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# RNA pathogen detection with one-step reverse transcription PCR and strand-displacement based signal amplification†

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A novel detection method for RNA pathogens based on one-step reverse transcription PCR is introduced here. This method utilized the reverse transcriptase activity and the 5′-nuclease activity of TaqM1 DNA polymerase to transform target RNA into cDNA. The following PCR process released a fragment from the 5′ end as a specific probe. Afterwards this fragment triggered a strand-displacement based signal amplification to release large amounts of G-quadruplex DNAzymes. All the probes applied in our method were unmodified DNA oligonucleotides. The detection results could be reported without sophisticated instruments either in the colorimetric way through oxidizing ABTS or in the fluorometric way by using tyramine as substrate. This approach could successfully detect HIV-1 in a blood sample and it has a linear concentration range of 6 fM to 60 pM.

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## Introduction

Many RNA pathogenic viruses are able to cause notable human diseases and threaten public health.[1,2] For example, HIV-1 (Human Immunodeficiency Virus) is one of the serious worldwide health issues, with more than 2.7 million new infections and 1.8 million deaths occurring in 2011.[3,4] According to the latest WHO statistics (July 2010), the H1N1 influenza virus has killed more than 18 000 people since it appeared in April 2009.[5] For these reasons, the development of an accurate, sensitive detection method for the monitoring of RNA viruses is very necessary and urgent for controlling the spread of these infectious diseases.

Besides traditional immunological assays, such as antigen/peptide ELISA assays and antibody-dependent cellular cytotoxicity assays,[6] nucleic acid assays are the most reliable diagnosis techniques used for pathogenic detection, which is based on the invention of the PCR[7] (polymerase chain reaction) and the confirmation of the genome sequences of viruses. With PCR magnification, trace amounts of DNA could be amplified and hundreds of thousands of copies of a target DNA sequence were produced across several orders of magnitude. However in contrast to DNA detection, detecting RNA normally needs two types of enzymes, a non-thermostable reverse transcriptase and a thermostable DNA polymerase,[8] to perform two-step reverse transcription PCR. Then, a recombinant DNA polymerase from *Thermus thermophilus* (Tth) was found to exhibit very efficient reverse transcriptase (RT) activity in the presence of $Mn^{2+}$ ions.[9] With this enzyme, one-step reverse transcription PCR can be carried out easily, but due to the presence of $Mn^{2+}$ is unsuitable for many biotechnological applications. Recently, a mutated Taq DNA polymerase with reverse transcriptase activity and nuclease activity, named TaqM1, was screened by Prof. Andreas Marx's group.[10] Their results demonstrated the usefulness of TaqM1 for fast and reliable one-step RNA pathogen detection.

In the middle of the 1990s, real-time reverse transcription PCR (RT-PCR) was invented and became the method of choice for RNA quantification.[11] After that, two common methods for detection of products in real-time PCR were built: non-specific fluorescent dyes that intercalate with any double-stranded DNA (SYBR Green;[12,13] YOYO-1[14]), and sequence-specific DNA probes like TaqMan probes.[15] In the system using TaqMan probes, an intact TaqMan probe contains a fluorophore and a quencher covalently attached to both ends of the target strand respectively, with no fluorescence being detected due to fluorescence resonance energy transfer (FRET). During the PCR extension steps, a DNA polymerase, which harbors an active 5′–3′ nuclease domain, degrades the DNA stretch of the fluorescence probe that is annealed to the target strand,[16] resulting in increased fluorescence. The main limitation of the TaqMan method is the need for expensive reagents and equipment. In our previous work, we reported a similar TaqMan approach for the colorimetric detection of the PCR product.[17] The approach took

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advantage of the 5′ nuclease activity of Taq DNA polymerase to release a G-quadruplex element embedded in the probe. In the presence of alkali metal ions the G-quadruplex DNAzyme can bind with hemin, possessing a peroxidase-like activity which catalyzes the oxidation of $ABTS^{2-}$[18–21] by $H_2O_2$. However, this method could only be used for the detection of DNA targets for qualitative purposes. To expand the DNAzyme based assay to the RNA pathogen and realize the accurate quantitative real-time detection, a novel RNA detection method with one-step reverse transcription PCR and strand-displacement based signal amplification[22,23] was introduced herein (Fig. 1).

## Experimental

### 1 Reagents and apparatus

The oligonucleotides (Table S1, ESI†) and LB medium powder were purchased from Sangon Biotech (Shanghai, China). Hemin was purchased from Alfa Aesar; Trans1-T1 phage resistant chemically competent cell, T4 polynucleotide kinase, Taq DNA polymerase, Taq buffer and dNTP were purchased from TransGen Biotech (Beijing, China). Ni–agarose His tag protein purification kit was bought from Beijing CoWin Biotech Co., Ltd. Bsm DNA polymerase (large fragment) and Nb. Bpu10I was bought from Fermentas. [γ-$^{32}$P]ATP was purchased from Furui Biological Engineering (Beijing, China). ABTS was purchased from Wolsen (Xi an, China), $H_2O_2$ was purchased from Bodi Chemical Holding Co., Ltd. (Tianjin, China). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA). The TaqM1 plasmid was provided by Andreas Marx's group in Konstanz University and HIV-1 blood samples were provided by Wuhan University. The detection of the fluorescence produced by oxidized tyramine·HCl was performed using a Varioskan Flash (Thermo Scientific). The excitation wavelength was 320 nm and emission wavelength was 410 nm. About 15 readings with a 0.5 min interval were recorded. PCR was performed on a C1000 thermal cycler (Bio-Rad).

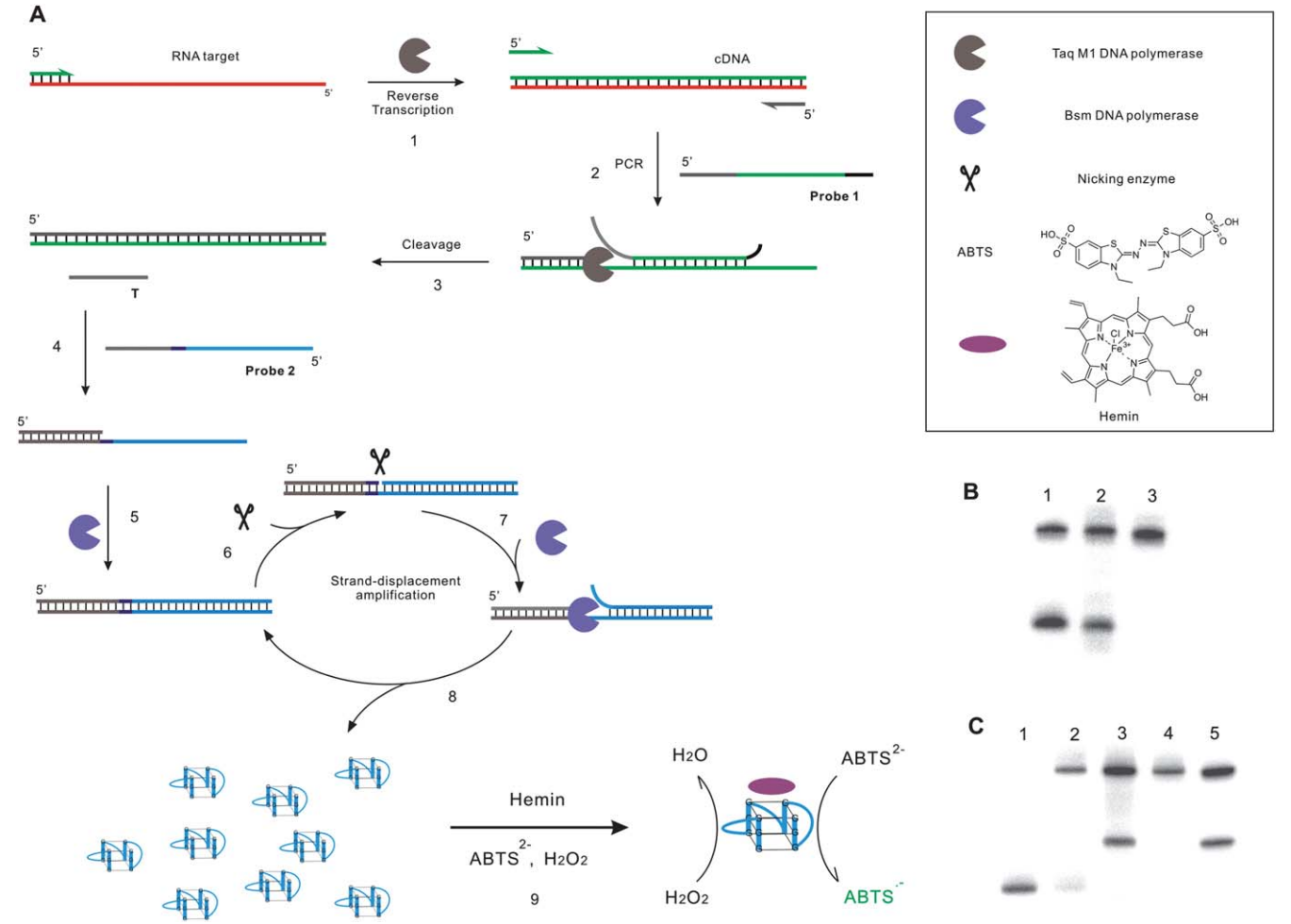


**Fig. 1** One-step RT-PCR and strand-displacement based signal amplification system. (A) The principle of the one-step RT-PCR and strand-displacement based signal amplification system. (B) PAGE-gel analysis of isotope experiments to prove the cleavage of probe 1 in the PCR reaction. Lane 1: 5′ $^{32}$P-labeled probe 1 and a 18 nt segment T as markers; lane 2: 5′ $^{32}$P-labeled probe 1 was added to the PCR reaction containing HIV cDNA; lane 3: 5′ $^{32}$P-labeled probe 1 was added to the PCR reaction without HIV cDNA. (C) PAGE-gel analysis of isotope experiments to verify the strand-displacement amplification. Lane 1: 5′ $^{32}$P-labeled fragment T as marker. Lane 2: the extension reaction of $^{32}$P-labeled T on probe 2 by mesophilic DNA polymerase. Lane 3: [α-$^{32}$P]dATP was added to the strand-displacement amplification reaction that contains T, probe 2, mesophilic DNA polymerase and nicking enzyme. Lane 4: [α-$^{32}$P]dATP was added to the negative control reaction that contains 5′ $^{32}$P-labeled probe 2, mesophilic DNA polymerase and a nicking enzyme. Lane 5: 5′ $^{32}$P-labeled probe 2 and a 25 nt G-quadruplex DNAzyme as markers.

### 2 Preparation of TaqM1 polymerase

The TaqM1 plasmid was transformed into an *E. coli* Trans1-T1 Phage Resistant Chemically Competent Cell. Clones were picked from plates (100 μg ml$^{-1}$ ampicillin) and grown in 40 ml LB medium (100 μg ml$^{-1}$ ampicillin) until the $OD_{600}$ was about 1.0. TaqM1 polymerase was expressed by inducing with 2 mM IPTG for 12 h. After centrifugation and washing three times with 1× Taq buffer (20 mM Tris–HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$), lysis was achieved using a 500 μl 1× Taq buffer containing 1 mg ml$^{-1}$ lysozyme. The lysate was incubated at room temperature for 30 min followed by heat denaturation of all non-thermostable proteins at 75 °C for 15 min. After centrifugation, the lysate was filtered through a 0.45 μm membrane and purified using a Ni–agarose His tag protein purification kit.

### 3 Colorimetric detection of cDNA or RNA

At the beginning, the PCR/RT-PCR was performed in the presence of a HIV cDNA/RNA template which was isolated from the blood sample (see ESI†). 0.5 μM forward primer, 0.5 μM reverse primer, 0.15 μM probe 1, 200 μM dNTP, 5 units of TaqM1 polymerase, 1× Taq buffer (20 mM Tris–HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$) and Milli-Q water was added to the 50 μl final volume. The PCR procedure was as follows: 95 °C for 1 min; 95 °C 20 s, 51 °C 40 s, 59 °C 40 s, 30 cycles. For RNA, a reverse transcription step of 65 °C for 15 min was added before PCR. Then, 10 mM $MgSO_4$, 0.35 μM probe 2, 8 units Bsm DNA polymerase (large fragment) and 5 units Nb. Bpu10I were added to the former PCR products. Then continued the second amplification at 37 °C for 1.5 h. After the PCR, 1.2 μM hemin, 3.8 mM ABTS and 1.5 mM $H_2O_2$ were added. The color could be observed after the addition of hemin, ABTS and $H_2O_2$.

### 4 Fluorometric detection of RNA with tyramine

The RT-PCR was processed as described above. After RT-PCR and isothermal amplification, 90 mM $(NH_4)_2SO_4$, 0.2 μM hemin, 0.8 mM tyramine·HCl, and 0.8 mM $H_2O_2$ were added to the 50 μl product. The real-time fluorometric detection was carried out in a Varioskan Flash (Thermo Scientific) machine (30 °C) and around 15 readings with a 0.5 min interval were recorded. The excitation wavelength was 320 nm and emission wavelength was 410 nm.

## Results and discussion

### 1 Principles of the RT-PCR system

The principle of our detection method is depicted in Fig. 1A. TaqM1 DNA polymerase was used here. The advantages of TaqM1 DNA polymerase to realize the one-step reverse transcription PCR and the cleavage of the probe with the 5′ nuclease activity were combined in our system. In the presence of the target RNA, one of the PCR primers was designed to be complimentary to a part of the RNA sequence, which could be extended by TaqM1 DNA polymerase to obtain cDNA (Step 1, Fig. 1A). The PCR amplification of cDNA could be accomplished by the same DNA polymerase. A 56 nt DNA probe 1 (green part) that is partially complimentary to the amplified sequence was introduced into the PCR reaction (Step 2, Fig. 1A). As the TaqM1 polymerase has 5′ to 3′ exonuclease activity, it will digest probe 1 from the hybridized position to leave a short DNA fragment T (Step 3, Fig. 1A). A short uncomplimentary sequence was added to the 3′ end of probe 1 intentionally to avert the undesired extension of probe 1 on cDNA caused by DNA polymerase. After PCR amplification, the released fragment T from probe 1 could hybridized to another DNA probe 2 (Step 4, Fig. 1A), which consisted of three parts from 3′ to 5′ end: the antisense sequence of T (gray), the recognition site of the nicking endonuclease (purple) and the antisense sequence of peroxidase-mimicking DNAzyme (blue). Therefore, the released short DNA sequence T could serve as a primer for mesophilic DNA polymerase to replicate the sequence of probe 2 (Step 5, Fig. 1A). As a short DNA fragment which contains the recognition site of a nicking endonuclease has been designed into probe 2, the cleavage of the double strand DNA product by the nicking enzyme could generate a nick site for the initiation of extension (Step 6, Fig. 1A). The single-stranded DNAzyme sequence will be displaced when DNA polymerase carries out the extension (Step 7 and 8, Fig. 1A). The extension on the template again yields a double-stranded domain that has the recognition site for the nicking endonuclease. A nicking event would follow next. Thus, extension, cleavage and strand displacement can be repeated continuously in cycles and release a large amount of short DNAzyme sequences which could form a G-quadruplex structure. Once hemin is added into the reaction mixture after amplification, the DNAzyme could oxidize the colorless ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) into a green colored product in the presence of $H_2O_2$ (Step 9, Fig. 1A). Thus, the detection result could be read through direct observation of the color change of reaction mixture.

### 2 Colorimetric detection of HIV cDNA or RNA

In this paper, HIV RNA was used as target RNA. A specific region (gag–pol) of the HIV virus was selected as the target sequence for pathogen detection. Two PCR primers were designed to amplify the targeted sequence specifically, and one of them could serve as a prime to obtain the cDNA through reverse transcription. For the reason that the HIV RNA is not as stable as DNA, cDNA reverse transcripted from HIV RNA was used as the template for the PCR to prove the feasibility of our strategy at the beginning of our experiment. The middle domain of probe 1 was designed according to the target sequence, and 16 nt and 9 nt DNA sequences were added to the 5′ and 3′ of this domain respectively to obtain the intact probe. In order to investigate whether TaqM1 DNA polymerase could cleave probe 1 and release the segment T, the 5′ $^{32}$P-labeled probe 1 was added into the PCR reaction mixture. As expected, the PAGE analysis indicated that an 18 nt fragment T was released from probe 1 in the PCR reaction containing the target cDNA (Lane 2, Fig. 1B). Comparatively, the PCR reaction without a target sequence revealed no cleavage of probe 1 (Lane 3, Fig. 1B). When TaqM1 DNA polymerase encounters the hybridized

 

complementary duplex between the amplified target and probe 1, the 5′ to 3′ exonuclease function of the polymerase results in the cleavage of the probe after the second position of this double-stranded region, which is located at position 18 of the probe. This result is consistent with the previous research work on the 5′-nuclease activity of wildtype Taq DNA polymerases.[16,17] To realize the strand-displacement based signal amplification, Bsm DNA polymerase, which lacks both 5′ to 3′ exonuclease activity and 3′–5′ proof-reading ability and has a strong strand displacement activity, was chosen as the mesophilic DNA polymerase to replicate the DNA sequence, with the addition of the nicking enzyme Nb. Bpu10I which can recognize a specific duplex sequence and cut one strand was applied in our method. Probe 2 was built up conveniently through combining the antisense sequence of T, the recognition site of the corresponding nicking enzyme and the antisense of DNAzyme sequence. To verify whether the strand-displacement based signal amplification could process successfully or not, several isotope labeling experiments were carried out. Firstly, 5′ $^{32}$P-labeled T was proven to be successfully extended by Bsm DNA polymerase (Lane 2, Fig. 1C). The isotope labeled [α-$^{32}$P] dATP was added to the strand-displacement amplification reactions (Fig. 1A). A new band which corresponds in size to the DNAzyme appeared on the denaturing PAGE gel (Lane 3, Fig. 1C). However, this band couldn't be found in the negative control reaction where the primer T was absent (Lane 4, Fig. 1C). The following colorimetric reaction verified the accumulation of the DNAzyme from the strand-displacement amplification by adding hemin, $H_2O_2$, and ABTS (Fig. S2, ESI†).

It is important to mention that the isothermal amplification reaction occurred in the PCR buffer, which indicates that TaqM1 polymerase, Bsm polymerase and the nicking endonuclease were compatible in the same buffer. Also, we found that $Mg^{2+}$ ions play an important role in the extension and nicking reactions. To improve the catalytic efficiency of the mesophilic DNA polymerase and the nicking enzyme, the effect of the concentrations of $Mg^{2+}$ ions was investigated over the range of 0 mM to 10 mM. The green color of the final colorimetric reaction increased dramatically with the concentration of $Mg^{2+}$ over the range of 0 mM to 10 mM, and it reached a plateau at the concentration of 10 mM (Fig. S1, ESI†). Therefore, 10 mM was selected as the optimized $Mg^{2+}$ concentration in the reaction buffer for the strand-displacement amplification. After PCR and the isothermal strand-displacement amplification, hemin, ABTS, and $H_2O_2$ were added and the color signals of the different concentrations of HIV cDNA were recorded (Fig. 2). The color intensities are proportional to the concentration of the HIV cDNA in all samples. With an increasing concentration of HIV cDNA, the color of the reaction solutions presented a trend from light green to dark green, and the negative control remained colorless. As little as 600 fM target DNA molecules could be detected by the naked eye in 50 μl reaction mixture. Then different concentrations of HIV RNA isolated from blood samples (see ESI†) was used as a template for the RT-PCR and used for isothermal amplification under the optimal conditions. The color signals were also observed by the naked eye, which confirmed our expectations (Fig. S5, ESI†).

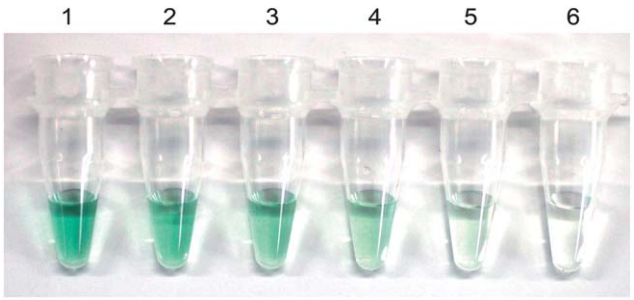


**Fig. 2** Colorimetric detection of the reaction product using different concentrations of target HIV cDNA. Tubes 1–6: containing 6 nM, 600 pM, 60 pM, 6 pM, 600 fM, 0 M of HIV cDNA respectively.

## 3 Fluorometric detection of HIV RNA with tyramine

To get better quantitative results, ABTS was substituted for tyramine because it can be oxidized by G-quadruplex DNAzyme to form a dimeric phenol which is a fluorophore that can be excited at 320 nm to produce a strong fluorescent signal at 410 nm.[24,25] After RT-PCR and isothermal strand-displacement amplification, hemin, tyramine·HCl, and $H_2O_2$ were added to the reaction mixture. A time-dependent fluorometric graph was obtained and the relationship between the concentration of HIV

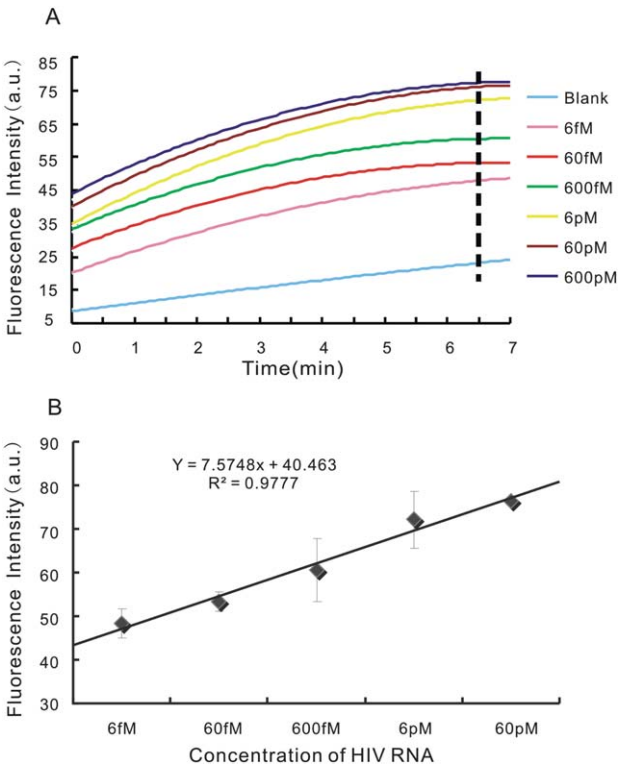


**Fig. 3** Fluorometric detection of HIV RNA. (A) Time-dependent fluorometric detection of different concentrations of target HIV RNA based on one-step RT-PCR and strand-displacement amplification. (B) Fluorescence intensities at 6.5 min were plotted against the number of HIV RNA molecules present. The solid line indicates a linear least squares fit between 6 fM and 60 pM of HIV RNA molecules, and the correlation equation is $FI = 7.5748\log(C_{RNA}) + 40.463$ ($R^2 = 0.9777$). The error bars were determined by the standard deviation (SD) of the triplicate data.

RNA and the fluorescence was studied. As shown in Fig. 3, the fluorescence was proportional to the concentration of HIV RNA over the range of 6 fM to 60 pM. The calibration curve indicates that there is a good linear relationship between fluorescence intensity and the concentration of the target molecule. The correlation equations are $FI = 7.5748\log(C_{RNA}) + 40.463$ with a correlation coefficient of 0.9777 in the range from 6 fM to 60 pM. The fluorescent signal of 6 fM HIV RNA is two times that of the negative control with no HIV RNA molecules present.

## Conclusions

In conclusion, we have demonstrated a novel colorimetric/fluorometric detection method for RNA using one-step reverse transcription PCR and strand-displacement based signal amplification. This method utilized the reverse transcriptase activity and the 5′-nuclease activity of TaqM1 DNA polymerase to transcribe target RNA into cDNA and then release a fragment from the 5′ end of a specific probe during the PCR process. The released fragment triggered the following signal amplification though a strand-displacement amplification to release a large amount of G-quadruplex DNAzymes, which could bind hemin and catalyze the oxidation of $ABTS^{2-}$ or tyramine·HCl with $H_2O_2$. As the TaqM1 polymerase has similar polymerization and 5′–3′ exonuclease activities as the Taq wild-type DNA polymerase, our method can be used not only for the detection of RNA molecules, but also for DNA targets. We have verified our method in the real application through successful detection of the HIV virus from blood samples. The colorimetric RNA pathogen detection based on our method could be carried out in a setting-limited laboratory, as a normal PCR machine is the minimal requirement for the assay. Additionally, more accurate quantitative measurements could be obtained through a fluorometric method by the aid of a microplate reader or a fluorometer.



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