Substrate Specificity of Taq Polymerase

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Abstract:

Reverse Transcriptase Polymerase chain reaction (RT-PCR) is widely used to detect the presence of RNA based pathogens as well as other tasks that require converting RNA to DNA. In general, the RT-PCR reaction is inefficient and time consuming due to the requirement of having to use a two enzyme system consisting of both a heat resistant polymerase and a heat sensitive reverse transcriptase. The reverse transcriptase is always denatured and can never be recycled. A comprehensive study was carried out to investigate the effect of modifying reaction conditions in a PCR mix to hopefully achieve RT-PCR from Taq polymerase alone. A control RT-PCR was setup where template consisted of either RNA, DNA or both and the same set of primers would amplify either a 840bp gDNA fragment or 724/586 bp mRNA coding sequences which lacked the introns as the primers were coded in the flanking exons. Having a way to determine if RNA is being reverse transcribed and subsequently amplified is thus distinguished from gDNA amplification by fragment size. Positive and negative controls using a standard RT-PCR mix showed this to be the case and so the project headed towards modifying reactions conditions of normal PCR using a recombinant Taq so as to get it to reverse transcribe and subsequently amplify RNA.

Introduction:

•Enzymes are nano-machines of nature. They are usually made up of proteins which are made up of amino acids however they can also be made up of certain nucleic acids such as the ribozyme which is an enzyme made up of both protein and RNA (1).

•In general a single enzyme will catalyze a single reaction for a single or very limited number of substrates. The precise substrate chosen by the enzyme and the exact chemical reaction carried out will depend heavily on the enzyme but also the reaction conditions that the enzyme is facing at the time of the reaction (2).

•The activity of the enzyme depends on many factors. Among these factors are substrate concentration, enzyme amount, temperature, presence or absence of co-factors. For instance if the substrate is very scarce then the enzyme can only turnover what is available which means activity will be limited. In contrast, if the substrate is abundant then the enzyme is saturated with an unlimited number of substrate molecules waiting to be turned over into product and activity is maximized (3).

•Different cofactors like metal ions such as magnesium and manganese as well as calcium play important roles in stabilizing specific enzymes so that catalysis can take place (2).

•Not all DNA polymerases are limited to using DNA as a template; unlike the reverse transcriptases which strictly use RNA as a template to produce DNA it was hypothesized that the standard DNA polymerases may have a degree of reverse transcriptase activity associated with some of them. This was discovered as early as 1973 when Dr. Loeb reported that *E.coli* DNA polymerase 1 had *in vitro* reverse transcriptase activity.

•Following that discovery Jones & Foulkes in 1989 (4) and Tse & Forget in 1990 (5) also reported that *Thermus aquaticus* (Taq) polymerase also had *in vitro* reverse transcriptase activity. Recently, another report also showed that the genetically modified DNA polymerase can act as DNA polymerase, RNA polymerase and reverse transcriptase (6).

•In this study we will attempt to test the hypothesis that by altering the reaction conditions we will be able to alter the template specificity of a recombinant Taq DNA polymerase.

Materials & Methods:

•Human Embryonic Kidney 293 cell line (HEK 293) was used in this experiment. Cells were maintained as monolayer cultures and incubated in a water-jacketed incubator (Fisher Scientific, Pittsburgh PA) at 37° C with 96% humidity and 5% CO_2. The cell culture medium contains Minimum Essential Medium (MEM) (Invitrogen Corp., Gibco) and 5~10% (v/v) Fetal Bovine Serum (FBS, PAA Laboratories Inc.).

•The isolation of genomic DNA (gDNA) and total RNA were performed using Norgen RNA/ DNA/ Protein Isolation Kit. The procedures for isolating RNA and gDNA is illustrated in Figure 1. The gDNA was then treated with 10 units of RNase T1 at room temperature for 30 min and further purified using Norgen PCR Clean Up Kit. The integrity of purified DNA samples were examined by 1.2% agarose gel (Figure 2. A). The total RNA was treated with 10 units of DNase (RNase-free) at room temperature for 30 min and further purified using Norgen Total RNA Isolation Kit. The integrity of purified RNA samples were examined by 1.5% formaldehyde agarose gel (Figure 2. B).

•A homemade recombinant Taq polymerase (designated as NorTaq) was used in this study.

•In this study, the amplification of human adenovirus 5 (Ad5) early region 1 (E1) gDNA and complementary DNA (cDNA) was investigated as HEK 293 cell line consistently expresses this gene. The sequence of the Ad5 E1A forward primer is 5'-ACACCG-GGACTGAAAATGAG-3'; the sequence of the Ad5 E1A reverse primer is 5'-AAGGACCGGAGTCACAGCTA-3'. The amplification of E1A gDNA will give an 840 bp band and the amplification of E1A cDNA will give bands of 724, 586, 507, 369, and 249 bp. The splicing patterns of Ad5 E1A and the locations of the primers is shown in Figure 3.

•Reverse transcription (RT) reactions were performed using pure DNA samples, pure RNA samples, or DNA/RNA mixture as templates. For an RT reaction, 5.0 uL of gDNA, 5.0 uL of RNA, or 2.5uL gDNA plus 2.5uL RNA was added. The reverse transcriptase used in this study is SuperScript II (SS2, Invitrogen). The RT reactions were carried out with or without 0.5 uL of SS2, and with or without 0.5 uL of NorTaq as controls, as shown in Figure 4. The RT reaction was carried out at 25° C for 5 min, 42° C for 1.5 h, and 70° C for 15 min.

•All the PCR reactions were performed using the iCycler PCR machine (Bio-Rad). The gDNA/ RNA/ cDNA template was amplified at 94°C for 3 min. The heat cycle involved the annealing at 58° C for 30 sec, the extension of 72°C for 90 sec, and the denaturation at 94°C for 30 sec, with 30 cycles. The reaction was held at 72°C for 5 min followed by incubation at 4°C.

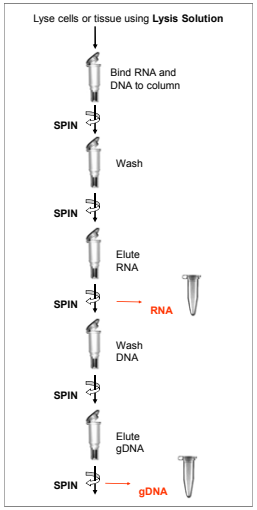

Figure 1. Illustration of total RNA and gDNA isolation procedure.

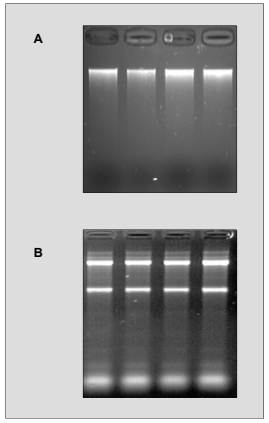

Figure 2. Integrity of gDNA and RNA templates. A) The integrity of gDNA treated with RNase was examined on a 1.2% agarose gel. B) The integrity of total RNA treated with DNase was examined on a 1.5% formaldehyde agarose gel.

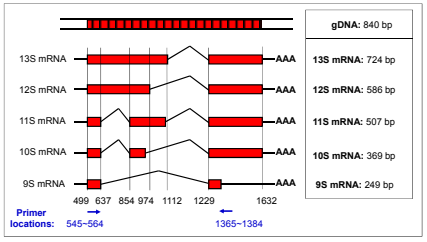

Figure 3. Splicing patterns of Ad5 E1A and the primer locations. The exons are represented as red rectangles, the introns as carets, and the poly (A) signal shown as "AAA". The numbers of amino acids of the protein fragments are indicated above the red rectangles. The forward primer is located at 545-564 bp and the reverse primer is located at 1365-1384 bp of Ad5 sequence. The PCR of gDNA should generate an 840bp band, and the PCR of cDNA should generate five bands. The sizes of the expected PCR products are shown to the right of the splicing map.

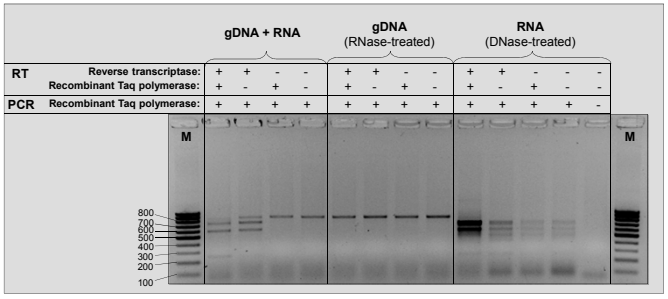

Figure 4. The PCR amplification results. For a 20 uL-RT reaction system, 5.0 uL of gDNA, 5.0 uL of RNA, or 2.5 uL of gDNA plus 2.5 uL of RNA was added. The RT reactions were carried out with or without SuperScript II (SS2), with or without NorTaq, as indicated above the lanes. Two micro-liter of each RT reaction was then amplified by PCR. All the PCR reactions were performed using recombinant NorTaq except for the last lane which contains RNA template but without NorTaq. M: Norgen PCR Sizer.

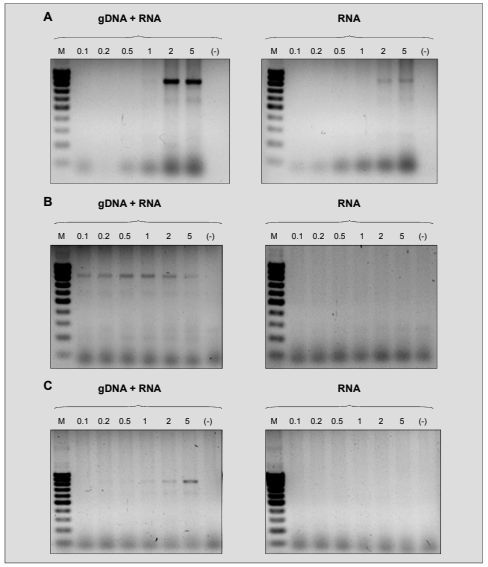

Figure 5. Effect of different reaction conditions on RNA-specific amplification. The RNA and gDNA/RNA were used for the reaction condition study. A) The effect of various amounts of NorTaq (from 0.1 to 5 uL, approximately 5,000 units/mL) on RNA-specific amplification. The adding of 2 and 5 uL of NorTaq generated some weak bands of E1A transcripts. B) The effect of various concentrations of $MgCl_2$ RNA-specific amplification. The magnesium-free PCR buffer was used and various amounts (from 0.1 to 5 uL) of 50 mM $MgCl_2$ were added to the reaction. The amplification did not show much difference with various $MgCl_2$ concentrations. C) The effect of various amounts of template RNA and DNA/RNA (from 0.1 to 5 uL) RNA-specific amplification. The band intensities increased with escalating volumes of nucleic acid template.

Conclusions:

•The PCR amplification results demonstrated the reverse transcriptase activity of the recombinant NorTaq. Noticeably, the amplification of RNA with SS2-NorTaq+ and SS2-NorTaq- generated clearly visible bands, suggesting the recombinant Taq can take RNA as the template.

•The RNA and RNA/gDNA were used for the reaction condition study in order to investigate the optimal reaction conditions of RNA-specific amplification. The band intensities increased with escalating amounts of NorTaq and DNA/RNA template. Some E1A mRNA specific bands can be observed, although these bands were faint. These results suggested that the NorTaq can amplify the RNA at a low level. It is of our further interests to carry on studies to investigate the optimal reaction conditions at which the recombinant Taq can only amplify RNA in the presence of gDNA.

•It would be also intriguing to investigate the configuration of the mRNA-specific recombinant Taq through structural biology approaches such as X-ray crystallography and nuclear magnetic resonance (NMR), which will provide detailed spatial information for site-specific mutations to generate recombinant Taq that can only amplify RNA.

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