in the reaction. The lengths of these dsDNA products are varied as the NFRP primer can bind multiple positions along the telomeric repeats on the NTS substrates. Accordingly, a ladder of products with 6 bp increments starting from a minimum length of 38 bp is predicted (Figures S16 and S17). In lane 5, six laddered main bands (38, 44, 50, 56, 62, and 68 bp) were observed, consistent with the prediction based on TPC8 model. There is also an additional band with a slower migration rate appeared in lane 5. According to the similar observations in the SDA reactions,[21] we assumed that this band might come from the unnicked strands (duplexes II and III, both are 84 bp) of the highest-yield products (the band at 56 bp) (Figure S15). Lane 4 of the HeLa cancer cell assay showed a ladder of five bands, indicating at least seven telomeric repeats were added to the NTS primer by telomerase, in which the corresponding bands located in similar positions of TPC8 model and the band at 56 bp appeared more intense than the others. However, longer telomerase extension products were not visualized, unlike in the gel image observed by the TRAP assay. This may be explained by the high K^+ concentration in the EXPIATR buffer (100 mM), which is good for telomerase activity but decreases the overall length of telomerase products when compared with the TRAP buffer (K^+ concentration = 63 mM).[20] To prove that this unique gel pattern comes from genuine telomerase signals, a control experiment was performed by isolating the telomerase incubation step from the amplification step, so that independent incubation conditions could be used to achieve a higher degree of telomerase processivity. We incubated the cell extracts with dNTPs and NTS primers at 30 °C for 40 min in TRAP buffer, heat-inactivated at 85 °C, and then applied the extracts to the EXPIATR amplification reaction. The gel from this control

dx.doi.org/10.1021/ja309198j | J. Am. Chem. Soc. 2013, 135, 1661–1664

experiment showed almost the same pattern as the EXPIATR except for the appearance of several faint bands of larger products due to the improved incubation conditions (Figure S15). This control experiment demonstrated that such banding patterns formed as the result of the amplification of telomerase products.

Telomerase is a unique enzyme that it is a reverse transcriptase endogenous to human cells and requires an internal RNA component to work as a template.[5c] That the assay was sensitive to the pre-incubation of cell extracts with ribonuclease (deoxyribonuclease-free) is further evidence that EXPIATR is a specific assay for telomerase activity (Figure S19). Moreover, to demonstrate that it is a general and reliable method for telomerase detection, another cancer cell line MDA-MB-231 (Figure S20) and a normal cell line MRC-5 (Figure S21) were tested. As expected, the tests on MRC-5 cell did not generate any fluorescent signal before the signal of negative control due to the lack of telomerase activity in normal cells; whereas MDA-MB-231 cancer cells showed positive telomerase activity. The relative telomerase activities (RTAs) of the extracts equivalent to 200 cells from different samples were quantified on the basis of a relative standard curve method (see Supporting Information) referenced from the TRAP assay[22] (Figure 5). We found that,

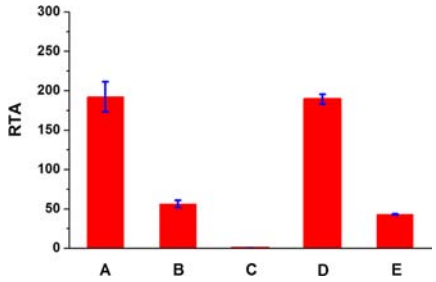

Figure 5. RTAs of cell extracts equivalent to 200 cells from HeLa (A), MDA-MB-231 (B), MRC-5 (C), and of their mixtures: HeLa/MRC-5 (D) and MDA-MB-231/MRC-5 (E).

after mixing with the same amount of extracts from MRC-5 normal cells, the telomerase activity of HeLa and MDA-MB-231 cancer cells can still be accurately expressed by the assay.

The nonspecific signal is always an inevitable problem of nucleic acid isothermal amplification and also the PCR method.[23] In this EXPIATR assay, we found that by adjusting the temperature of telomerase incubation stage, the nonspecific signals can be significantly delayed. For example, the best specificity was achieved when the incubation was carried out at 30 °C (Figure S12), presumably due to the relatively high activity of telomerase and relatively low activity of Nt.BspQI NEase and Bst 2.0 polymerase at this temperature. The high activity (80%) of Nt.BspQI NEase at 37 °C may induce some nonspecific products at the incubation stage, and Bst 2.0 polymerase, which is designed to be activated around 45 °C, should also be more stable (in its inactive form) at 20/30 °C rather than 37 °C.

In conclusion, a new approach, EXPIATR, is introduced to detect telomerase/telomeres from crude cell extracts that obviates the need for multiple steps of thermal cycling and expensive instruments and also achieves ultrafast detection: ~25 min to detect telomerase activity equivalent to a single HeLa cancer cell. Compared with the 1.5−2 h time of the RT-TRAP method,[6a] the EXPIATR assay markedly shortens the time needed for reliable telomerase detections. As an almost universal marker for human cancer, telomerase is a promising target for cancer therapeutics. Sensitive, reliable, and quick detection of telomerase is important for related studies. Owing to its high-throughput, isothermal, ready-to-go, and cost-efficient features, EXPIATR shows great potential for future use in clinical tests.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental methods and data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

yweizmann@uchicago.edu

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was supported by the University of Chicago.

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