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SPECIAL ISSUE

Zero-Background Helicase-Dependent Amplification and Its Application to Reliable Assay of Telomerase Activity in Cancer Cell by Eliminating Primer–Dimer Artifacts

Feng Chen,[a] Dexin Zhang,[b] Qing Zhang,[a] Xiaolei Zuo,[c] Chunhai Fan,[c] and Yongxi Zhao*[a]

Primer–dimer artifacts resulting from unintended template-independent primer–primer interactions often hinder the specific amplification of nucleic acids. We demonstrate, for the first time, zero-background helicase-dependent amplification (HDA), with low concentrations of both ATP and dNTPs. This strategy achieved the reliable evaluation of telomerase activity in cancer cells by eliminating primer–dimer artifacts, which have plagued many previous methods with reduced specificity. We found that the performance of the telomerase assay by zero-background HDA was negatively affected by highly concentrated cellular proteins. This inhibitory effect is attributed to the binding of DNA templates to proteins, thus making them unavailable for polymerases. However, gold nanoparticles were demonstrated to highly attenuate such inhibition by abundant proteins, and to enhance the assay sensitivity and reliability when the reaction was performed with concentrated cell extracts.

Introduction

Helicase-dependent amplification (HDA) is a powerful amplification technique used for the exponential accumulation of target nucleic acids. DNA helicase separates double-stranded DNA (dsDNA) and produces single-stranded templates for primer hybridization and subsequent extension, thus mimicking in vivo DNA replication.[1] The enzymatic unwinding of dsDNA enables simple DNA amplification under isothermal conditions, and omits the complicated thermocycling process (heat denaturation, annealing, extension) that is required in PCR. HDA is regarded as a promising alternative to PCR, especially in resource-limited settings and for on-site testing,[2–4] and has been applied to a broad range of applications, such as pathogen diagnosis[5–8] and SNP genotyping.[9–11]

Early HDA experiments were performed at low temperature (≈37 °C) because of the use of the *Escherichia coli* UvrD helicase, and required two accessory proteins (MutL, and a ssDNA-binding protein) to facilitate amplification.[1] Considerable effort has been directed towards improving HDA performance. Kong and co-workers cloned and purified a thermostable UvrD helicase from *Thermoanaerobacter tengcongensis* to support HDA reactions at higher temperatures (60–65 °C); this achieved improved amplification efficiency, even in the absence of accessory proteins, with simplified reaction components.[12] A new bifunctional protein has been engineered by fusing a helicase with a DNA polymerase for the successful amplification of longer fragments (up to 2.3 kb).[13] Treating the target genomic DNA with an endonuclease prior to amplification was shown to significantly enhance both the speed and sensitivity of HDA.[14] In addition, primers with 5′ termini enriched in A or C (rather than T or G) were shown to be favored in HDA, and led to much more efficient amplification.[15] Nevertheless, primer–dimer artifacts resulting from template-independent primer–primer interactions were often observed, even after careful primer design. This common problem challenges the specific accumulation of target nucleic acids and leads to misinterpretation of detection results.[16,17]

Recently, Zhang's group reported a novel target-converted HDA assay for the ultrasensitive detection of transcription factors without the problems of primer–dimer artifacts.[18] They used a single primer that was complementary to the stem of a designed hairpin template for the HDA reaction, thus eliminating primer–dimer nonspecific amplification. However, exonucleases-catalyzed complete digestion of the hairpin templates followed by heat inactivation of exonucleases was required, in order to prevent background amplification in the absence of target. Moreover, this method was not applicable for both amplification of other nucleic acids and the biosensing of various non-nucleic-acid targets, because of the hairpin template. As HDA-based applications continue to evolve, the de-

[a] F. Chen, Q. Zhang, Prof. Dr. Y. Zhao
Key Laboratory of Biomedical Information Engineering of Education Ministry, School of Life Science and Technology, Xi'an Jiaotong University
Xianning West Road, Xi'an, Shaanxi 710049 (P. R. China)
E-mail: yxzhao@mail.xjtu.edu.cn

[b] Dr. D. Zhang
Department of Respiratory Medicine
The Second Affiliated Hospital of Medical College, Xi'an Jiaotong University
Xiwu Road, Xi'an, Shaanxi 710049 (P. R. China)

[c] Prof. Dr. X. Zuo, Prof. Dr. C. Fan
Radiation Facility, CAS Key Laboraotory of Interfacial Physics and Technology, Shanghai Institute of Applied Physics, Chinese Academy of Sciences
Yuquan Road, Shanghai 201800 (P. R. China)

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velopment of general and simple strategies for further HDA improvements remains an urgent need.

Here, we investigated the effects on HDA performance of several important factors, including enzyme concentration, reaction temperature, and the concentrations of adenosine triphosphate (ATP, a cofactor for UvrD DNA helicase) and deoxynucleotide triphosphates (dNTPs). Reducing enzyme concentration slowed the amplification, yet the primer–dimer artifacts still existed. Increasing reaction temperature also did not reduce primer–dimers, but accelerated this nonspecific amplification and reduced its difference to template-specific amplification. To our surprise, primer–dimer artifacts were eliminated by simultaneously adjusting the concentrations of ATP and dNTPs to relatively low levels. This modified HDA was tested for zero-background detection of telomerase activity in cell extracts without primer–dimer nonspecific products. Primer–dimer artifacts were clearly observed in many previously reported methods, including the gold standard method, telomere repeat amplification protocol (TRAP), and these degraded telomerase assay specificity.[16,17,19–22] Furthermore, we demonstrate that gold nanoparticles (AuNPs) reduce the inhibitory effect of concentrated cellular proteins on zero-background HDA, and improve the sensitivity and reliability of the telomerase assay when amplification reactions are performed in protein-rich samples.

Results and Discussion

The primers/template system used in PCR-based TRAP[23] was selected as a model system to demonstrate zero-background HDA (without primer–dimer artifacts). TRAP was developed for the detection of telomerase activity; however, primer–dimer artifacts in TRAP lowered the detection specificity. Previous studies have reported that protein concentration has significant effects on the HDA reaction, and that rapid amplification was achieved at a high concentration of the enzyme mix.[14,24] We doubted whether decreasing enzyme concentration could contribute to reducing nonspecific primer–dimer products. We carried out HDA reactions with a series of enzyme mix concentrations in the presence or absence of synthetic template (time-course fluorescence curves in Figure 1 A). For both template-triggered (specific) and template-independent (nonspecific) amplification, the speeds dropped as the enzyme mix concentrations decreased. When the enzyme mix concentration was 0.14× (0.25 μL IsoAmp enzyme mix in 25 μL reaction mixture), no fluorescence signal for specific amplification was observed, despite the elimination of primer–dimer artifacts, thus indicating failure of the amplification process.[1] Agarose gel (Figure 1 A) and melting curve analyses (Figure 1 B) confirmed these results. It is important to note that the primer–dimer had the same length as that of the specific amplified product in our primers/template system (a short synthetic template).[16] As primer–primer complexes are unstable (easily dissociated at high temperatures),[25] we studied the effect of reaction temperature on nonspecific HDA reactions. Unfortunately, high reaction temperatures (up to 65 °C) accelerated not only template-based specific amplification but also nonspecific amplification, and reduced the difference in kinetics between these two amplification reactions (Figure S1 in the Supporting Information). The rapid template-based amplification can be attributed to the maximum activity of enzyme mix at 65 °C;[12] but accelerated nonspecific amplification was unexpected. Because the melting temperature of the primer–primer complexes is much less than 65 °C, stable primer–primer complexes should not exist at this temperature. Thus, we suggested this efficient nonspecific amplification resulted from the formation of only transient primer–primer duplexes. Transient duplexes are also available for polymerization reaction.[26,27]

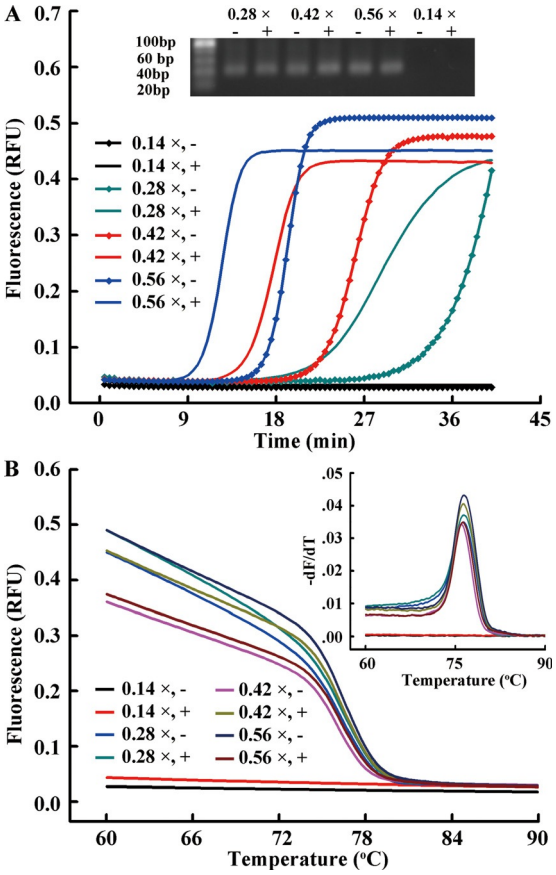


Figure 1. Effect of enzyme mix concentration on HDA performance. A) Fluorescence curves of different enzyme mix concentrations; corresponding gel electrophoresis analysis. B) Corresponding melting curves. The symbols − and + represent the absence and presence of template, respectively. An enzyme mix concentration of 0.14× indicates 0.25 μL IsoAmp enzyme mix in 25 μL reaction mixture.

In HDA, ATP is the energy source for helicase to separate DNA duplexes, by breaking the hydrogen bonds. (This is not involved in other amplification strategies, such as PCR and strand displacement amplification.)[28–37] Thus, we speculated ATP concentration might have an effect on HDA performance. To our surprise, as ATP concentration was reduced from 3 to 1.8 mM, the primer–dimer artifacts reduced significantly (Figure 2 A), and specific amplification was also reduced. Based on signal-to-noise ratios, 2.1 mM ATP was selected for the following experiments.

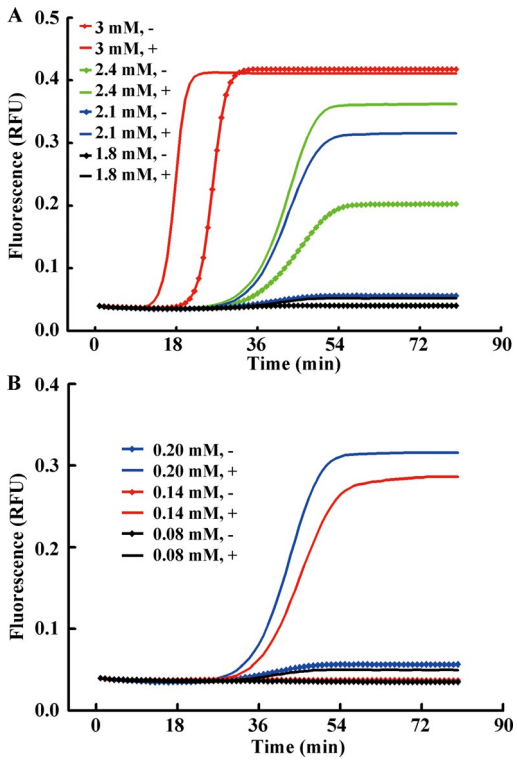
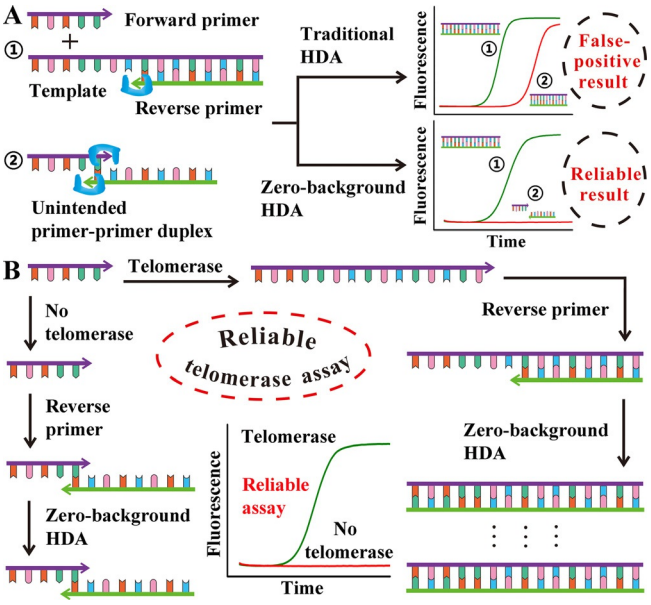


Figure 2. Effects of A) ATP concentration and B) dNTP concentration on HDA performance. The symbols − and + represent the absence or presence of template, respectively.



Scheme 1. A) Difference between conventional HDA and zero-background HAD. B) Reliable telomerase assay by zero-background HDA without interference from primer–dimer artifacts. The blue symbol represents DNA polymerase, arrows on primers indicate the 3′ terminus.

In order to further reduce the primer–dimer artifacts, dNTP concentration was also optimized: primer–dimer artifacts were eliminated when the dNTP concentration was down to 0.14 mM (Figures 2B and S2). Although specific yield was also slightly lower, this concentration was chosen. Overall, for the first time, we demonstrated zero-background HDA to eliminate primer–dimer artifacts by adjusting the concentrations of both ATP and dNTPs to relatively low levels. The major differences between traditional and zero-background HDA are outlined in Scheme 1A.

Next, zero-background HDA was used for the analysis of telomerase activity. Human telomerase is a ribonucleoprotein that maintains telomere length by adding tandem repeats (TTAGGG) to the ends of chromosomes; it is closely associated with cellular immortality and carcinogenesis.[33–40] Upregulation (or reactivation) of telomerase activity has been observed in the vast majority of human tumors (85–90%).[40,41] Telomerase activity is relatively low (or undetectable) in most normal somatic cells. Accordingly, telomerase is regarded as a common cancer marker for early diagnosis and prognosis. We used telomerase substrate (TS) primer to perform telomerase-catalyzed extension reactions.[42–45] A small number of TS primers are extended with 3′ telomeric repeats in the presence of telomerase (Scheme 1B). These extension products can act as templates for DNA amplification by zero-background HDA. First, reverse primers hybridize with the templates to initiate polymerization, thereby forming duplex templates. Each duplex is separated by DNA helicase to generate two single-stranded templates, then reverse and forward primers (TS primers) hybridize to the corresponding single-stranded templates to initiate two polymerization reactions, thereby producing two duplex templates. These newly synthesized duplex templates again act as substrates for DNA helicase, thus entering the next round of DNA amplification. This chain reaction results in exponential amplification of the telomerase extension products. Fluorescent dye SYBR Green I (specific for dsDNA) was used to monitor the amplification reaction over time. In the absence of telomerase, no extension products or DNA amplicons are generated, and primer–dimer nonspecific amplification is eliminated in zero-background HDA.

The feasibility of the proposed method for telomerase detection was tested with HeLa cell extracts: there was an exponential increase in the fluorescence signal, whereas no fluorescence enhancement was observed in the sample without HeLa cell extract (Figure S3). To evaluate the sensing performance of zero-background HDA, a series of samples containing various amounts of HeLa cell extract were examined. The fluorescence intensity increased with cell number, thus indicating that cell number was directly proportional to the amplified product (Figure 3A). Fluorescence intensities were plotted against the logarithm of cell number (Figure 3B), and the resulting standard curve showed a linear correlation over 100–1000 cells, with the correlation equation $F = 0.273 \log(\text{cell number}) - 0.441$ ($R^2 = 0.986$). To demonstrate the general applicability of zero-background HDA for telomerase detection, other cancer cell lines (MDA-MB-231 and A549) and a noncancerous cell line (MRC-5) were tested. As expected, both MDA-MB-231 and A549 cells showed telomerase activity (Figure 4), whereas MRC-5 cells did not induce significant fluorescence, because of the lack of telomerase activity in these cells. Heat-inactivated

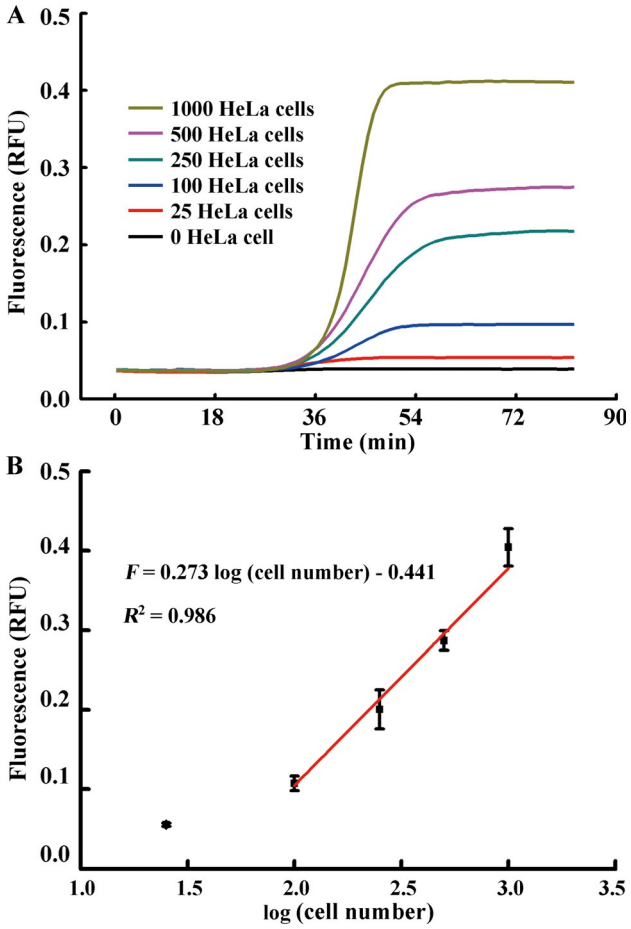


Figure 3. Telomerase activity in human HeLa cells detected by zero-background HDA. A) Fluorescence curves for different cell numbers. B) Linear relationship between the fluorescence intensity and the logarithm of cell number.

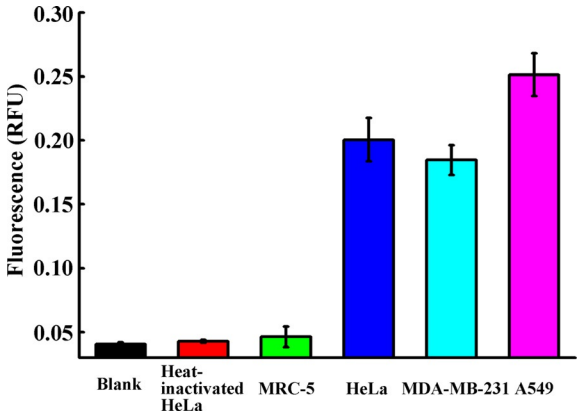


Figure 4. Evaluation of telomerase activity in 250 cells from different cell lines. The errors were calculated from three independent experiments.

HeLa cells extracts also did not generate any obvious fluorescence response. All these results demonstrate the telomerase-specific zero-background amplification reaction. Moreover, our telomerase assay eliminated the primer–dimer artifacts that were observed in previously reported methods.[16,17,19–22] (Some of these methods achieved reduced yield of primer–dimer artifacts,[16,19,22,46] but primer–primer nonspecific amplification still occurred.)

Despite the excellent performance of zero-background HDA, we found that the telomerase assay was inhibited when the HeLa cell extract was mixed with a large amount of normal MRC-5 cell extract (Figure 5 A). Similar phenomena were reported in previous studies.[16,19] The abundant cellular proteins in highly concentrated cell extracts have been shown to be powerful inhibitors of the amplification reactions, by binding to the DNA templates thus making them unavailable for polymerases.[19] Treatment of normal cell lysate with proteinase K prior to mixing with the cancer cell extracts can eliminate this inhibitory effect,[19] and this was found also for the zero-background HDA telomerase assay (Figure 5 A). However, telomerase can also be degraded and inactivated when the samples are pretreated with proteinase K, thus making this approach impractical.

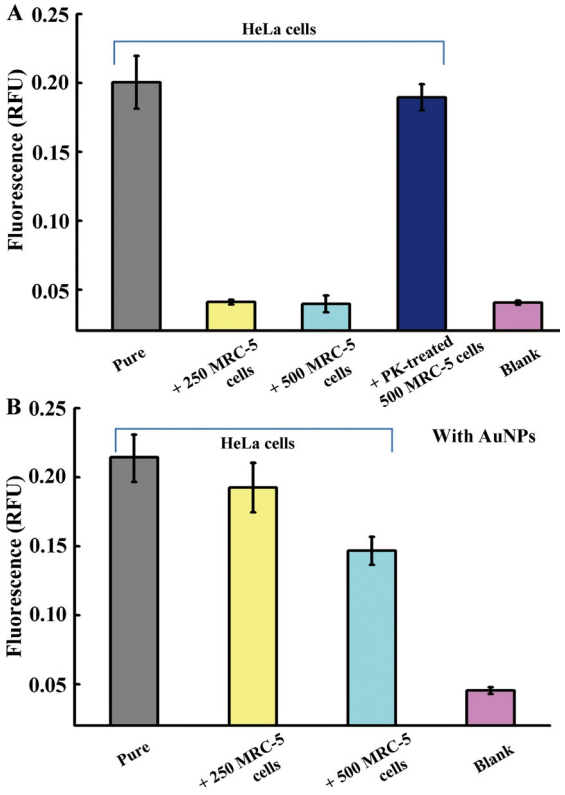


Figure 5. A) Negative effect of highly concentrated cell extracts (250 HeLa cells) was on zero-background HDA performance in the telomerase assay. PK: proteinase K. B) AuNP resistance to the inhibition. The errors were calculated from three independent experiments. The concentration of AuNPs was 0.3 nM.

Inspired by a previous study that utilized AuNPs to enhance the sensitivity and reliability of the telomerase assay in protein-rich samples,[19] we investigated the effect of AuNPs on the inhibition on zero-background HDA caused by abundant proteins. An AuNP-based zero-background HDA system was developed by adding an appropriate concentration of AuNPs.

In the presence of extract from 250 normal (MRC-5) cells, the mixed samples triggered high amplification signals (Figure 5B). Even with 500 normal cells, the fluorescence response of the AuNP-based assay was significantly enhanced compared to the unmodified assay (Figure 5A). These results demonstrate that addition of AuNPs can overcome the inhibitory effect from abundant normal cells on zero-background HDA, and improve assay sensitivity and reliability. The AuNP effect has been attributed to strong adsorption of lysate proteins onto the surface of the particles,[16,19] did thereby preventing interference with DNA templates.

Conclusions

We report zero-background HDA for the reliable evaluation of telomerase activity in cancer cells. By lowering the concentrations of both ATP and dNTPs, the proposed strategy successfully eliminated nonspecific primer–dimer artifacts, which have produced false-positive results in many previously reported methods, including the gold standard method, TRAP. Furthermore, AuNPs were demonstrated to be capable of reducing inhibition by abundant proteins on zero-background HDA reactions, and improving the sensitivity and reliability when the assay was performed in concentrated cell extracts. The excellent performance of the developed strategy makes it a promising solution for the direct measurement of telomerase activity in protein-rich samples, with accurate results. Although the technique was demonstrated by using the model primer/template system for telomerase assay in TRAP, it is anticipated that the method has potential applications in various fields. Our ongoing work will investigate the feasibility of zero-background HDA for multiplex DNA amplification and whole-genome amplification, with more than one set of primers and even random primers.

Experimental Section

Materials: The dNTPs mix, ATP, DNA marker, and RNase inhibitor were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). An IsoAmp II Universal tHDA Kit was obtained from New England Biolabs. TRAPeze CHAPS Lysis Buffer was purchased from Merck Millipore. Gold nanoparticles (5 nm) were purchased from Sigma–Aldrich. The oligonucleotides, synthesized by Sangon Biotechnology (Shanghai, China) were Forward primer (5′-AAT CCG TCG AGC AGA GTT-3′), Reverse primer (5′-CCC TTA CCC TTA CCC TTA CCC TAA-3′, and Synthetic template (5′-AAT CCG TCG AGC AGA GTT AGG GTT AGG GTT AGG GTT AGG G-3′).

Zero-background HDA: A standard zero-background HDA reaction mixture (25 μL) contained 10× annealing buffer II (2.5 μL: $MgSO_4$ (1 μL, 100 mM), NaCl (2 μL, 500 mM)), Reverse primer (1 μL, 5 μM), Forward primer (1 μL, 5 μM), ATP (1 μL, 52.5 mM), dNTPs (1 μL, 3.5 mM each), IsoAmp Enzyme Mix (0.75 μL), and 5×SYBR Green I (1 μL). The assay was incubated at 60 °C in LightCycler 96 System (Roche) with fluorescence monitoring at 60 s intervals. (The reaction mixture for conventional HDA is similar except for the concentrations of ATP (1 μL, 75 mM) and dNTPs (1 μL, 5 mM).)

Preparation of cell extracts: HeLa cells were cultured in Dulbecco's modified Eagle's medium with fetal calf serum (10%) and maintained in a humidified atmosphere (5% CO_2) at 37 °C. Other cell lines were cultured in their recommended culture media. Cells were collected in the exponential phase of growth. Then, 1×10^6 cells were transferred into an RNase-free 1.5 mL Eppendorf tube and washed twice with ice-cold PBS (pH 7.4) with centrifuging (2000 rpm, 10 min, 4 °C). Pelleted cells were resuspended in ice-cold 1×CHAPS Lysis Buffer (200 μL), incubated on ice for 30 min, then centrifuged (12000 rpm, 20 min, 4 °C). The supernatant was transferred into a fresh tube and stored at −80 °C. Heat-inactivated cell extracts were prepared by incubating cell extract at 85 °C for 15 min. Centrifuging was performed in a 5415R Centrifuge (Eppendorf).

Zero-background HDA for telomerase activity assay: All solutions were prepared and diluted in RNase-free sterilized water. Cell extracts were suspended in 1×CHAPS lysis buffer. For the telomerase extension reaction, cell extract (6 μL) was added to a solution (10 μL) containing Forward primer (1 μL, 10 μM), dNTPs (1 μL, 2 mM each), and 10×telomerase extension reaction buffer (1 μL: Tris·HCl (20 mM, pH 7.9), $MgCl_2$ (1.5 mM), EGTA (1 mM), KCl (63 mM), Tween 20 (0.05%)). The mixture was incubated at 37 °C for 30 min. Then, 2 μL of the resulting solution was mixed with of 10×Annealing buffer II (2.5 μL), Reverse primer (1 μL, 5 μM), Forward primer (1 μL, 4 μM), ATP (1 μL, 52.5 mM), dNTPs (1 μL, 3.5 mM each), of IsoAmp Enzyme Mix (0.75 μL) and 5×SYBR Green I (1 μL). The mixture (total 25 μL) was incubated at 60 °C and monitored by the LightCycler 96 System.

Gel electrophoresis: Reaction products were analyzed by 3.5% agarose gel electrophoresis in TAE buffer (0.5×) at 50 V for 70 min. The gel was imaged by a G:BOX Imaging System (Syngene, Cambridge, UK).

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