Journal of Molecular Diagnostics, Vol. 9, No. 5, November 2007

DOI: 10.2353/jmoldx.2007.070012

Development of a Novel One-Tube Isothermal Reverse Transcription Thermophilic Helicase-Dependent Amplification Platform for Rapid RNA Detection

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The high complexity and cost of polymerase chain reaction-based molecular diagnostics sometimes limits their use in the clinical diagnostics setting. A new helicase-based isothermal amplification method offers an alternative to standard polymerase chain reaction, allowing amplification and detection of specific DNA sequences at a constant reaction temperature without thermocycling equipment. Herein, we describe the development of a novel one-tube isothermal reverse transcription-thermophilic helicase-dependent amplification (RT-tHDA) platform for RNA target detection based on the already established tHDA system. The RT-tHDA platform is highly sensitive and specific for a variety of RNA targets tested, including purified RNA molecules, armored RNA particles, and RNA virus. Moreover, rapid one-step RT-tHDA can be achieved by inclusion of an extreme thermostable single-stranded DNA binding protein in the reaction, resulting in one millionfold amplification of Ebola virus-armored RNA in less than 10 minutes. This RT-tHDA method expands on the known methods to amplify specific RNA targets and results in an easily prepared and contained platform. ***(J Mol Diagn 2007, 9:639–644; DOI: 10.2353/jmoldx.2007.070012)***

Amplification of RNA targets is an important part of nucleic acid amplification. It is especially useful in diagnostic applications for RNA-based pathogens including RNA viruses and for monitoring the levels of gene expression, eg, dysregulation of cancer genes.[1–3] Since the copy number of mRNA transcripts of an expressed gene is generally greater than the gene itself, amplification of RNA targets may greatly enhance diagnostic sensitivity and overcome problems caused by inadequate sensitivity in some amplification reactions. Among the current RNA amplification techniques, RT-PCR is the most used one for detection of RNA targets. RT-PCR is often performed in two sequential steps. A reverse transcriptase is used in the first step to convert the RNA target to cDNA that is in turn used as the substrate for amplification in a conventional PCR catalyzed by a thermophilic DNA polymerase in the second step. RT-PCR can also be performed in one step using a single polymerase such as Tth polymerase for both reverse transcription and DNA polymerization.[4] These thermostable DNA-dependent DNA polymerases exert significant reverse transcriptase activity in appropriate reaction conditions. Amplification of RNA targets may also be performed in one temperature without temperature cycling through combination of reverse transcription and an isothermal amplification platform such as loop-mediated isothermal amplification, nucleic acid sequence-based amplification (NASBA), transcription-mediated amplification, or strand displacement amplification.[5–7]

Helicase-dependent amplification (HDA) is a unique isothermal DNA amplification technique that has been developed by using a DNA helicase enzyme to unwind double-stranded DNA. HDA amplifies target sequences delineated by two specific primers similar to PCR. Unlike the heat denaturation used in PCR, a DNA helicase is used to separate two strands of a duplex DNA. The activity of the helicase enzyme substitutes for the high temperature denaturation step present in PCR and allows isothermal DNA amplification.[8,9] HDA has been used to isothermally amplify target sequences from bacteria without prior heat denaturation.[9] Using helicase to denature the target DNA allows the HDA platform to be a truly isothermal amplification technology.[9] Thermophilic HDA (tHDA) amplifies nucleic acid targets efficiently at 65°C and requires less protein components than the mesophilic HDA platform, which is performed at 37°C. The tHDA platform has been successfully used in pathogen DNA detection with a high detection sensitivity.[8]

In this article, we expanded the tHDA platform for RNA molecule amplification and developed a one-tube isothermal reverse transcription-tHDA (RT-tHDA) platform in which a thermostable reverse transcriptase works coordinately with the tHDA platform to amplify RNA targets.

Partially supported by Small Business Innovation Research grant GM077780 and by funding from the Department of Homeland Security.

Accepted for publication August 10, 2007.

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Inclusion of an extreme thermostable single-stranded DNA-binding protein (ET SSB) in the RT-tHDA reaction dramatically increased its reaction speed and enabled the development of a one-step RT-tHDA platform for rapid detection of RNA targets.

Materials and Methods

Ebola virus-armored RNA was purchased from Asuragen, Inc. (Austin, TX), Enterovirus Clear QC Panel was purchased from Argene (North Massapequa, NY), amplification primers were purchased from Operon Biotechnologies, Inc. (Huntsville, AL), EvaGreen real-time double-stranded DNA detection dye was purchased from Biotium, Inc. (Haywood, CA), Transcriptor reverse transcriptase was purchased from Roche Applied Science (Indianapolis, IN), StrataScript 5.0 and AffinityScript reverse transcriptases were purchased from Stratagene (La Jolla, CA), and ThermoScript reverse transcriptase, Thermo-X reverse transcriptase, ROX real-time reference dye, the TOPO-TA cloning kit, and One Shot TOP10 cells were purchased from Invitrogen (Carlsbad, CA). ET SSB is commercially available at BioHelix Corp. (Beverly, MA). The purification of Tte-UvrD helicase was described previously.[8] All other enzymes were from New England Biolabs (Ipswich, MA).

Primer Design and Screening

RT-tHDA primers used in this study were designed by Primer3 program, found at *http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi*. The parameters in Primer3 were selected as follows to fit the RT-tHDA reactions. Product size ranged from 80 to 120 bp, because RT-tHDA is not efficient outside of this range. Product Tm ranged from 68 to 75°C with the optimal temperature at 71°C. Primer size ranged from 24 to 30 bases with the optimum at 27 bases. Primer Tm ranges from 62 to 74°C with the optimal temperature at 68°C. Primer GC% ranged from 30 to 60% with the optimum at 45%. At least three pairs of primers for each of the RNA targets described in this study were selected from the output of Primer3. All primer sequences were analyzed with BLASTN (*http://www.ncbi.n/m.nih.gov/BLAST/*) for specificity. The performance of these primer sets was evaluated using the one-tube RT-tHDA protocol. The primer set with the highest detection sensitivity and the least nonspecific amplification for each RNA target was then chosen. Optimization of the general RT-tHDA condition such as adjustment of the concentration of $MgSO_4$, NaCl, primers, and modification of the primers with small molecules like biotin and dinitrophenol was performed as necessary to further improve performance as labeling with these small molecules can affect the amplification efficiency of the primers.

Development of the One-Tube RT-tHDA Platform

In the one-tube RT-tHDA system, cDNA copies were generated and amplified from an RNA target concurrently using a helicase, a reverse transcriptase, and a DNA polymerase. To develop this system, a combination of buffers and enzymes were tested to determine the requirements of the one-tube protocol. In brief, a series of thermostable reverse transcriptases including ThermoScript RT, Thermo-X RT, Transcriptor RT, AffinityScript RT, and StrataScript 5.0 RT were tested in protocols performed in one tube with one or two steps. Concurrently, the reaction conditions were optimized to a novel one-tube buffer system to allow maximum performance out of all enzymes simultaneously. This involved a modification of the Thermopol II (Mg^{2+}-free) buffer (New England Biolabs) to a new buffer containing only KCl and Tris-HCl over a pH range of 8.2 to 8.8 at 25°C.

The RT-tHDA reaction is dependent on the combination of two reaction mixtures, A and B. Mixture A contained the RNA sample and 100 to 600 nmol/L of each primer in a 1× RT-tHDA annealing buffer (10 mmol/L KCl and 20 mmol/L Tris-Cl, pH 8.8, at 25°C) in a volume of 25 μl. Mixture B contained the same 1× annealing buffer as well as 8 mmol/L $MgSO_4$, 80 mmol/L NaCl, 0.8 mmol/L dNTPs, 6 mmol/L ATP, 200 ng of Tte-UvrD helicase, 20 U of Bst polymerase, and either 1.05 U of ThermoScript or 4.0 U of StrataScript reverse transcriptase in a volume of 25 μl.

Amplification was performed in either a one-step or two-step isothermal reaction. In the one-step RT-tHDA, mixtures A and B were combined and incubated at 65°C for 120 minutes. In the two-step RT-tHDA reaction, mixture A alone was placed at 65°C for 3 minutes to decrease the nonspecific amplification and then placed on ice to cool. After cooling, mixture B is mixed with mixture A, and the complete reaction is placed at 65°C for 120 minutes. Amplified products were separated on 2% agarose gels stained with ethidium bromide. Sensitivity of the assay was assayed based on whether a band of correct target size could be amplified by the RT-tHDA system from a specific starting amount of RNA template.

RT-tHDA Detection of Ebola Virus Armored RNA

A pair of Ebola virus (subtype Zaire) nucleoprotein gene specific primers, evF3 (5′-TTCAAAAGTCGGGCGAAAGGAGCATA-3′) and evR3 (5′-TCTTGCAAGGACCGCCAAGGTAAAAA-3′), were used in the RT-tHDA reactions with an expected amplified product size of 105 bp. RT-tHDA was performed in two steps as described above with 300 nmol/L of each primer (evF3 and evR3) and 10-fold serial dilutions of Ebola virus-armored RNA starting from 3.2×10^6 particles down to 3.2 particles per reaction using 4 U of StrataScript 5.0 Multiple Temperature reverse transcriptase. Following the incubation, 10 μl of the amplified products were separated on 2% agarose gels stained with ethidium bromide. The specificity of the amplified products from the RT-tHDA reaction was further examined by DNA sequencing. DNA fragments generated from the RT-tHDA reaction were first cloned into pCR II-TOPO using the TOPO-TA cloning kit and then transformed into One Shot TOP10 competent cells according to the manuals provided by Invitrogen. Plasmid DNAs extracted from ten transformed colonies were prescreened for the correct insert size and then sequenced.

RT-tHDA Detection of Human GAPDH Gene

A pair of human GAPDH gene-specific primers was used to amplify the exon/intron boundary region of the human *GAPDH* gene in the RT-tHDA reactions with an expected amplified product size of 95 bp. The forward primer LGAPDHF3 (biotin-5′-ACTCTGGTAAAGTGGATATTGTTGCCA-3′) is located in the third exon of human GAPDH gene, whereas the reverse primer GAPDHR3 (5′-TTTGCCATGGGTGGAATCATATTGGAA-3′) is located in the fourth exon of human *GAPDH* gene. RT-tHDA was performed in two steps as described above with 150 nmol/L of each primer of LGAPDHF3 and GAPDHR3 and 10-fold serial dilutions of human total RNA (BD Biosciences) starting from 200 ng down to 0.2 pg in Mixture A. Mixture B contained 7.0 mmol/L $MgSO_4$ rather than 8.0 mmol/L. Following the reaction, 10 μl of the amplified products were separated on 2% agarose gels stained with ethidium bromide.

RT-tHDA Detection of Enterovirus

A pair of Enterovirus-specific primers, LEVF1A (biotin-5′-TCCTCCGGCCCCTGAATGCGGCTAAT-3′) and LEVR1 (dinitrophenyl-5′-CACGGACACCCAAAGTAGTCGGTTCC-3′), which target the conserved sequences located in the 5′-untranslated region of the viral genome,[10] were used in the RT-tHDA reactions with an expected amplified product size of 116 bp. Unpurified Enterovirus samples from the Enterovirus Clear QC Panel were used as the template in the RT-tHDA reactions. The panel consists of three freeze-dried supernatant cultures quantified by the manufacturer and resuspended with pure water according to the provided instructions to yield known concentrations of virus particles per ml. RT-tHDA was performed in two steps as described above in the presence of 75 nmol/L of each primer of LEVF1A and LEVR1; 3.5 mmol/L $MgSO_4$; 2000, 200, and 20 copies of enterovirus; and 4 U of StrataScript 5.0 Multiple Temperature reverse transcriptase. Following the incubation, 10 μl of the amplified products were separated on 2% agarose gels stained with ethidium bromide.

Real-Time RT-tHDA Detection of Ebola Virus-Armored RNA

Real-time RT-tHDA was set up the same way as the one-step RT-tHDA for Ebola virus-armored RNA as described above with the addition of 2× ROX reference dye and 0.4× EvaGreen double-stranded DNA detection dye into mixture B. Real-time RT-tHDA reactions were run on 100-fold serial dilutions of Ebola virus-armored RNA as the template including 500,000, 5000, and 50 copies per reaction as well as a non-template negative control. Real-time reactions were performed on an ABI 7000 series real-time detection system (Applied Biosystems, Foster City, CA) programed for 60 cycles of 65°C for 1 hour and 55 minutes and 66°C for 5 minutes, resulting in 120 minutes at 65°C, yet allowing for calculation of Ct. Amplified products were confirmed to be correct through use of the melting curve analysis and comparison of the actual fragment size with the predicted size through agarose gel electrophoresis. Following the initial tests of sensitivity and speed using real-time RT-tHDA, the effects of ET SSB (BioHelix) were examined in the RT-tHDA system. When present, 100 ng of ET SSB was included in mixture A. The doubling time (t_d) for the RT-tHDA reaction was calculated using the real-time RT-tHDA data by comparing the time required by different starting masses to reach the uniform Ct. A simple logarithmic function allowed for calculation of t_d, which could be further used to estimate the time required for millionfold amplification.

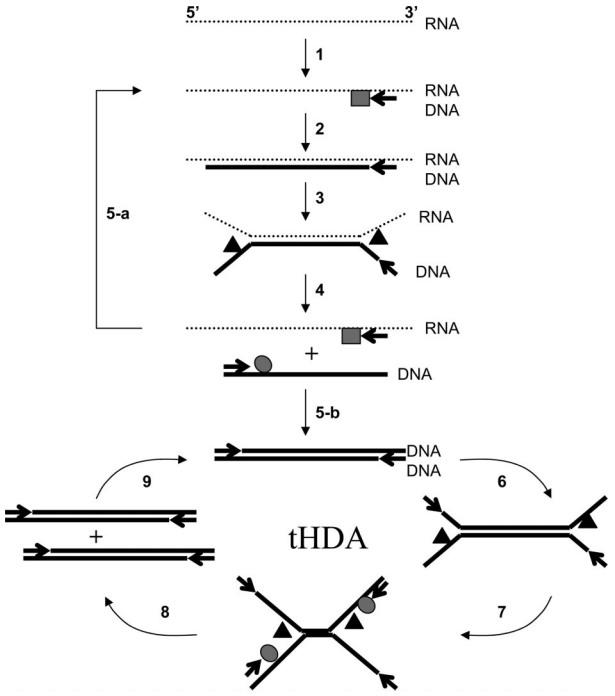

Figure 1. Schematic diagram showing the RT-tHDA reaction. The reverse transcription and amplification steps occur concurrently and independent of each other as they rely on different enzymes and nucleic acid substrates. **Arrows** indicate specific primers, **circles** indicate DNA polymerase, **squares** indicate reverse transcriptase, and **triangles** indicate helicase. The first-strand cDNA is first synthesized by a reverse transcriptase (steps 1 and 2). The RNA-DNA hybrids from the reverse transcription are then separated by UvrD helicases generating single stranded (ss) RNA and DNA templates (steps 3 and 4). The ssRNA enters next round of RT reaction (step 5-a) generating more first strand cDNA. The ssDNA was converted into double-stranded DNA by the DNA polymerase (step 5-b) and amplified concurrently in the tHDA reaction (steps 6 through 9). This process repeats itself to achieve exponential amplification of the RNA target sequence.

Results

Development of the One-Tube RT-tHDA Platform

The mechanisms underlining the one-tube RT-tHDA are described in Figure 1. In this system, cDNA copies were generated and amplified from a RNA target concurrently using a helicase, a reverse transcriptase, and a DNA polymerase. This process repeats itself to achieve exponential amplification of the RNA target sequence.

A novel one-tube buffer system was developed to support both the reverse transcription and tHDA reactions. The new buffer system was a modification of the

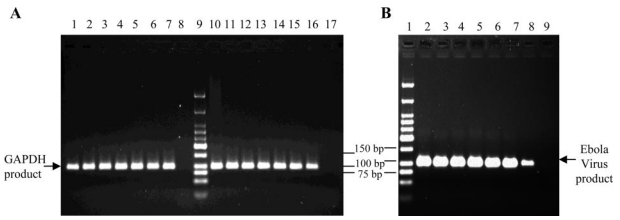

Figure 2. Development of a one-tube isothermal RT-tHDA platform with high detection sensitivity for RNA targets. The one-tube isothermal RT-tHDA was performed as described in Materials and Methods with a pair of specific primers targeting human *GAPDH* gene (**A**) or Ebola virus NP gene (**B**). Ten microliters of the 50-μl RT-tHDA products was then separated on a 2% agarose gel. **A:** Detection of human *GAPDH* gene. A ThermoScript reverse transcriptase from Invitrogen (**lanes 1** to **8**) or a StrataScript 5.0 Multiple Temperature reverse transcriptase from Stratagene (**lanes 10** to **17**) was used in the RT-tHDA experiments with 10-fold serial dilutions of human total RNA starting from 200 ng down to 0.2 pg as the template. The amounts of input human total RNA per 50-μl reaction are: **lanes 1** and **10:** 200 ng; **lanes 2** and **11:** 20 ng; **lanes 3** and **12:** 2 ng; **lanes 4**, **13**: 200 pg; **lanes 5** and **14:** 20 pg; **lanes 6** and **15:** 2 pg; **lanes 7** and **16:** 0.2 pg; and **lanes 8** and **17:** 0 pg. **Lane 9:** Two hundred-fifty nanograms of Low Molecular Weight DNA Ladder (New England Biolabs). **B:** Detection of Ebola virus-armored RNA. 10-fold serial dilutions of Ebola virus-armored RNA starting from 3.2×10^6 particles down to 3.2 particles were used as the template in the RT-tHDA experiment. The amounts of input Ebola virus-armored RNA per 50-μl reaction are: **lane 2:** 3.2×10^6 particles; **lane 3:** 3.2×10^5 particles; **lane 4:** 3.2×10^4 particles; **lane 5:** 3.2×10^3 particles; **lane 6:** 3.2×10^2 particles; **lane 7:** 3.2×10 particles; **lane 8:** 3.2 particles; and **lane 9:** 0 particle. **Lane 1:** Two hundred-fifty nanograms of Low Molecular Weight DNA Ladder (New England Biolabs).

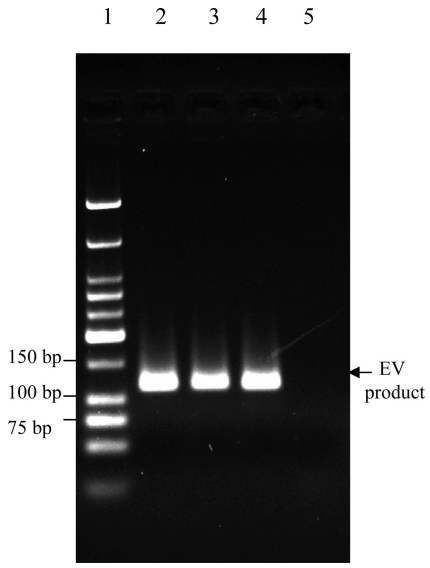

Figure 3. Detection of Enterovirus by RT-tHDA. RT-tHDA was performed by using a pair of enterovirus-specific primers and 2 μl of each of the Enterovirus Clear QC Panel (Argene) as template. Ten microliters of the 50-μl RT-HDA products was then separated on a 2% agarose gel. The amounts of input enterovirus RNA per 50-μl reaction are: **lane 2:** 2000 copies; **lane 3:** 200 copies; **lane 4:** 20 copies; and **lane 5:** 0. **Lane 1:** 250 ng of Low Molecular Weight DNA Ladder (New England Biolabs).

Thermopol II (Mg-free) buffer (New England Biolabs) by eliminating $(NH_4)_2SO_4$ and Triton X-100 that showed adverse effects on the one-tube RT-tHDA reaction in the Thermopol II buffer (data not shown). The performance of the one-tube RT-tHDA was also dependent on both the type and the amount of reverse transcriptase used in the RT-tHDA reaction. All of the commercially available thermostable reverse transcriptases tested including ThermoScript RT, Thermo-X RT, Transcriptor RT, AffinityScript RT, and StrataScript 5.0 RT worked in the one-tube RT-tHDA setting but with different efficiency. RT-tHDA worked best with ThermoScript RT in a concentration of 20 to 40 U/ml and StrataScript 5.0 RT in a concentration of 32 to 126 U/ml (data not shown).

Performance of the One-Tube RT-tHDA Platform

The performance of the one-tube RT-tHDA platform was evaluated using a variety of RNA targets as the template. Amplification of human *GAPDH* gene in the one-tube protocol was successful over a range of 200 ng down to 0.2 pg per reaction of starting total RNA using both the ThermoScript RT and the StrataScript RT (Figure 2a). The RT-tHDA one-tube protocol was also successful in the detection of Ebola virus-armored RNA particles over a range of concentrations from 3.2×10^6 down to 3.2 particles per reaction of starting mass (Figure 2b). No nonspecific amplification was observed from the RT-tHDA reactions described above. To examine further the specificity of the amplified products from the RT-tHDA reaction, DNA fragment generated from the RT-tHDA reaction of Ebola virus-armored RNA was cloned into pCR II-TOPO (Invitrogen) as described in Materials and Methods. The sequenced inserts from plasmid DNA extracted from 10 separate transformed colonies all matched the armored RNA sequence. Taken together, these two data sets show that with relatively pure RNA samples the RT-tHDA system works robustly in a broad range of input RNA template. The RT-tHDA system is also extremely sensitive with negligible production of nonspecific secondary products.

The ability of the RT-tHDA to successfully amplify real virus particles was examined through the use of Enterovirus Clear QC Panel as template as described above. The particles were used directly without the RNA purification step. These data show successful amplification sensitivity to at least 20 virus particles per reaction (Figure 3). Concentrations below this value were not tested.

Effects of SSB on One-Step RT-tHDA

To examine the effect of SSB on RT-tHDA, 100 ng of the protein was added to the reaction mixture A in the one-step protocol. The addition of an ET SSB resulted in a significant increase in amplification speed and sensitivity by the one-step reaction schemes down to 50 copies of target Ebola virus-armored RNA per reaction when measured with real-time analysis (Figure 4).

The velocity of the one-step RT-tHDA system was measured through the modification of a fluorescent dye-based real-time tHDA detection method we developed previously (W. Tang and H. Kong, unpublished data). Confirmation of amplification of the specific product was based on the dissociation curve at the end of the real-time run. Real-time RT-tHDA was performed in the same

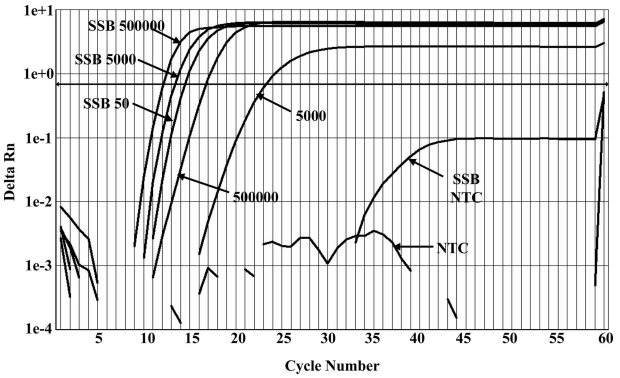

Figure 4. Amplification plot from an example of real-time RT-tHDA. The effects of SSB on RT-tHDA in a one-step protocol were evaluated. The reactions indicated are the NTC; 500,000; 5000; and 50 copies of input Ebola virus-armored RNA per 50-μl reaction with the presence of 100 ng of ET SSB, and the NTC; 500,000; and 5000 copies of input Ebola virus-armored RNA per 50-μl reaction in the absence of ET SSB. The amplification of 50 copies of Ebola virus-armored RNA per 50-μl reaction without ET SSB resulted in the formation of primer-dimers and was subsequently removed from the graph for clarity. The signal detected from the SSB-NTC is well below threshold and is the result of primer-dimer formation. NTC, no template control.

one-step manner above, with and without ET SSB present in the reaction mixtures, allowing insight as to whether ET SSB enhances specificity, velocity, or both. It was observed that in the one-step real-time procedure, the amplification curves and corresponding Ct were shifted significantly to the left indicating an increase in reaction speed (Figure 4). The magnitude of the shift does not seem to be directly correlated with the template concentration. It was further observed that sensitivity was greatest in the one-step reactions containing ET SSB, as these were the only reactions to successfully amplify 50 copies of Ebola virus target per reaction in the real-time reactions (Figure 4). The plateau phase was reached well before the end of the 2-hour amplification time in the presence of ET SSB.

The change in the velocity of the reaction due to the addition of ET SSB was calculated based on the differences in time between 500,000 and 5000 copies of input Ebola virus target to reach the threshold in the one-step reactions. It is clear that the addition of ET SSB enhances the reaction speed and has great influence on the one-step RT-tHDA reaction. The presence of ET SSB reduced t_d from 1. 06 $\pm$ 0.59 minutes to 0.37 $\pm$ 0.09* minutes and reduced the time to millionfold amplification from 21.17 $\pm$ 11.69 minutes to 7.43 $\pm$ 1.72* minutes calculated from three independent experiments (*$P \leq 0.01$).

Discussion

The need for rapid and sensitive reverse transcription-based assays is becoming increasingly necessary for current detection of mRNAs and diagnosis of RNA viral pathogens (eg, HIV, West Nile virus, and enterovirus). The development of an isothermal one-tube one-step RT-tHDA platform allows the user to perform specific RNA amplification using simple instruments such as a water bath or heating block. When the data are collected in a real-time system, the user can know the result in as little as 30 minutes. The real-time tHDA method can be used for both qualitative and quantitative analysis through examination of the dissociation curve and use of a series of known standards, respectively. A hallmark of the RT-tHDA system is the ability of the reverse transcriptase and helicase/polymerase mechanisms to work simultaneously and independently of each other (Figure 1). This allows continued linear amplification of the cDNA by the RT reaction and exponential amplification of the DNA by the tHDA reaction. This will continue to be of benefit until either the RNA template is degraded by an RNase or until exponential amplification products far exceed linearly produced cDNAs. This unique feature of RT-tHDA is based on the character of UvrD helicase, which unwinds DNA-RNA hybrids more efficiently than it does duplex DNA.[11]

Examination of the sensitivity and performance of the one-tube RT-tHDA has shown that the reaction has a limit of detection of 0.2 pg of total human RNA per reaction for GAPDH detection and 3.2 copies of Ebola virus-armored RNA per reaction (Figure 2). This indicates that the amplification reaction is highly sensitive and is capable of amplifying the target sequence even when it is present in a very low copy number. Viruses are isolated along with host nucleic acids and may not be abundant in the purified sample, particularly early in an infection; high sensitivity may lead to earlier detection and initiation of proper treatment. There is also the possibility of earlier detection of altered cellular function as abnormal cells may be low in abundance and therefore produce few detectable RNAs. Should the amplification prove sufficiently sensitive, it may be possible to detect altered cellular function at a much earlier stage.

The speed of the RT-tHDA reaction is further enhanced by single-stranded DNA binding protein (SSB). The reaction is able to perform millionfold amplification in less than 10 minutes in the one-step reaction. The ability to perform this procedure in a single step with only a water bath at the proper temperature lends itself to diagnostics whereby a product can be detected on a lateral flow strip or similar +/− detection system in under an hour after sample preparation. The use of ET SSB has also been shown to have an enhancing effect on PCR and tHDA (data not shown). ET SSB has been reported to have functions in DNA processing, repair, and duplication. It is generally believed that ET SSB functions by binding ssDNA and destabilizing DNA duplexes and recruiting polymerases to initiate duplication or repair.[12] In amplification protocols where the specific target is initially in low abundance, the addition of ET SSB would increase the probability of primers and polymerase meeting properly on the target strand, thus increasing the amplification rate early in the reaction.

Acknowledgments

We thank Melissa Millar for her contributions to primer screening of Ebola virus target. We are grateful to Dr. Hyun-Jin Kim for the purification of Tte-UvrD helicase and ET SSB. We also thank New England Biolabs for providing the enzymes and reagents used in this study.

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