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Innate Reverse Transcriptase Activity of DNA Polymerase for Isothermal RNA Direct Detection

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Supporting Information

ABSTRACT: RNA detection has become one of the most robust parts in molecular biology, medical diagnostics and drug discovery. Conventional RNA detection methods involve an extra reverse transcription step, which limits their further application for RNA rapid detection. We herein report a novel finding that *Bst* and Klenow DNA polymerases possess innate reverse transcriptase activities, so that the reverse transcription step and next amplification reaction can be combined to one step in isothermal RNA detection. We have demonstrated that *Bst* and Klenow DNA polymerases could be successfully used to reverse transcribe RNA within 125-nt length by real time RT-PCR and polyacrylamide gel electrophoresis (PAGE). Our findings will spur the development of a myriad of simple and easy to use RNA detection technologies for isothermal RNA direct detection. This will just meet the future needs of bioanalysis and clinical diagnosis to RNA rapid detection in POC settings and inspection and quarantine.

RNA not only can be the carrier of genetic information, but also the executant of life functions in living cells.[1−3] Thus, RNA as an important research target has attracted more and more attention owing to its great value in molecular biology,[4] medical diagnostics[5] and disease therapy.[6] Several common methods for RNA detection have been developed, such as reverse transcription-PCR (RT-PCR),[7−9] rolling-cycle amplification (RCA),[10,11] nucleic acid sequence-based amplification (NASBA),[12−14] modified invader amplification[15] and loop-mediated isothermal amplification (LAMP).[16,17] These methods involve an extra step of reverse transcription, in which RNA is reverse transcribed into the complementary DNA (cDNA) strand using an additional reverse transcriptase.[18] The cDNA strand serves as an initiator for next amplification reactions. This will no doubt bring inconveniences to the user in finding optimum assay conditions being permissive for all of the enzymes being used, increasing experimental costs and reaction times, adding additional steps and the probability of contamination.[19,20] These aforementioned limitations are completely incompatible with point-of-care (POC) or field use,[21] and not appropriate for the detection of inner RNA sequence in live cells.

There was a surprising discovery in our lab that DNA polymerase of Klenow fragment (3′ → 5′ exo$^-$) (Klenow exo$^-$) exhibits innate reverse transcriptase activity, which have been exploited to directly detect microRNA.[22] Motivated by our ongoing interest in applying innate reverse transcriptase activity of Klenow exo$^-$, we sought to explore the possibility of other DNA polymerases as reverse transcriptase to be used in RNA detection.

To verify if *Bst* DNA polymerase, large fragment (*Bst* LF) and Vent (exo$^-$) DNA polymerase (Vent exo$^-$) had innate reverse transcriptase activities, 50 nt hepatitis C virus (HCV) RNA was synthesized as a template (sequence listed in Supplementary Table S1). The classical AMV reverse transcriptase (AMV) was used as a positive control here. HCV RNA was reverse transcribed by Vent exo$^-$, AMV and *Bst* LF, respectively, then the reaction products were further digested by RNase H, followed by denaturing polyacrylamide gel electrophoresis (PAGE). If RNA was successfully reverse transcribed, hybrid of RNA and cDNA would generate. With addition of RNase H, RNA was digested, and only cDNA bands appeared on gel. As shown in Figure 1, there was only primer

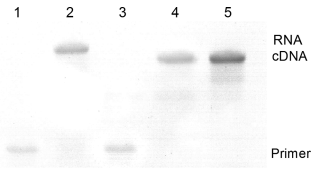

Figure 1. 17.5% denaturing PAGE of cDNAs from HCV RNA by different DNA polymerases. Lane 1: Primer; Lane 2: HCV RNA; Lanes 3, 4, and 5: Reverse transcription products from Vent exo$^-$, AMV and *Bst* LF, respectively.

band appeared in Lane 3. This result showed no cDNA generated, so Vent exo$^-$ had no reverse transcriptase activity. Noticeably, there were the same cDNA bands appeared in Lane 4 and 5. Thus, *Bst* LF possessed innate reverse transcriptase activity like AMV.

We further assess reverse transcriptase activities of a few more commonly used DNA polymerases by real-time RT-PCR using the 50 nt HCV RNA as target. The reverse transcription reaction was first performed by different enzymes at their

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optimum conditions. Then, the diluted reverse transcription products were used as template for real-time PCR. As shown in Figure 2a, when AMV, DNA polymerase I, large (Klenow)

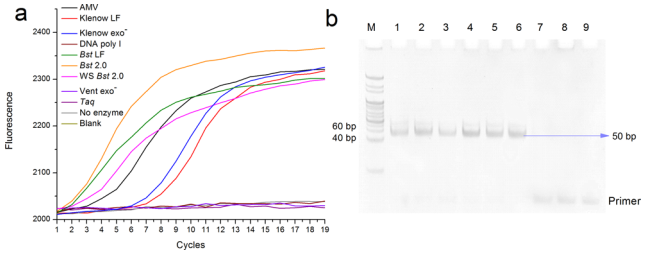

Figure 2. Verification of reverse transcriptase activities of multiple DNA polymerases. (a) Verification of reverse transcriptase activities by real-time RT-PCR. (b) Native PAGE of the RT-PCR products. Lane 1: AMV; Lane 2: Klenow exo⁻; Lane 3: Klenow LF; Lane 4: *Bst* LF; Lane 5: *Bst* 2.0; Lane 6: WS *Bst* 2.0; Lane 7: DNA poly I; Lane 8: Vent exo⁻; Lane 9: *Taq*; M: 20-bp DNA ladder.

fragment (Klenow LF) and Klenow exo⁻, *Bst* LF, *Bst* 2.0 DNA polymerase (*Bst* 2.0) and *Bst* 2.0 WarmStart DNA polymerase (WS *Bst* 2.0) were used to reverse transcribe, respectively, all of their corresponding fluorescence intensities of PCR significantly increased. This result indicated that cDNA templates for real-time PCR were produced, showing the five types of DNA polymerases like AMV possessing reverse transcriptase activities. However, no change of fluorescence intensity was observed, when DNA polymerase I (*E. coli*) (DNA poly I), Vent exo⁻ or *Taq* DNA polymerase (*Taq*) was added, which implied that these three types of DNA polymerase had no reverse transcriptase activities of their own. Surprisingly, the fluorescence signals of RT-PCR by *Bst* LF, *Bst* 2.0 and WS *Bst* 2.0 appeared earlier than that of AMV. Thus, the reverse transcriptase activities of a series of *Bst* DNA polymerase were superior to that of AMV at the tested length of 50 nt.

Native PAGE of the real time RT-PCR products was performed (Figure 2b). RT-PCR products of 50 bp, among 40- and 60-bp band of the ladder, appeared in Lanes 1−6, when AMV, Klenow exo⁻, Klenow LF, *Bst* LF, *Bst* 2.0, and WS *Bst* 2.0, respectively, were used to reverse transcribe. This result demonstrated that successful transcription of RNA generated cDNA as a template for RT-PCR, showing Klenow exo⁻, Klenow LF, *Bst* LF, *Bst* 2.0, and WS *Bst* 2.0 possessed innate reverse transcriptase activities like AMV. On the contrary, there were no RT-PCR products generated in Lane 7−9, which implied DNA Poly I, Vent exo⁻, and *Taq* could not reverse transcribe RNA to cDNA, and had no innate reverse transcriptase activities. This electrophoresis result was consistent with that of real time RT-PCR above-described.

To assess the reverse transcription ability to RNA of different lengths, we designed different primers for reverse transcribing different lengths of 16S rRNA from the extracted total RNA of *Escherichia coli* (*E. coli*) K-12. As shown in Figure 3, real time RT-PCR was performed for 65-, 125- and 262-nt RNA, respectively. The reverse transcription abilities of Klenow LF, Klenow exo⁻, *Bst* LF, *Bst* 2.0, and WS *Bst* 2.0 were comparable with that of AMV for 65-nt RNA in Figure 3a. When the target was increased to 125-nt RNA, the levels of their reverse transcription abilities were decreased to a certain extent (Figure 3b). When being increased to 262-nt RNA, their reverse transcription abilities were decreased to approximately a thousand to one of that of AMV according to increasing

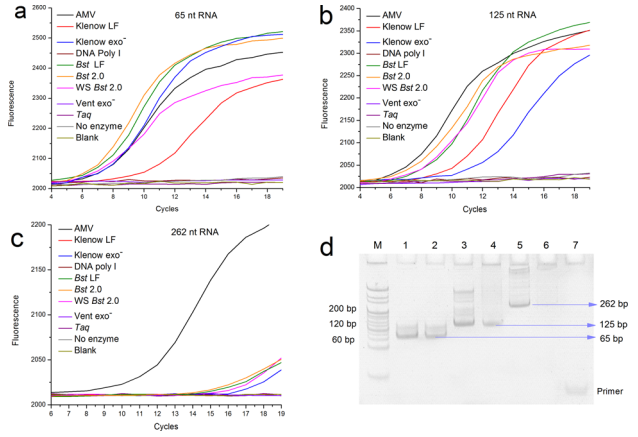

Figure 3. Real-time RT-PCR for RNA of different lengths from *E. coli* K-12 by different DNA polymerases. (a) 65 nt RNA (b) 125 nt RNA (c) 262 nt RNA (d) Native PAGE of the RT-PCR products for AMV and *Bst* LF. Lanes 1, 3, and 5: The products of RT-PCR by AMV from 65-, 125-, and 262-nt RNA, respectively; Lanes 2, 4, and 6: The products of RT-PCR by *Bst* LF from 65-, 125-, and 262-nt RNA, respectively; Lane 7: Without addition of enzyme for conversion of RNA template to cDNA that served as a target for RT-PCR; M: 20-bp DNA ladder. The primers were listed in Supplementary Table S1.

about 10 cycles for tested DNA polymerases compared with AMV when fluorescence signal appeared (Figure 3c). Also, the reverse transcriptase activities of tested DNA polymerases were decreased with the increase of the length of RNA target, and there were only low reverse transcriptase activity until 262-nt RNA template. The electrophoresis for the products of RT-PCR was consistent with the fluorescence curves (Figure 3d).The products of RT-PCR by AMV and *Bst* LF from 65-, 125-, and 262-nt RNA templates, respectively, were analyzed by native PAGE. As seen in Figure 3d, the same RT-PCR products appeared in Lane 1 and 2, and Lane 3 and 4. This result showed that the reverse transcription ability of *Bst* LF was comparable with that of AMV for 65- and 125-nt RNA templates. However, with the increase of RNA template to 262-nt, RT-PCR product was only formed in Lane 5, and weak product appeared in Lane 6. This also verified that *Bst* LF was only low reverse transcriptase activity for 262-nt RNA template. Without addition of enzyme for conversion of RNA template to cDNA that served as a target for real time PCR, there was no products in Lane 7, showing high quality of extracted RNA and the templates of real time PCR indeed were from reverse transcription of extracted RNA in Lane 1−6.

In order to verify the effectiveness of cDNA from the extracted RNA as template in the subsequent amplification experiment, 10-fold dilution of 65-nt cDNA from the conversion of the extracted RNA of *E. coli* K-12 by WS *Bst* 2.0 was detected by RT-PCR (Figure 4). The fluorescence curves showed good regularity with the increase of amount of cDNA (Figure 4a), demonstrating that WS *Bst* 2.0 could be used to reverse transcribe RNA instead of common reverse transcriptase for RT-PCR. The time corresponding to the maximum slope in the fluorescence curve as well as the point of inflection (POI) was linearly related to the negative logarithmic (lg) moles of cDNA in the tested concentration range from 10 fmol to 1 amol (Figure 4b). The correlation equation was found to be POI = −51.553−4.00547 lgA (mol) (A was the moles of cDNA, R^2 = 0.9912). Thus, the cDNA reverse transcribed from the extracted RNA by WS *Bst* 2.0 can be used

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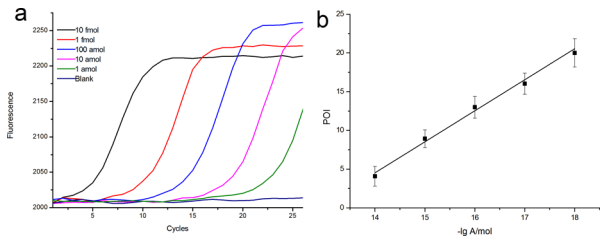

Figure 4. (a) The real-time RT-PCR for 10-fold dilution of cDNA from RNA extracted from *E. coli* K-12 by WS *Bst* 2.0. (b) The relationship between the POI values and the negative logarithmic values of the amount of cDNA (error bars showed mean standard deviation of three determinations).

as an effective template in the subsequent amplification experiments.

Bst LF, *Bst* 2.0, and WS *Bst* 2.0 work at 65 °C, and it is more beneficial for RNA detection because their thermostabilities can alleviate secondary structure of RNA,[19] compared to currently available reverse transcriptases that work at 42 °C. Especially, this will contribute to detect some important bacterial and viral targets, which can be highly structured. *Bst* DNA polymerases are currently the most common DNA polymerases in isothermal nucleic acid amplification. On the basis of *Bst* DNA polymerases, a lot of isothermal amplification methods have been developed,[23−25] in which LAMP is highly specific and highly sensitive, producing amplicons from as few as several copies targets. Therefore, more RNA detection methods with high specificity and sensitivity would successively be developed according to our findings.

Rapid detection of RNA, especially from various infectious agents such as Ebola virus, will be very beneficial for inspection and quarantine. Available methods of RNA detection usually involve an extra inverse transcription step,[26,27] which does not meet the future needs for time-consuming. Our findings can make the inverse transcription and amplification occur together, realizing single enzyme and one-pot for RNA detection, which shorten reaction time and lower experimental cost. Therefore, our findings will spur the development of a myriad of RNA detection technologies to meet the demand of RNA rapid detection to aid clinical diagnosis and the detection of specific nucleic acid sequences for understanding fundamental biology.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b08144.

Experimental details and data. (PDF)

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Notes

The authors declare no competing financial interest.

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