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


Abstract: Primer-dimer artifacts resulting from unintended template-independent primer-primer interactions often hinder the specific amplification of nucleic acids. Herein, we demonstrated the zero-background helicase-dependent amplification (HDA) with low concentrations of both ATP and dNTPs for the first time. This strategy achieved the reliable evaluation of telomerase activity in cancer cell via eliminating primer-dimer which always plagued many previously reported methods with reduced specificity. And we found that the telomerase assay performance of zero-background HDA was negatively affected by the concentrated cellular proteins. This inhibitory effect was attributed to the binding of DNA templates to proteins, making them unavailable for PCR. HDA has been regarded as a promising process (heat denaturation and extension, which mimics DNA replication in vivo) for the exponential accumulation of nucleic acids. Herein, we demonstrated the zero-dependent amplification (HDA) is a powerful amplification technique used for the exponential accumulation of interested nucleic acids. A DNA helicase is employed to separate double-stranded DNA (dsDNA) and produce single-stranded templates for primer hybridization and subsequent extension, which mimics DNA replication in vivo. The enzymatic unwinding of dsDNA enables simple DNA amplification under isothermal conditions, and omits the complicated thermocycling process (heat denaturation, annealing and extension) that is required in PCR. HDA has been regarded as a promising alternative to PCR especially in resource-limited settings and for on-site testing, and has been expanded for broad applications such as pathogen diagnosis and SNP genotyping.

The early HDA was performed at low temperature (~37 °C) due to utilizing the Escherichia coli UvrD helicase, and required two accessory proteins (MutL and single-stranded DNA-binding protein) to facilitate amplification reaction. Considerable effort has been directed towards the improvement of HDA performance. Kong et al. cloned and purified a thermostable UvrD helicase from Thermoanaerobacter tengcongensis to support HDA reactions at higher temperatures (60-65 °C), demonstrating heightened amplification efficiency even in the absence of accessory proteins with simplified reaction components. A new bifunctional protein has been engineered via fusing helicase with DNA polymerase for the successful amplification of longer fragments up to 2.3 kb. Treating the target genomic DNA with an endonuclease prior to amplification was proved to significantly enhance both the speed and sensitivity of HDA. In addition, primers with 5’-termini enriching A or C rather than T or G was favored in HDA, and could lead to much more efficient amplification reactions. Nevertheless, primer-dimer artifacts resulting from template-independent primer-primer interactions were often observed even via careful primer design. This common problem challenges the specific accumulation of target nucleic acids and leads to relevant misinterpretations of detection results.

Recently, Zhang’s group reported a novel target-converted HDA assay for the ultrasensitive detection of transcription factors without the interference of primer-dimer artifacts. They used only one kind of primer that was complementary to the stem of a designed hairpin template for the HDA reaction, thus eliminating the primer-dimer type nonspecific amplification. However, exounucleases-catalyzed complete digestion of the hairpin templates followed by heat inactivation of exounucleases was required to prevent background amplification in the absence of target. Moreover, this method was not applicable for both the amplification of other nucleic acids and the biosensing of various non-nucleic acid targets because of a given hairpin template. As HDA-based applications continue to evolve, the development of general and simple strategies for further improvements of HDA performance remains an urgent need.

Herein, we investigated the effect of several important factors, including enzymes concentration, reaction temperature, adenosine triphosphate (ATP) concentration, and deoxynucleotide triphosphates (dNTPs) concentration, on HDA performance. The decrease of enzymes concentration slowed down the amplification speed, yet the primer-dimer artifacts still existed. Increasing reaction temperature also couldn’t reduce primer dimers, but accelerated this nonspecific amplification and reduced its difference to template-triggered specific amplification. To our surprise, primer-dimer artifacts were eliminated by simultaneously adjusting the concentrations of ATP (as a cofactor for UvrD DNA helicase) and dNTPs to relatively low levels. Then, the modified HDA was applied to the zero-background detection of telomerase activity in cell extracts without the interference of primer-dimer nonspecific products. As

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In HDA, ATP is used as the energy donor for helicase to separate DNA duplex via breaking the hydrogen bonds, which is not involved in other amplification strategies (e.g., PCR and strand displacement amplification). Thus, we speculated ATP concentration might present particular effect on HDA performance. As ATP concentration decreased from 3 mM to 1.8 mM, to our surprise, the primer-dimer artifacts were significantly reduced (Figure 2A). It can be seen that the specific amplification was also inhibited. Based on the highest signal to noise ratio, the ATP concentration of 2.1 mM was selected for the following experiments. To further reduce the primer-dimer artifacts, the concentration of dNTPs as substrates for DNA synthesis was optimized. As depicted in Figure 2B and Figure S2, the primer-dimer artifact was eliminated when the dNTPs concentration was down to 0.14 mM. Though the yield of specific amplified products was also slightly lowered, this dNTPs concentration was chosen. Overall, for the first time, we demonstrated the zero-background HDA to eliminate primer-dimer artifacts were observed in TRAP assay and lowered the detection specificity. Previous studies have reported that the concentrations of proteins had significant effects on the outcome of HDA reaction, and rapid amplification was achieved at high concentration of enzyme mix. In contrast, we doubted whether the decrease of enzyme mix concentration could contribute to reducing nonspecific primer-dimer products. HDA reactions using a series of enzyme mix concentration in the presence and the absence of synthetic template were simultaneously carried out, and the corresponding real-time fluorescence curves were shown in Figure 1A. It can be seen that the speeds of both template-triggered specific amplification and template-independent nonspecific amplification dropped as the enzyme mix concentration decreased. When the enzyme mix concentration was down to 0.14 ×, no fluorescence signal of specific amplification was observed despite the elimination of primer-dimer artifacts, indicating the failure of the amplification process. The gel electrophoresis analysis (Figure 1A) and melting curve analysis (Figure 1B) also confirmed the consistent results. It is notable that the primer dimer has the same length as that of the specific amplified product in the used primers/template system due to using short synthetic template. Considering that nonspecific primer-primer complexes are unstable and easily dissociated at high temperatures, we studied the effect of reaction temperature on nonspecific HDA reactions. Unfortunately, elevated reaction temperature up to 65 °C accelerated not only template-triggered specific amplification but also template-independent nonspecific amplification, and reduced the difference in kinetics between these two amplification reactions (Figure S1). Such rapid template-triggered amplification could be attributed to the maximum activity of enzyme mix at 65 °C, whereas accelerated nonspecific amplification was unexpected. Since the melting temperatures of the primer-primer complexes are much lower 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 which are also available for polymerization reaction except stable duplexes.

In this work, the primers/template system used in PCR-based TRAP was selected as a model system to demonstrate the zero-background HDA without the interference of primer-dimer artifacts. TRAP has been developed as the gold standard method for the detection of telomerase activity. However, primer-dimer artifacts were clearly observed in many previously reported methods including the gold standard method telomere repeat amplification protocol (TRAP), and degraded the telomerase assay specificity. Furthermore, we demonstrated that gold nanoparticles (AuNPs) reduced the inhibitory effect of concentrated cellular proteins on zero-background HDA, and improved the sensitivity and reliability for telomerase assay when amplification reaction was performed in protein-rich samples.

**Results and Discussion**

In this work, the primers/template system used in PCR-based TRAP was selected as a model system to demonstrate the zero-background HDA without the interference of primer-dimer artifacts. TRAP has been developed as the gold standard method for the detection of telomerase activity. However, primer-dimer artifacts were clearly observed in many previously reported methods including the gold standard method telomere repeat amplification protocol (TRAP), and degraded the telomerase assay specificity. Furthermore, we demonstrated that gold nanoparticles (AuNPs) reduced the inhibitory effect of concentrated cellular proteins on zero-background HDA, and improved the sensitivity and reliability for telomerase assay when amplification reaction was performed in protein-rich samples.

**Figure 1.** Effect of enzyme mix concentration on HDA performance. (A) Real-time fluorescence curves of different enzyme mix concentrations. Inset is the corresponding gel electrophoresis analysis result. (B) Corresponding melting curves. The symbols - and + represent the absence of template and presence of template, respectively.
dimer artifacts by adjusting the concentrations of both ATP and dNTPs to relatively low levels. And the major difference between traditional HDA and zero-background HDA is briefly depicted in Scheme 1A. Subsequently, the zero-background HDA was used for the analysis of telomerase activity. Human telomerase is a ribonucleoprotein that maintains telomere length by adding tandem repeat (TTAGGG) to the end of chromosome, and is in strong association with cellular immortality and carcinogenesis. Up-regulation or reactivation of telomerase activity has been observed in a vast majority of human tumors (~85% - 90%). In contrast, 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. Here, telomerase substrate (TS) primer is used to perform telomerase-catalyzed extension reaction. As illustrated in Scheme 1B, a minor number of TS primers are extended with telomeric repeats at 3’-ends in the presence of telomerase. These telomerase extension products can act as templates for the DNA amplification stage by zero-background HDA. Firstly, reverse primers hybridize with the templates to initiate polymerization reaction, forming duplex templates. Each duplex template is separated by DNA helicase to generate two single-stranded templates. Then reverse primer and forward primer (TS primer) can each hybridize to corresponding single-stranded template to initiate two polymerization reactions, producing two duplex templates. These newly synthesized duplex templates again act as substrates for DNA helicase, entering the next round of DNA amplification. Eventually, such a chain reaction results in exponential amplification of the telomerase extension products. Fluorescent dye SYBR Green I, specific for dsDNA, was used to real time monitor the amplification reaction. Oppositely, in the absence of telomerase, no telomerase extension products as well as following DNA amplicons is generated. And the primer-dimer nonspecific amplification is eliminated in the zero-background HDA. The feasibility of the proposed method for telomerase detection was firstly demonstrated. The HeLa cells extracts triggered an exponential increase of fluorescence signal (Figure S3, red line), whereas no visible fluorescence enhancement was observed in the curve of blank sample without HeLa cells extract (Figure S3, black line). To evaluate the sensing performance of zero-background HDA, a series of samples containing various HeLa cell extracts were examined. As shown in Figure 3A, the fluorescence intensity gradually increased with the increase of cell number, indicating that the cell number is directly proportional to the amplified products of zero-background HDA. When the fluorescence intensity were plotted against the logarithm of cell number (Figure 3B), the resulting standard curve showed a linear correlation in the range of 100 - 1000 HeLa cells with the correlation equation of \[ F = 0.273 \log(\text{cell}) \]
Figure 3. Detection of telomerase activity in human HeLa cells by zero-background HDA. (A) Real-time fluorescence curves triggered by different cell numbers. (B) Linear relationship between the fluorescence intensity and the logarithm of cell number.

Figure 4. Evaluation of telomerase activity from different cell lines. All the cell numbers were 250.

Figure 5. (A) Negative effect of highly concentrated cell extracts on zero-background HDA performance for telomerase assay. PK is the abbreviation for proteinase K. (B) AuNP-based resistance to the inhibition. The number of HeLa cell was 250.
DNA templates and making them unavailable for polymerases.\textsuperscript{19} Treating the normal cell lysates with proteinase K prior to mixed with cancer cell extracts could eliminate such inhibitory effect on the amplification reaction,\textsuperscript{19} which was also confirmed by the zero-background HDA based telomerase assay (Figure 5A). Nevertheless, telomerase can also be degraded and inactivated when the real samples are pretreated with proteinase K, making this approach impractical.

Inspired by the previous study that utilized AuNPs to enhance the sensitivity and reliability for telomerase assay in protein-rich samples,\textsuperscript{19} we then investigated the resistance of AuNPs to the inhibition on zero-background HDA caused by abundant proteins. An AuNP-based assay was developed by adding appropriate concentration of AuNPs to the zero-background HDA system. As shown in Figure 5B, in the presence of extracts of 250 normal cells, the mixed samples triggered high amplification signals. Even for the mixed samples with 500 normal cells, the fluorescence response of the AuNP-based assay was also significantly heightened compared to that of the unmodified assay (Figure 5A). These results demonstrated the addition of AuNPs could effectively resist the inhibitory effect from abundant normal cells on zero-background HDA, supporting the improvement of assay sensitivity and reliability. The origin of the AuNP effect has been attributed to the strong adsorption of proteins from the cell lysate onto the surface of charged nanoparticles,\textsuperscript{16,19} which prevented the impairment of DNA templates.

Conclusions

In summary, we have reported the zero-background HDA for the reliable evaluation of telomerase activity in cancer cell. By lowering the concentrations of both ATP and dNTPs, the proposed strategy successfully eliminated the nonspecific primer-dimer artifacts which produced false-positive results in many previously reported methods including the gold standard method TRAP. Furthermore, AuNPs were demonstrated to be capable of reducing the inhibition of abundant proteins on the zero-background HDA reactions, and improving the sensitivity and reliability when assay was performed in concentrated cell extracts. These excellent performances of the developed strategy make it a promising solution for the direct measurement of telomerase activity in protein-rich samples with accurate results. Although this work was demonstrated by using the model primer/template system for telomerase assay in TRAP, it can be anticipated that the developed method has broad 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). IsoAmp® II Universal HDA Kit was obtained from New England Biolabs Ltd. (Beijing, China). TRAPEze® 1 x CHAPS Lysis Buffer was purchased from Millipore (Bedford, MA, USA). Gold nanoparticle was purchased from Sigma-Aldrich (St. Louis, MO, USA). All oligonucleotides were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China). The sequences were (1) Forward primer, 5’-AAT CGG TCG AGA AGA GTT-3’; (2) Reverse primer, 5’-CCC TTA CCC TTA CCC TTA CCC TAA-3’; (3) Synthetic template, 5’-AAT CGG TCG AGC AGA GTT AGG GTG AGT AGG GTT AGG GTT AGG G-3’.

Zero-background HDA: A standard zero-background HDA reaction mixture (25 μL) contained 2.5 μL of 10 × Anneling buffer II, 1 μL of 100 mM MgSO₄, 2 μL of 500 mM NaCl, 1 μL of 5 μM Reverse primer, 1 μL of 5 μM Forward primer, 1 μL of 52.5 mM ATP solution, 1 μL of 3.5 mM dNTPs, 0.75 μL of IsoAmp® Enzyme Mix and 1 μL of 5 × SYBR Green I. The assay was incubated at 60 °C in LightCycler® 96 System (Roche Applied Science, Mannheim, Germany) for the real-time fluorescence monitoring at an interval of 60 s. The reaction mixture of traditional HDA were similar to those of the zero-background HDA except the different concentrations of ATP (1 μL of 75 mM) and dNTPs (1 μL of 5 mM).

Preparation of telomerase extracts: HeLa cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and maintained in a humidified atmosphere (95% air and 5% CO₂) at 37 °C. Other cell lines were cultured similarly in their recommended culture media. Cells were collected in the exponential phase of growth. Then, 1 × 10⁶ cells were transferred into a RNase-free 1.5 ml Eppendorf tube and washed twice with ice-cold phosphate buffered saline (pH = 7.4) by centrifuging at 2000 rpm for 10 min at 4 °C. The deposited cells were resuspended in 200 μL of ice-cold 1 × CHAPS Lysis Buffer, and incubated on ice for 30 min before centrifugation at 12000 rpm for 20 min at 4 °C. Subsequently, the supernatant was carefully transferred into a fresh tube and stored at -80 °C before use. Similar protocol was used for the preparation of telomerase extracts from other cells. The heat-inactivated cell extracts were prepared by incubating cell ex-tracts at 88 °C for 15 min.

Zero-background HDA for telomerase activity assay: All solutions were prepared and diluted by RNase-free sterilized water. Telomerase extracts from HeLa or other cells were diluted with 1 × CHAPS Lysis Buffer. To perform telomerase extension reaction, 6 μL of telomerase extracts was added to the solution containing 1 μL of 10 μM Forward primer, 1 μL of 2 mM dNTPs, and 1 μL of 10 × telomerase extension reaction buffer (20 mM Tris-HCl, 1.5 mM MgCl₂, 1 mM EGTA, 63 mM KCl, 0.05% Tween 20). The mixture up to 10 μL was incubated at 37 °C for 30 min. Then, 2 μL of the resulting solution of telomerase extension reaction was mixed with 2.5 μL of 10 × Anneling buffer II, 1 μL of 100 mM MgSO₄, 2 μL of 500 mM NaCl, 1 μL of 5 μM Reverse primer, 1 μL of 4 μM Forward primer, 1 μL of 52.5 mM ATP solution, 1 μL of 3.5 mM dNTPs, 0.75 μL of IsoAmp® Enzyme Mix and 1 μL of 5 × SYBR Green I. Finally, this mixture, with a total volume of 25 μL, was performed at 60 °C and monitored by LightCycler® 96 System.

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

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A zero-background helicase-dependent amplification strategy was developed by eliminating primer-dimer artifacts for the reliable evaluation of telomerase activity in cancer cell.