DOI: 10.1002/cbic.201100592

Two-Step, PCR-Free Telomerase Detection by Using Exonuclease III-Aided Target Recycling

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Telomerase, the enzyme responsible for telomere maintenance, is found in the large majority of human tumors and serves as an important target for cancer diagnostics and the development of new anti-cancer therapeutics.[1] The most widely employed assay for the detection of telomerase activity, the PCR-based telomeric repeat amplification protocol (TRAP assay) developed in 1994 by Kim and co-workers,[1b] uses PCR to amplify and gel electrophoresis to detect the six-base ladder produced when telomerase elongates a primer oligonucleotide.[1b] Although the TRAP assay has seen widespread application (indeed, its use revolutionized the studies of the telomerase enzyme[1b]), it remains a complex, time-consuming process prone to false positives that arise due to artifactual amplification (e.g., primer-dimers)[2] and false negatives to the PCR inhibitors/interferants often present in protein-rich clinical samples.[3] In response, recent years have seen the development of alternative, PCR-free telomerase assays, including detection via polyvalent oligonucleotide/gold nanoparticle conjugates or via the "catalytic beacon amplification" provided by the use of telomere-generated DNAzymes as catalytic labels.[4] None of these methods, however, have seen broad adaptation, perhaps because they, too, require cumbersome, multi-step processes that are not significantly more convenient than the TRAP assay they are designed to replace.

We have recently developed a simple, PCR-free method for the detection of low concentration, single-stranded DNA targets that requires only a single fluorescence measurement for its readout.[5] The signal amplification method used in this assay, termed exonuclease III-aided target recycling (EATR), employs the enzymatic activity of exonuclease III,[5–6] a sequence-independent nuclease that preferentially degrades double-stranded DNA with blunt or recessed 3′ termini, to digest target-bound molecular beacons. This digestion not only segregates the molecular beacon's quencher–fluorophore pair, leading to significantly enhanced fluorescence, but also liberates the intact target strand, which is then free to bind a fresh molecular beacon and start the process anew (Figure 1). The enhanced signaling produced by this catalytic cycle leads to greatly improved sensitivity relative to traditional molecular beacons, and does so without any significant restriction in the choice of target sequence.[7] Here we report a modification of this exonuclease-amplified approach that improves its speed and sensitivity, and the adaptation of the modified assay to the sensitive, two-step detection of telomerase activity directly in crude cellular extracts.

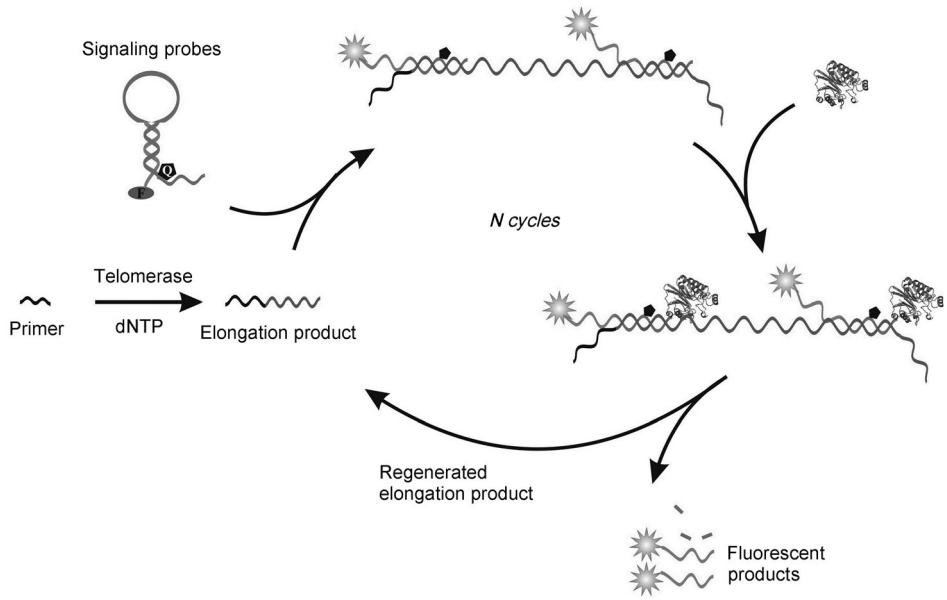

Figure 1. We have developed a sensitive, PCR-free method for detecting telomerase and thus telomerase activity. In the assay telomere binding opens a fluorophore/quencher-modified stem-loop probe to form a blunt 3′ termini. The blunt-end cleavage activity of exonuclease III degrades this probe, causing an increase in fluorescence and liberating the telomere target. The released target is then free to hybridize with a second probe, whence it can start the cycle anew, leading to significant signal amplification.

In the prior implementation of our exonuclease-amplified DNA assay we employed a "tailed" molecular beacon, a stem-loop DNA sequence modified with a CAL Fluor Red 610 (FR610) fluorophore at its 5′ terminus and a black hole quench-

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201100592.

er (BHQ) at an internal position, as the signaling probe. Upon the hybridization with a target sequence the stem of the molecular beacon opens, forming a double-stranded structure with a blunt 3′ terminus that serves as an exonuclease III substrate. This, in turn, leads to the aforementioned stepwise digestion of the molecular beacon and liberation of the target molecule. While sensitive, this first generation assay suffered from a potentially significant limitation: although it achieves a 20 aM (2×10^{-17} M) detection limit when performed at 4 °C, the assay requires approximately 24 h to equilibrate at this temperature. At room temperature, in contrast, the assay equilibrates in minutes, but significant exonuclease-driven digestion of the free signaling probe under these conditions increases the assay's background fluorescence, degrading its sensitivity by many orders of magnitude (Supporting Information, Figure S1, left).[5] Here we circumvent this problem by the incorporation of digestion-resistant locked nucleic acids (LNA)[8] in the 5′ strand of the molecular beacon's stem. This chimeric LNA–DNA signaling probe is resistant to nonspecific digestion at 37 °C (Supporting Information, Figure S1, right), allowing us to achieve femtomolar detection limits in minutes (Figure 2A and B).

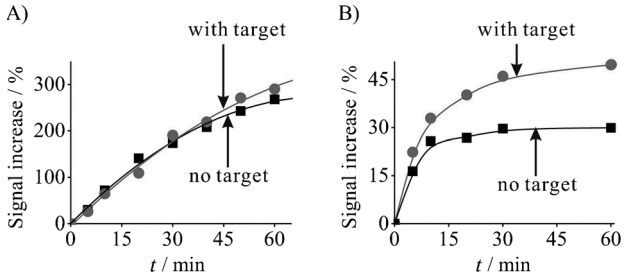

Figure 2. The use of chimeric LNA–DNA probes significantly improves the limit of detection of our assay relative to an assay employing a simple DNA probe. A) Using a DNA probe we see no significant difference in the rate or magnitude of the fluorescence change upon the addition of a synthetic DNA target (30 fM). B) In contrast, the reduced background degradation associated with a chimeric probe leads to a readily detectable change in fluorescence.

Specifically, with this modification our assay detects as low as 30 fM (3×10^{-14} M) of a synthetic DNA target after just 60 min of incubation (Figure 2B).

To employ our new exonuclease-amplified assay in the monitoring of telomerase activity we have fabricated a LNA–DNA chimeric signaling probe specific for the detection of elongated telomeres (Figure 1). Using this we have developed a simple, two-step PCR-free assay consisting of a telomerase elongation step performed in a lysis/elongation buffer followed by the above-described amplified detection performed in a second buffer optimized for exonuclease activity. As expected, when we perform this assay with a sample of ~8000 telomerase-positive cells we observe robust, 50% increase in fluorescence intensity (Figure 3A). No obvious fluorescence increase is observed with negative controls comprising either lysis buffer alone or an equivalent number of telomerase inactive (heat treated) cells in lysis buffer. The assay is sensitive enough to detect telomerase from as few as 30 breast cancer

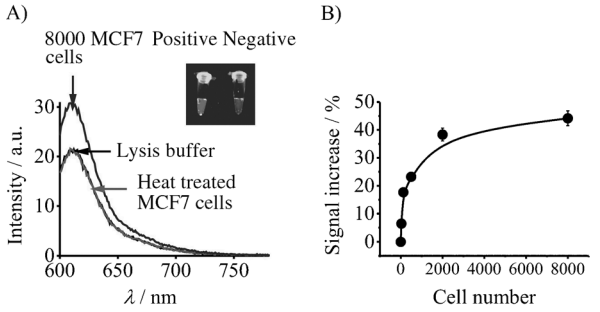

Figure 3. Our assay readily and quantitatively detects the telomerase activity in the telomerase positive breast cancer cell line MCF7. A) For example, the fluorescence associated with a sample containing ~8000 MCF7 cells is more than 40% greater than that observed for cell-free lysis buffer (black line) or heat-inactivated cells (gray line). Inset: The fluorescence increase associated with the telomerase positive cells is so great that it is easily visible to the naked eye. B) The observed fluorescence rises monotonically with cell number with a limit of detection being the telomerase activity present in approximately 30 MCF7 cells.

cell-line cells, above which we observed a monotonic increase in emission intensity with increasing cell numbers until saturation is reached at a few thousand cells (Figure 3B). Indeed, at the level of 8000 cells the assay response is so strong that the change in emission is visible as a naked-eye color change (Figure 3A, inset). In comparison, when a DNA probe lacking the LNA modification is used, it becomes impossible to distinguish between telomerase-negative and telomerase-positive samples due to the significant increase in background fluorescence associated with these probes (Figure S2, Supporting Information).

Our assay is specific for telomerase activity and does not respond to, for example, the endogenous telomeres in crude cell extracts. To demonstrate this we have challenged our assay with a sample containing ~8,000 HMEC cells, a human mammary epithelial cell line that, although containing endogenous telomeric termini equivalent in our assay to ~5 fM, lacks the telomerase enzyme. As expected, we observed no significant increase in fluorescence intensity over background (Figure 4).

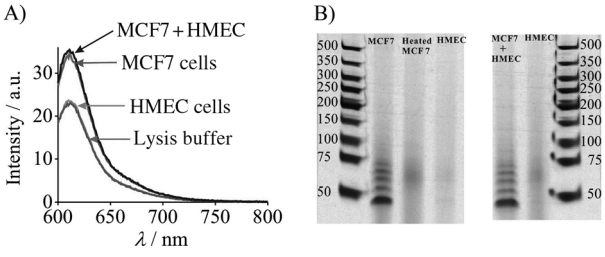

Figure 4. A) Our assay is specific: we do not observe any significant increase in fluorescence when the assay is challenged with ~8000 human mammary epithelial cells (HMEC), which lack endogenous telomerase activity. In contrast, samples containing an equal number of telomerase-positive MCF7 cells in isolation or in the presence of a similar number of HMEC cells produce a robust positive response. B) Shown are TRAP assay results, confirming that while MCF7 cells are telomerase-positive, neither HMEC cells nor the heat-treated MCF7 cells used in Figure 3 produce any detectable telomerase activity. It is worthwhile to note that, while both assays required 2 h from start to finish, the confirmatory TRAP assay requires far more effort, skill and resources.

Our assay is likewise selective enough to detect cancer cells in the presence of noncancerous cells; using it we can readily detect the endogenous telomerase activity of MCF7 cells against a background of an equal number of telomerase-negative HMEC cells (Figure 4 A).

The 30-cell detection limit of our assay approaches those of the PCR-based TRAP assay[1b,3] and more recently described gold nanoparticle-based amplification assays.[3,4i,j] However, our assay achieves its sensitive detection without employing the complicated denaturation-annealing cycles or gel analysis required by the TRAP assay[1b] or the multiple self-assembly steps required to perform the gold nanoparticle-based amplification assay.[3,4i,j] All that is required is to add modified LNA–DNA chimera and exonuclease III to the telomerase elongation products; after this we can easily read the relevant emission color change by naked eye. Given that there is a general need for the highly sensitive telomerase detection within complex samples, we believe that our current sensor platform may prove a viable alternative solution to this problem.

Experimental Section

Materials: Exonuclease III and NEB buffer 1 were purchased from New England Biolabs (Ipswich, MA) and used as received. The fluorophore/quencher-labeled, all-DNA molecular beacons and synthetic target DNAs were synthesized by Biosearch Technologies, Inc. (Novato, CA), purified by HPLC, and confirmed by mass spectrometry. The sequences of these oligonucleotides are as follows: All-DNA molecular beacon probe: 5′-(CAL Fluor Red 610)-AGG AAG ACG TAC GTA TCT TCC T-(BHQ)-TTT GTT C-3′; Synthetic target DNA: 5′-GAA CAA AAG GAA GAT ACG TAC GTC TAA AATC-3′; DNA molecular beacon probe: 5′-(CAL Fluor Red 610)-TAG GGT TAC TAA CCC TAA CCC TAT-(BHQ)-A CCC TAA CCC TAA CCT TT-3′.
The fluorophore/quencher-labeled LNA–DNA chimeras were synthesized by Biosynthesis. (Lewisville, TX), purified by HPLC, and confirmed by mass spectrometry. The sequences of these oligonucleotides were: Modified LNA-DNA chimera: 5′-(CAL Fluor Red 610)-*AGGAAG*ACGTACGTATCTTCCT-(BHQ)-TTTGTTC-3′ (bases in italics are LNA); modified LNA-DNA chimera for telomerase detection: 5′-(CAL Fluor Red 610)-*TAG GGT TA*C TAA CCC TAA CCC TAA CCC T-(BHQ)-AA CCC TAA CCT TT-3′ (bases in italics are LNA).
Cancer cell (MCF 7 cell line, ATCC, VA) culture is based on the subculture protocol for MCF 7 from ATCC. Human mammary epithelial cell (HMEC, Lonza Walkersville Inc, MD) culture is based on the subculture protocol for HMEC from Lonza Walkersville Inc.

Telomerase extraction: The cell pellet was resuspended in CHAPS Lysis buffer (1×, ATCC, VA, 200 μL 10^5–10^6 cells^{-1}). The suspension was then incubated on ice for 30 min and centrifuged at 12000 *g* for 20 min at 4 °C. Finally, the supernatant was collected into a fresh tube and stored at −80 °C.

Telomerase elongation: Different concentration of telomerase and the reaction mixture (from TeloTAGGG Telomerase PCR ELISA kit, Roche, Cat. No. 11 854 666 910, which includes Tris buffer, telomerase primers and nucleotides) were mixed at 30 °C for 1 h.

The assay: The exonuclease III amplification was performed in NEB buffer 1 (1×, 50 μL) which contains stem-loop DNA probe (2 μM), exonuclease III (100 units) and varying concentrations of DNA target or telomerase elongation products. The reaction was maintained at 37 °C for 2 h. Fluorescence was measured on a Peltier temperature-controlled Varian Cary Eclipse Fluorimeter (Varian, Inc) with the following settings: λ_{ex} = 590 nm, λ_{em} = 610 nm, 5 nm slit, PMT detector voltage = 650 V. The TRAP assay and electrophoresis process is based on the commercially available detection kit (Trapeze Telomerase Detection Kit, s7700, Chemicon International).

Acknowledgements

We thank Dr. Juwen Shen and members of our research group for helpful discussions. This work was funded by the NIH, ARO Institute for Collaborative Biotechnologies (ICB) and DARPA grant 00264048.

Keywords: cancer · nucleic acid detection · signal amplification · telomerase

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Received: September 20, 2011
Published online on November 28, 2011

