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Improved Ligation-Mediated PCR Method Coupled with T7 RNA Polymerase for Sensitive DNA Detection

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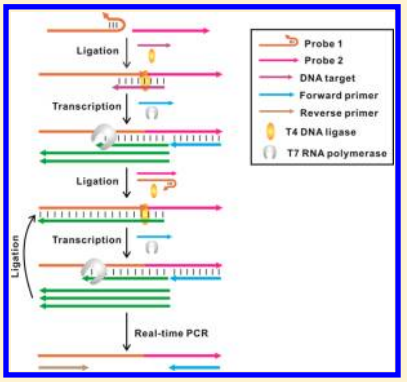
Supporting Information

ABSTRACT: The ligation-mediated polymerase chain reaction (PCR) method is widely applied for detecting short-length DNA target. The primary principle of this method is based on the linkage of two separated DNA probes as PCR templates via simultaneous hybridization with DNA target by DNA ligase. Even before taking into account low ligation efficiency, a 1:1 stoichiometric ratio between DNA target and the produced PCR template would put an intrinsic limitation on the detection sensitivity. In order to solve this problem, we have developed an improved ligation-mediated PCR method. It is designed such that a transcription reaction by T7 RNA polymerase is integrated into the ligation reaction. In this way, the produced joint DNA strand composed by two DNA probes can be used as a template both in the transcription reaction and the following PCR process. Then a great number of RNA strands containing the same sequence as DNA target are transcribed to act as a target to initiate new cyclic reactions of ligation and transcription. The results indicate that our proposed method can improve the detection sensitivity by ~2 orders of magnitude compared with the conventional ligation-mediated PCR method.



The sequence-specific DNA detection has attracted great scientific interest in a wide range of areas including molecular diagnostics,[1−3] environmental and food safety monitoring,[4,5] and biodefense application.[6,7] Since some DNA targets of interest may be present in trace amounts, it is highly imperative to develop ultrasensitive and routine methods for DNA detection. To date, many new types of DNA-detection methods based on optical and electrochemical techniques have been developed. For example, the nanomaterials with the unique optical, catalytic, and electronic features such as gold nanoparticles,[8−10] quantum dots,[11−13] carbon nanotubes,[14−16] and silicon nanowires[17−19] have been exploited as signal transducers for specific DNA analysis. Although these nanomaterial-based methods have made significant progress toward DNA detection, they might not be suited as a routine and universal tool in bioanalytical application. Because these approaches may suffer the repeatability problem stemming from the complicated and nonconventional setups of nanomaterial synthesis and conjugation and specialized expertise in nanomaterial handling; thus, it would prohibit the widespread application of these methods. Recently, various electrochemical DNA (E-DNA) sensors have been developed for sensitive DNA analysis.[20−24] E-DNA sensors have shown remarkable features of portable and inexpensive instrumentation, high sensitivity, as well as low fabrication cost; they are yet to be widely used as a routine method due to the nonconventional electrochemical platform. In addition, the fabrication of E-DNA sensors also needs specialized expertise in electrode polishing and modification, and the electrical signal is vulnerable to external perturbations. Undoubtedly, the polymerase chain reaction (PCR) technique shows a remarkable generality ascribing to its high stability, easy operation, and wide dynamic detection range. PCR-based methods have been widely considered as the gold standard for nucleic acid detection since 1985[25] and have significantly impacted the genetics and molecular biology. Although they are fairly sensitive due to the powerful amplification ability, their sensitivity in the range from 10^{-10} to 10^{-13} M[26,27] might not satisfy the needs in some cases, where trace amounts of DNA beyond the limited detection range are present. In this regard, there is still a great desire to improve the performance of PCR-based method in the sensitivity.

Different types of PCR-based methods have their own characteristics and areas of application. For the DNA target with a long length sequence, an ordinary PCR method can be directly applied to amplify the target to detectable levels. As for DNA target with a short length sequence, an alternative ligation-mediated PCR method can be employed. The key process in the ligation-mediated PCR method is DNA target-assisted specific ligation of DNA probes. The DNA probes are

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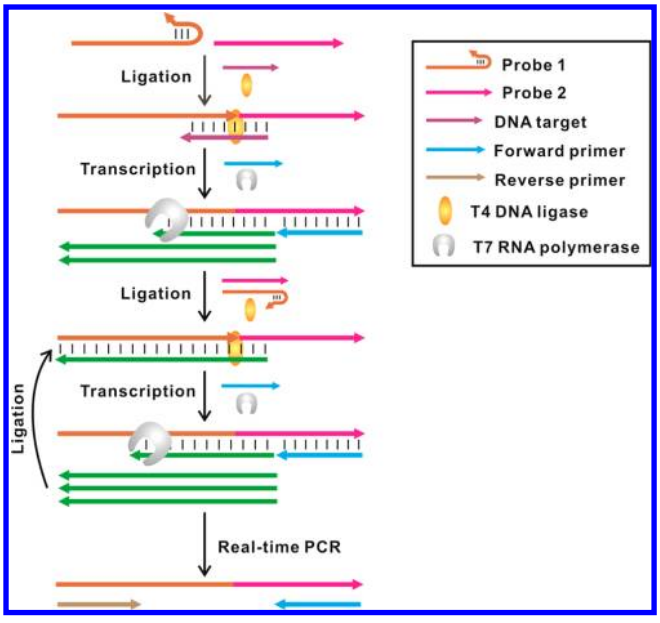
designed to be partly complementary to the half-sequence of DNA target and linked by DNA ligase to serve as a template for the following PCR process. It is worth noting that the sensitivity of this method is highly dependent on the ligation efficiency of DNA probes; however, it is much lower than those of other PCR-based methods due to the low ligation efficiency. Aiming to increase the sensitivity of the conventional ligation-mediated PCR method, here we have developed an improved version of the ligation-mediated PCR method by coupling T7 RNA polymerase. T7 RNA polymerase is a DNA-dependent RNA polymerase, and it catalyzes the synthesis of RNA in a 5′ to 3′ direction in the presence of a DNA template containing a T7 phage promoter. Taking advantage of this catalytic property of T7 RNA polymerase, we integrated a transcription step into the ligation step. Via the rational design of T7 promoter sequences encoded in one DNA probe and primer, the produced joint DNA strand is not only used as a template in the following PCR amplification reaction but also used as a template for the transcription reaction. With this design, a single DNA target does not only produce one template for PCR process but generate the one-to-multiple amplification effect. By combining the ligation reaction by DNA ligase, transcription by T7 RNA polymerase, and polymerase chain reaction by DNA polymerase, our proposed method can quantitatively detect a sequence-specific DNA as low as ~5 fM with a linear range spanning over 5 orders of magnitude, in which the sensitivity is improved ~100-fold in comparison with the conventional ligation-mediated PCR method.

RESULTS AND DISCUSSION

The working principle of the improved ligation-mediated PCR method for DNA detection is depicted in Scheme 1. The whole reaction system consists of two probes as templates, DNA target and two primers as triggers, three enzymes of T4 DNA ligase, T7 RNA polymerase, and DNA polymerase as mechanical activators, and intercalation dye SYBR Green I as signal output. One probe (named as probe 1, denoted in brown in Scheme 1) is designed to contain a stem-loop structure for the prevention of the self-ligation and the improvement of the discrimination ability for a single mutant in the target of interest. Another probe (named as probe 2, denoted in red in Scheme 1) is designed to encode with T7 promoter sequence for the transcription reaction and is modified with a 5′-phosphate group for the ligation reaction. Probes 1 and 2 are also designed to be partly complementary to the half sequence of DNA target (denoted in purple in Scheme 1) at the 3′- and the 5′-terminal, respectively. The proposed method comprises three primary processes, target-mediated ligation by T4 DNA ligase, transcription reaction by T7 RNA polymerase, and PCR amplification by DNA polymerase and involves two separated operation steps. In the first step, it is the aim to produce the PCR template by the joint action of T4 DNA ligase and T7 RNA polymerase. Upon the hybridization with DNA target, probe 1 and probe 2 are ligated via the formation of a phosphodiester bond between juxtaposed 5′ phosphate and 3′ hydroxyl termini by T4 DNA ligase. Then, a 25-mer forward primer (denoted in blue in Scheme 1) encompassing T7 promoter is annealed to the joint DNA strand in the complementary sequence of the T7 promoter downstream. T7 RNA polymerase recognizes the double-stranded promoter and transcribes a great amount of RNA strands (denoted in green in Scheme 1), which contains the same sequence as DNA target except that ribonucleotides and uridine replace the deoxyribonucleotides and thymine in target DNA. Subsequently, the produced RNA strands can act as the DNA target to initiate a new cycle of hybridization, ligation, and transcription, resulting in the production of a great amount of joint DNA strands. In the second step, all joint DNA strands with the universal primer-specific sequences are amplified by real-time PCR. Under the thermal cycling condition, the forward primer (denoted in blue in Scheme 1) and reverse primer (denoted in orange in Scheme 1) initiate a typical SYBR Green I-assisted PCR amplification reaction to produce double-strand DNA (dsDNA) products, into which free SYBR Green I molecules intercalate to generate significant fluorescence signal. Correspondingly, the fluorescence signal from intercalated SYBR Green I molecules in dsDNA products is directly proportional to the amount of DNA target present in the tested sample.

Scheme 1. Schematic Illustration of the Proposed Improved Ligation-Mediated PCR Method under the Participation of T7 RNA Polymerase for Short-Length DNA Detection



In a typical ligase-mediated PCR assay, the DNA target usually acts as a bridge to specifically ligate the DNA probes to produce a template for the following PCR process. Then the DNA target detection is directly converted to detect the ligation product, and correspondingly the sensitivity of target detection is dependent on the amount of ligated templates. Similarly, the first step of the ligation and transcription reactions in our method is also critical, aiming to produce the joint DNA strand as the PCR template. In order to achieve an optimal performance, we optimized the experimental conditions of the first step including the amounts of DNA probes, the amount of T7 RNA polymerase, the amount of NTPs, and the amount of T4 DNA ligase, which are directly related to the yield of the ligation products. The experimental details are described in the Supporting Information. The threshold cycle (C_t) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The threshold cycle change value of ΔC_t ($C_{t,\ blank} - C_{t,\ DNA\ target}$) between the sample and blank is employed to quantitatively evaluate the effect of the tested factors on the performance. On the basis of the working principle of the first step, it is clear to know that

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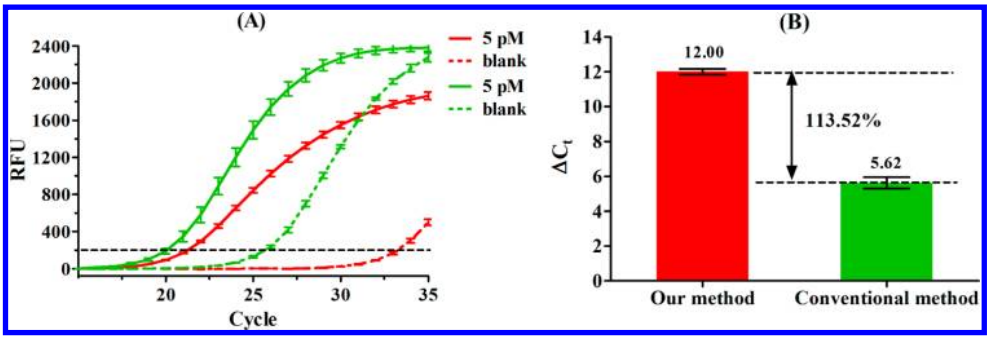

Figure 1. Performance comparison between our proposed method and conventional ligation-mediated PCR method. (A) The real-time fluorescence curves responses in the presence and absence of 5 pM T1 target by our proposed method with T7 RNA polymerase (denoted in red) and the conventional T4 DNA ligase-mediated PCR (denoted in green), respectively. (B) Bars represent the ΔC_t value based on the data (A). The error bars represent the standard deviations of three parallel tests.

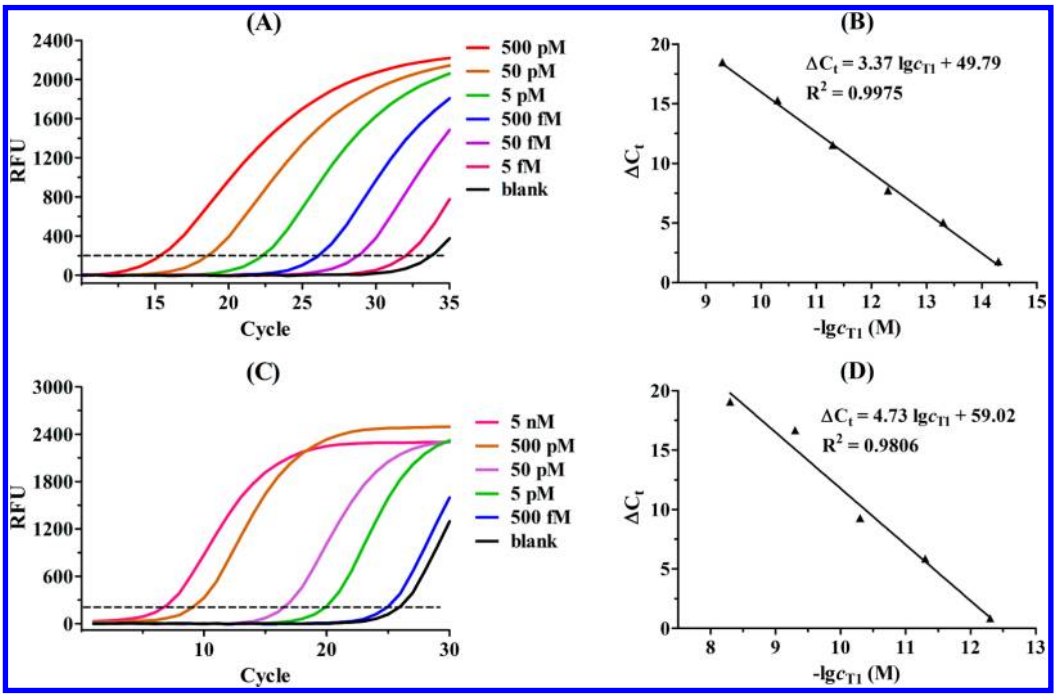

Figure 2. Sensitivity investigation for quantitative DNA detection by our proposed method and the conventional ligation-mediated PCR. (A) The real-time fluorescence curves were produced in the presence of T1 at different concentrations from 5 fM to 500 pM by our proposed method. (B) Plot of the relationship between ΔC_t value and the logarithm of the concentration of T1 based on the data (A). (C) The real-time fluorescence curves were produced in the presence of targets at different concentrations from 500 fM to 5 nM by the conventional ligation-mediated PCR. (D) Plot of the relationship between ΔC_t value and the logarithm of the concentration of T1 based on the data (C).

probe 1, probe 2, and the forward primer participate together and none of them can work effectively without each other. At same time in order to simplify the optimization steps, we chose probe 1, probe 2, and forward primer with the same concentrations in the first step. Four concentrations at 50 nM, 10 nM, 5 nM, and 1 nM were tested. As shown in Figure S1 in the Supporting Information, the high concentrations at 50 nM and 10 nM provided high background signals from the blank samples, resulting in small cycle changes in DNA target. On the other hand, the low concentration at 1 nM revealed a smaller change in ΔC_t than that of 5 nM. Thus, we chose the combination concentration of probe 1, probe 2, and the forward primer at 5 nM in the following experiments. In addition, we found that the optimum amounts of T7 RNA polymerase, NTPs, and T4 DNA ligase were 20 U, 2 mM, and 150 U, respectively (see Figures S2 and S3 in the Supporting Information).

Under the above optimized experimental conditions, we first investigated whether the introduction of T7 RNA polymerase could improve the ligation efficiency and increase the sensitivity as we expected. A DNA target of T1 at 5 pM and blank sample (no target present) were tested by our proposed method and the conventional ligation-mediated PCR method (see the detailed procedures in the Supporting Information), respectively. The time-course monitoring fluorescence curves in the presence and absence of T1 by these two methods are shown in Figure 1A. It was observed that both methods could distinguish the 5 pM T1 and blank sample, whereas our proposed method gave a remarkable dynamic range from ~21.5 to ~33.5 cycle, and the corresponding dynamic range in the conventional method is from ~20.0 to ~25.6 cycle. It is clearly observed a 113.52% increase in ΔC_t by our method (Figure 1B). It should be noted the C_t value not only relates to the initial concentration of PCR template but also to the components

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of the PCR reaction system. When the components of PCR reaction system change, the C_t value would also change (see Figure S4 in the Supporting Information).

Next, we investigated the sensitivity of the proposed method. Various amounts of T1 target of 5 fM, 50 fM, 500 fM, 5 pM, 50 pM, and 500 pM, were prepared from one stock solution and tested by our method, respectively. As depicted in Figure 2A, a series of significant time-course fluorescence curves were clearly observed upon the introduction of T1 target at different concentrations. The C_t value increased when the concentration of T1 target was decreased in the range from 500 pM to 5 fM, indicating that the fluorescence signals of thermal cycler were highly dependent on the T1 target concentration. The detectable concentration was as low as 5 fM ($>3\sigma$/slope, σ is the standard deviation of the blank solution, $n = 10$). Figure 2B shows an excellent linear relationship between the ΔC_t value and the logarithm base 10 (lg) of the amount of T1 target in the dynamic range of 5 orders of magnitude from 5 fM to 500 pM. The correlation equation obtained is $\Delta C_t = 3.37 \lg c_{T1} + 49.79$. More specifically, the calibration curve displayed an excellent linearity with a correlation coefficient R^2 of 0.9975. The resultant products in the presence of T1 target at different concentrations were also confirmed by nondenaturing polyacrylamide gel electrophoresis (PAGE) (see Figure S5 in the Supporting Information). As expected, the characteristic bands of reporter dsDNA fragments with the expected nucleotide length of 86 bp were clearly observed in the presence of T1 target (lanes 1−6). In contrast, the negative control of blank sample showed negligible bands (lane 7). To facilitate the comparison between our method and the conventional ligation-mediated PCR, we also investigated the sensitivity of the conventional method by testing the same DNA target of T1. Figure 2C,D indicates that the detection range of the conventional method is from 500 fM to 5 nM, and the correlation equation is $\Delta C_t = 4.73 \lg c_{T1} + 59.02$, with an R^2 of 0.9806. It is clearly seen that the conventional method gives a poorer performance in sensitivity, dynamic detection range, and linear relationship between target concentration and ΔC_t value when compared with our method. The high sensitivity achieved in the proposed method is attributed not only to the exceptional amplification power from PCR assay but also to signal amplification process based on T7 RNA polymerase-assisted transcription. The stability of this proposed method was investigated by testing the spiked T1 sample with 10 replicates (data not shown). The coefficient of variation of the C_t was calculated to be 1.14%, revealing a high precision of the proposed method. In addition, we spiked different amounts of T1 to test the accuracy of the proposed method. For an unknown concentration of sample, the concentration can be easily calculated from the above equation. The results are summarized in Table S2 in the Supporting Information, which shows that recoveries were in the range of 94.88−117.17%.

To validate the selectivity of the proposed method to the specific DNA detection, we conducted a comparison study on multiple-mismatch targets from perfect complementary target. Five kinds of targets including the perfect match target (T1), 1-base mismatch target (T2), 2-base mismatch target (T3), 3-base mismatch target (T4), and 12-base mismatch target (T5) were selected (see the detailed sequence in the Supporting Information). Each target was tested at two concentrations of 5 pM and 500 fM. Figure S6 in the Supporting Information exhibits the comparison result of ΔC_t values from these five target DNAs. As expected, it is observed that only the perfectly complementary T1 target gave the highest ΔC_t value. These results suggested that the proposed assay developed in this study allows for a good sequence specificity to discriminate single nucleotide polymorphism.

To investigate real application in biological samples, cell lysate was used as a model matrix for our assay. A short length target DNA from the respiratory syncytial virus (RSV) was selected as a model. RSV is a widespread virus that represents the leading cause of acute lower respiratory-tract infections in infants and young children worldwide. The sequence of RSV DNA target and two probes designed as templates were listed in Table S1 in the Supporting Information. We spiked different amount of RSV DNA target in the cell lysate with known concentration. As shown in Figure S7 in the Supporting Information, the proposed method can quantitatively detect RSV DNA target from 5 fM to 5 nM with a good linearity, similarly.

CONCLUSIONS

In summary, we take advantage of T7 RNA polymerase to develop an improved ligation-mediated PCR method for highly sensitive and selective detection of DNA target. This proposed method relies on the cyclic enzymatic reaction of ligation and transcription to produce the target-like RNA fragments and PCR templates simultaneously, which significantly enhances the ligation efficiency. It has demonstrated the remarkable advantages toward sensitivity and detection time. Under the participation of T7 RNA polymerase, it produces an amplified signal in the ligation step to achieve a high sensitive detection of DNA target as low as 5 fM, ∼100-fold increase in the sensitivity compared with the conventional ligation-mediated PCR method. Besides, it is worth to point out that the response of this method is rapid with a detection time of ∼60 min, which is faster than most ligation-based methods with the same sensitivity condition. This method employs the SYBR Green I as signal output, which is easy to prepare, convenient to operate with a good stability, and also has the potential for widespread use. On the basis of the distinctive features presented above, we believe the proposed assay holds great promise as a routine PCR method for short DNA analysis in ordinary laboratory studies and clinical diagnoses, where reproducible, selective, and ultrasensitive DNA analysis is critical.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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SUPPORTING INFORMATION

An Improved Ligation-Mediated PCR Method Coupled with T7 RNA Polymerase for Sensitive DNA Detection

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Materials. T4 DNA ligase and TransStart Top Green qPCR SuperMix were purchased from TransGen Biotech Co., Ltd. (Beijing, China). T7 RNA polymerase and NTPs were purchased from Thermo Fisher Scientific Co., Ltd. (Waltham, MA, USA). RNase Inhibitor and diethylpyrocarbonate (DEPC)-treated water were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). PAGE-purified DNA oligonucleotides were customized from Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). In order to create and maintain an RNase-free environment, all solutions in this work were treated with 0.1% DEPC and autoclaved. The tips and tubes are RNase-free and do not require pretreatment to inactivate RNases. The buffers used in cellular extracts were prepared as follows. Buffer A was 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl and 0.5 mM DTT. Buffer B was 20 mM HEPES (pH 7.9), 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5 mM DTT. Buffer C was 20 mM HEPES (pH 7.9), 20% (v/v) glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT.

Table S1. The sequence information of DNA oligonucleotides used in this work.

Name	Sequence* (5'-3')
T1	**TTGAGGTGCATGTTTGTGCC**
T2	**TTGAGGTGCAT<u>C</u>TTTGTGCC**
T3	**TTGAGGTGC<u>T</u>T<u>C</u>TTTGTGCC**
T4	**TTGAGGT<u>C</u>C<u>T</u>T<u>C</u>TTTGTGCC**
T5	**TT<u>CTC</u>GT<u>AGC</u>T<u>CA</u>TT<u>CACA</u>C**
Probe 1	**GGTATCCAGGGAAGTGGATACGAAGCGTTTCAGGCACAAACAT**
Probe 2	**Pi-GCACCTCAACGCGGTGACCCTATAGTGAGTCGTATTAGTGATC**
RSV DNA	**TATTTGCCCCATTTTT**
RSV-1	**GGTATCCAGGGAAGTGGATACGAAGCGTCGATAAAAATGG**
RSV-2	**Pi-GGCAAATACCTTTGCTTCGCTTCTATAGTGTCACCTAAAT**
Forward primer	**GATCACTAATACGACTCACTATAGG**
Reverse primer	**AGGGAAGTGGATACGAAGC**

* Underlined characters represent the different bases in the tested DNAs compared with DNA target of interest.

Ligation Reaction and Transcription Reaction. The mixtures for ligation reaction and transcription reaction were prepared separately on ice as part A solution and part B solution. The part A solution contains 5 nM probe 1, 5 nM probe 2, 5 nM forward primer, 0.5×transcription buffer (20 mM Tris-HCl, 30 mM $MgCl_2$, 5 mM DTT, 5 mM NaCl and 1 mM spermidine, pH 7.9), DNA target, and DEPC-treated H_2O in a volume of 5.25 μL. The part B solution contains 150 U T4 DNA ligase, 20 U T7 RNA polymerase, 2 mM nucleoside triphosphates (NTPs), 1×T4 DNA ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP and 25 μg/ml BSA, pH 7.5) and 8 U RNase Inhibitor in a volume of 4.75 μL. First, the part A solution was incubated at 95 °C for 2 min and at 25 °C for 10 min. Then, part A and part B were mixed, and incubated at 25 °C for 15 min with a volume of 10 μL. Following that, the mixture was put on ice for stopping the reaction.

Real-Time PCR Experiment. Real-time PCR experiment was performed on Bio-Rad CFX 96 Real-Time PCR instrument (BIO-RAD, USA). The PCR reaction mixture was prepared in a volume of 25 μL, containing the above 10 μL reaction mixture, 1×qPCR supermix, 200 nM forward primer, 200 nM reverse primer and DEPC-treated water. The PCR reaction was carried out by using hot start of 94 ºC for 30 s, followed by 40 cycles of 94 ºC for 5 s and 60 ºC for 30 s. Unless noted otherwise, all experiments in this work were repeated three times. The conventional ligation-mediated PCR assay performed in this work was also following the above procedures, just the part B did not contain T7 RNA polymerase, nucleoside triphosphates (NTPs), and RNase Inhibitor.

Cell Culture. Human hepatocellular carcinoma cell lines (HepG2) was obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin-streptomycin at 37ºC in a humidified 5% CO_2 incubator, respectively. The cells were grown with fresh medium at 50 mm glass-bottom dishes. Then the cells were harvested by trypsinization and washed with fresh medium for three times and suspended in fresh medium for following studies.

Preparation of Cellular Extracts. The cellular extracts were prepared as followed. Briefly, 10^7 cells were washed once with phosphate-buffered saline and twice with Buffer A. The cell pellet was suspended in Buffer B/0.1% Nonidet P-40 (20 μL per 10^7 cells). After incubating for 15 min on ice, the lyzed cellular suspension was briefly mixed on a vortex and centrifugated for 10 min at 4 ºC. Then supernatant was diluted with 80 μL per 10^7 cells of Buffer C and stored at -80 ºC for the following experiment.

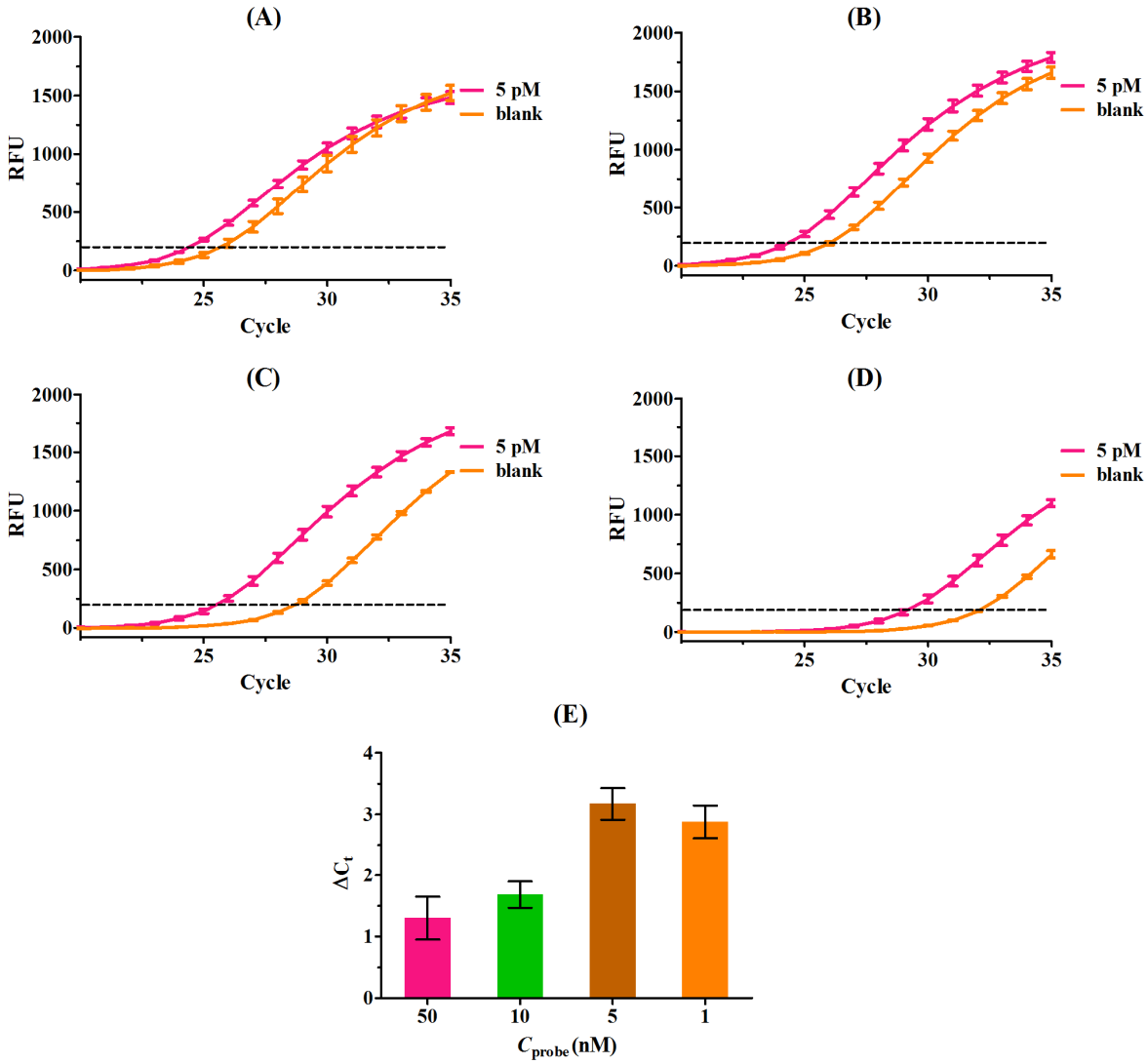

Figure S1. Optimization of the amounts of the probe 1, probe 2 and forward primer. The real-time fluorescence curves were produced by 5 pM T1 target and the blank. The amounts of the probe 1, probe 2 and forward primer were used as (A) 50 nM, (B) 10 nM, (C) 5 nM, and (D) 1 nM, respectively. The reaction mixture in the first step contained probe 1, probe 2, forward primer, target DNA, 100 U T4 DNA ligase, 20 U T7 RNA polymerase, 1 mM NTPs, 0.5× transcription buffer, 1× T4 DNA ligase buffer, 8 U RNase Inhibitor and DEPC-treated H_2O.

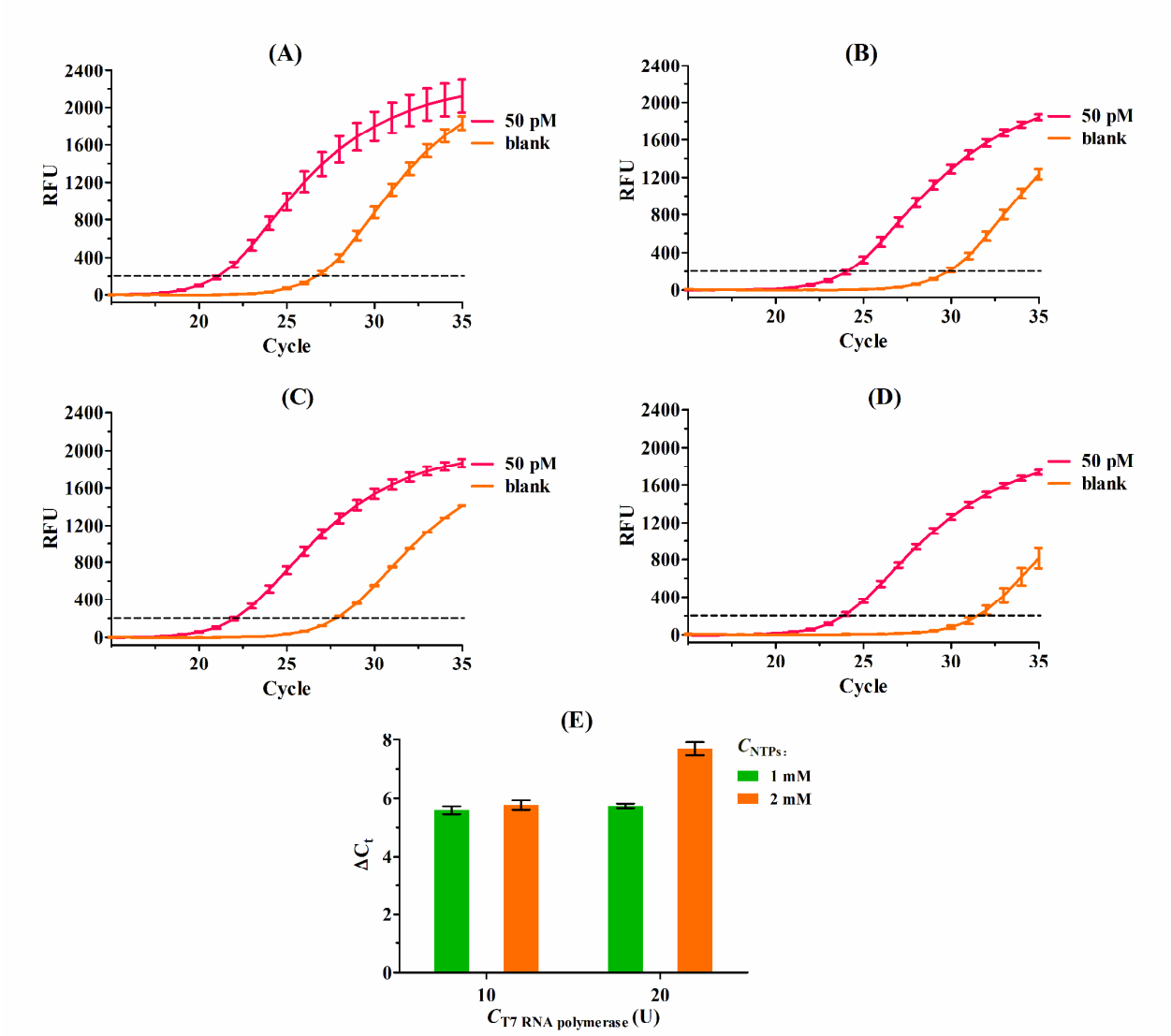

Figure S2. Optimization of the amount of T7 RNA polymerase and NTPs. The real-time fluorescence curves were produced by 50 pM T1 target and the blank. The amount of T7 RNA polymerase and NTPs were used as (A) 10 U and 1 mM, (B) 10 U and 2 mM, (C) 20 U and 1 mM, and (D) 20 U and 2 mM, respectively. The reaction mixture in the first step contained 5 nM probe 1, 5 nM probe 2, 5 nM forward primer, T1 target, 100 U T4 DNA ligase, T7 RNA polymerase, NTPs, 0.5× transcription buffer, 1× T4 DNA ligase buffer, 8 U RNase Inhibitor and DEPC-treated H_2O.

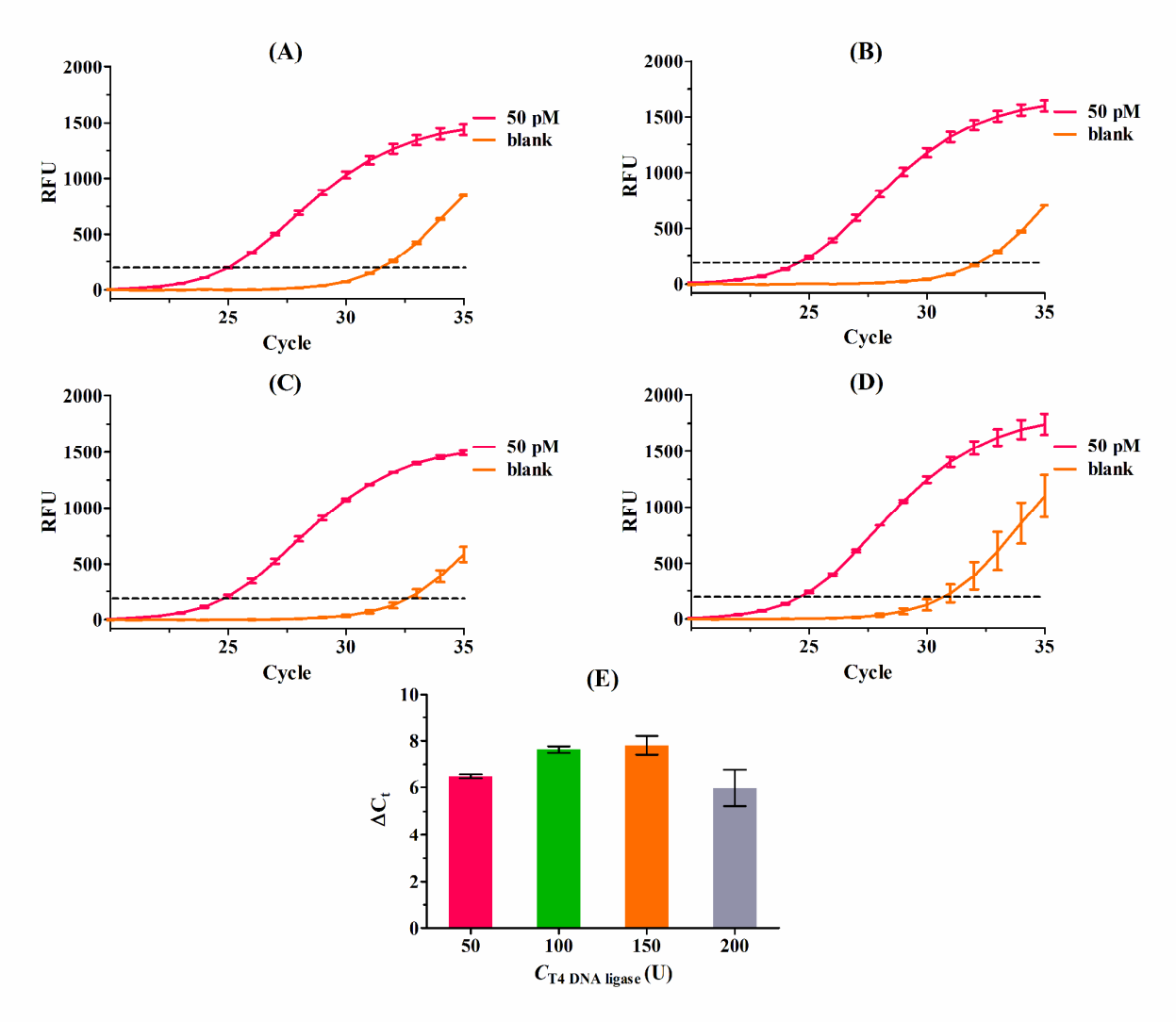

Figure S3. Optimization of the amount of T4 DNA ligase. The real-time fluorescence curves were produced by 50 pM target and the blank. The amount of T4 DNA ligase was used as (A) 50 U, (B) 100 U, (C) 150 U, and (D) 200 U. The reaction mixture in the first step contained 5 nM probe 1, 5 nM probe 2, 5 nM forward primer, T1 target, T4 DNA ligase, 20 U T7 RNA polymerase, 2 mM NTPs, 0.5× transcription buffer, 1× T4 DNA ligase buffer, 8 U RNase Inhibitor and DEPC-treated H_2O.

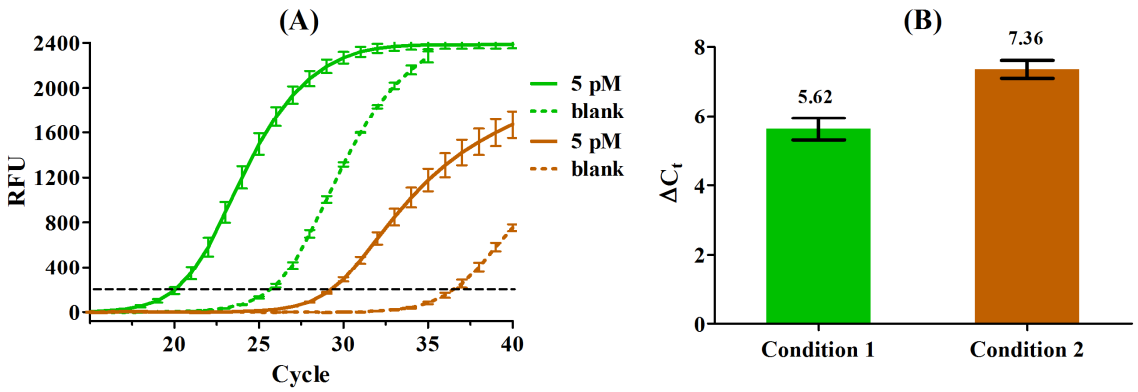

Figure S4. Investigation of the effect of the components of PCR reaction system on C_t value. (A) The real-time fluorescence curves responses in the presence and absence of 5 pM T1 target by the conventional ligation-mediated PCR (denoted in green, condition 1) and our proposed method except the addition of T7 RNA polymerase (denoted in brown, condition 2), respectively. (B) Bars represent the ΔC_t value based on the data (A). The error bars represent the standard deviations of three parallel tests.

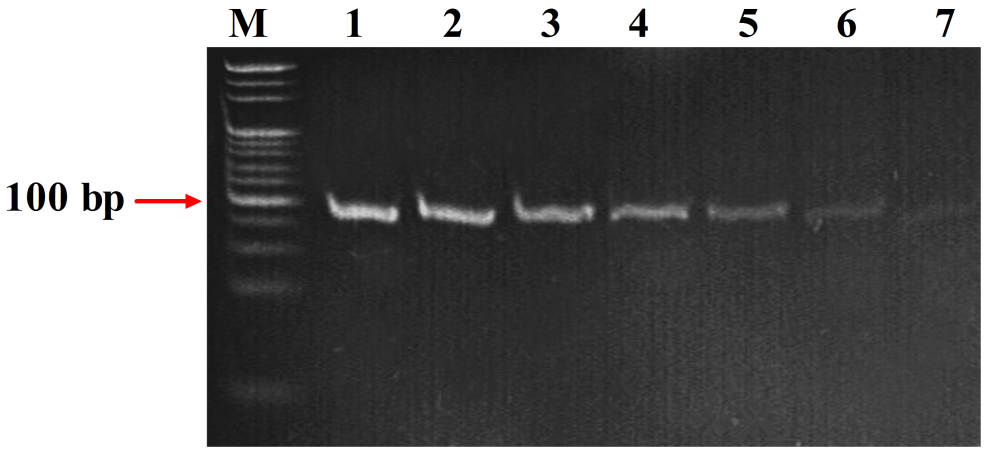

Figure S5. Image of non-denaturing PAGE of the PCR products by the proposed method in the presence of T1 at different concentrations and blank sample. Lane **M**: DNA ladder marker, lane **1**: 500 pM, lane **2**: 50 pM, lane **3**: 5 pM, lane **4**: 500 fM, lane **5**: 50 fM, lane **6**: 5 fM, lane **7**: blank sample. The red arrow indicates a DNA marker fragment with a length of 100 bp.

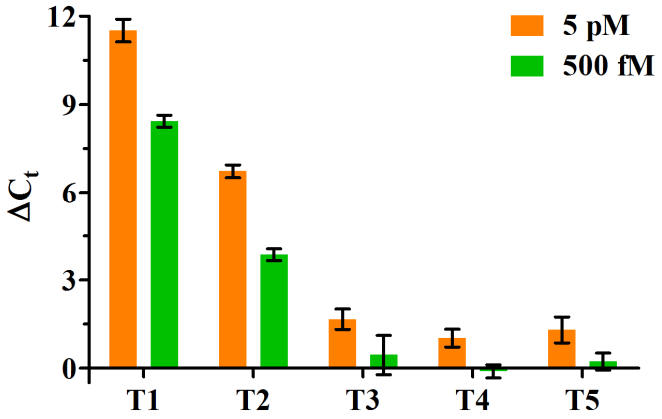

Figure S6. Sequence-specificity investigation of the proposed method upon the different DNA targets. Bars represent ΔC_t values from different DNA targets of T1, T2, T3, T4, and T5 at two concentrations of 5 pM and 500 fM, respectively.

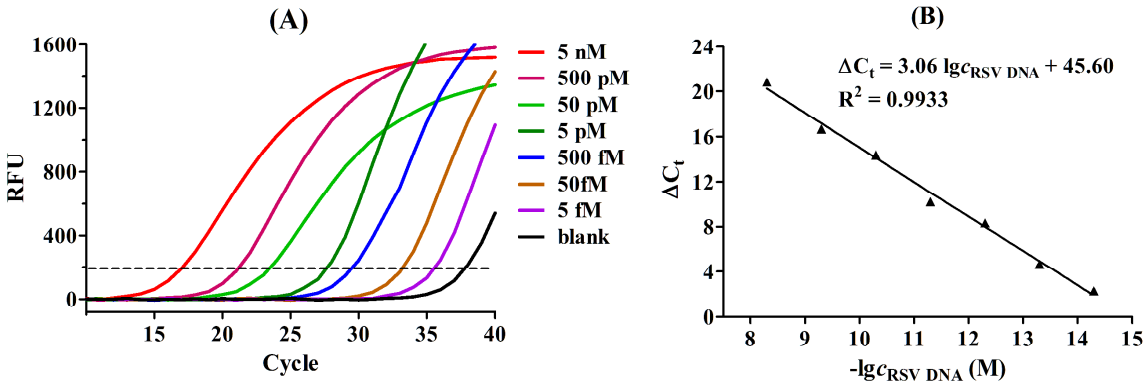

Figure S7. Quantitative analysis of the spiked RSV DNA target in cell lysate by our proposed method. (A) The real-time fluorescence curves were produced in the presence of RSV DNA target at different concentrations from 5 fM to 5 nM. (B) Plot of the relationship between ΔC_t value and the logarithm of the concentration of RSV DNA target based on the data (A).

Table S2. Recovery results of spiked T1 target at different concentration

Spiked amount (pM)	Detected amount (pM)	Recovery (%)	CV (%)
50.00	58.59	117.17	9.28
5.00	4.84	96.74	10.76
0.50	0.47	94.88	9.35