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PCR ACCESS! A Sensitive Single-tube, Two-enzyme System for RT-PCR

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The relative advantages of three RT-PCR methods are discussed, including detection sensitivity, reverse transcription temperature, fidelity and ease-of-use. The mixture of AMV and Tfl DNA Polymerase in Promega's Access RT-PCR System gives detection sensitivity equivalent to or better than the combination of MuLV and Taq DNA polymerases. Both viral reverse transcriptase-based systems were significantly more sensitive for amplification from total RNA than the rTth-based single-enzyme system.

Introduction

Numerous techniques have been developed to measure gene expression in tissues and cells. These include Northern blots, coupled reverse transcription and PCR amplification (RT-PCR), RNase protection assays, in situ hybridization, dot blots and S1 nuclease assays. Of these methods, RT-PCR is the most sensitive and versatile. The technique can be used to determine the presence or absence of a transcript, to estimate expression levels and to clone cDNA products without the necessity of constructing and screening a cDNA library.

As originally described, RT-PCR employed avian myeloblastosis virus (AMV) or Moloney murine leukemia virus (MMLV or MuLV) reverse transcriptases for first strand cDNA synthesis. Second strand cDNA synthesis and subsequent PCR amplification was performed with *Thermus aquaticus* (*Taq*) DNA polymerase.* Numerous one-step, one- buffer RT-PCR methods have been described (1-6). However, most RT-PCR protocols recommend a first strand cDNA synthesis reaction followed by inactivation of the reverse transcriptase and dilution of the first strand reaction mixture to eliminate inhibitory effects of the reverse transcriptase upon *Taq* DNA polymerase. This necessitates secondary additions to the reaction mix (*Taq* DNA polymerase, buffer and primers), increasing both hands-on time and the likelihood of introducing contaminants into the reaction (7).

The observation that some thermostable DNA polymerases (e.g., *Thermus thermophilus* (*Tth*) DNA polymerase*) demonstrate reverse transcriptase activity in the presence of manganese led to the development of protocols for single-enzyme reverse transcription and PCR amplification (8). The first commercial products required a reverse transcription reaction in the presence of manganese ions. The manganese was then chelated by the addition of EGTA, and amplification performed in the presence of magnesium. Again, this protocol necessitates secondary additions to the reaction tube.

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A more recent development has been the use of bicine buffers containing manganese ions with *Tth* DNA polymerase (9,10). The bicine buffer allows reverse transcription and subsequent DNA amplification to be performed without intermediate additions to the reaction mixture. The presence of manganese ions during the amplification cycles may reduce the fidelity of nucleotide incorporation (11-13). Consequently, the bicine buffer system is not recommended (by Perkin-Elmer) for experiments requiring high fidelity (7).

Promega's Access RT-PCR System incorporates AMV Reverse Transcriptase (AMV RT) for first strand cDNA synthesis and *Thermus flavus* (*Tfl*) DNA Polymerase for second strand cDNA synthesis and DNA amplification. The system includes an optimized single-buffer system that permits extremely sensitive detection of RNA transcripts without a requirement for buffer additions between the reverse transcriptase and PCR amplification steps.

Detection sensitivity

The detection sensitivities of three RT-PCR methods were compared by performing equivalent reactions with three commercially available products: a MuLV/*Taq* RNA PCR Kit, *Tth* RNA PCR Kit, and the Promega Access RT-PCR System (16). Figure 1 illustrates the key differences between these protocols.

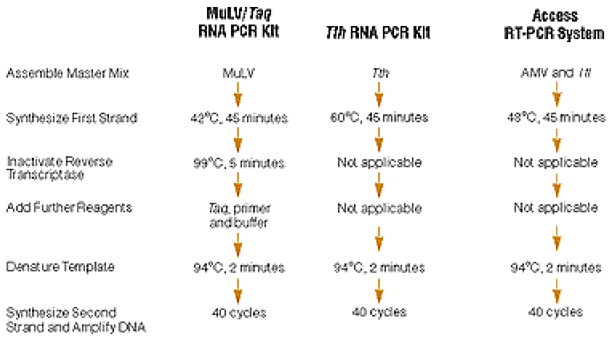
1. 

Figure 1. Outline of the RT-PCR reactions.

The two-enzyme methods using MuLV/*Taq* or AMV/*Tfl* were each 100- to 1,000-fold more sensitive than the one-enzyme *Tth* method for amplification of a target from total RNA (Figure 2). The 540bp beta-actin RT-PCR product was observed in 10pg of total RNA after 40 cycles with the MuLV/*Taq* RNA PCR Kit. The Promega Access RT-PCR System (AMV/*Tfl*) was approximately 10-fold more sensitive than the MuLV/*Taq* Kit in this experiment, detecting the beta-actin RNA in 1pg of total RNA after 40 cycles. In contrast, the limit of detection for the *Tth* RNA PCR Kit was 1,000pg of total RNA. The 540bp RT-PCR product was not observed in any of the control reactions. In addition, the beta-actin product was not observed in the reactions containing *Tfl* DNA Polymerase but no AMV RT. Thus, there is essentially no intrinsic reverse transcriptase activity associated with *Tfl* DNA polymerase under these reaction conditions. A recent study by Cusi *et al*. (17) compared the sensitivities of MuLV/*Taq* DNA polymerase and *Tth* DNA polymerase in an RT-PCR-based assay for detection of viral load. The authors reported that the combination of MuLV reverse transcriptase and *Taq* DNA polymerase was approximately 1,000-fold more sensitive than *Tth* DNA polymerase alone. Our observations using a mouse beta-actin target are consistent with their finding.

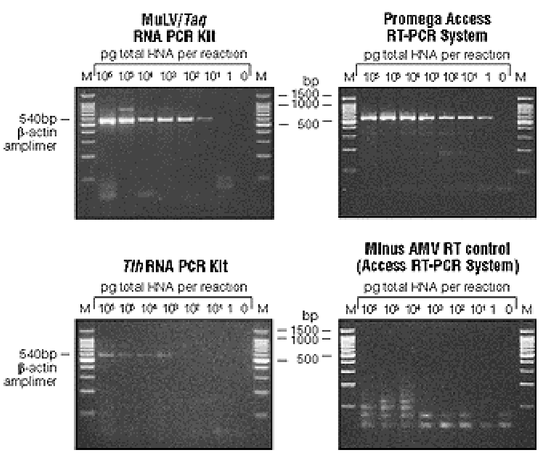
1. 

Figure 2. RT-PCR amplification of mouse beta-actin from total liver RNA. Total RNA was isolated from mouse liver using Promega's RNAgents® Total RNA Isolation System (Cat.# Z5110) as described in the instructions for use (14). The RNA preparation was treated with RQ1 RNase-Free DNase (1u/µg RNA) (Cat.# M6101) for 45 minutes at 37°C to eliminate residual contaminating DNA, then re-purified by phenol:chloroform extraction and ethanol precipitation. Serial 10-fold dilutions of the RNA were prepared in nuclease-free water. RT-PCR reactions containing the indicated amounts of total RNA were performed using the MuLV/*Taq* RNA PCR Kit, *Tth* RNA PCR Kit or Promega Access RT-PCR System as described in the manufacturer's protocol (7,15,16, Figure 1) using oligonucleotide primers complementary to the mouse beta-actin transcript. To demonstrate that the Access RT-PCR reverse transcription reaction was catalyzed by AMV RT, and not by reverse transcriptase activity associated with *Tfl* DNA Polymerase, an additional set of Access RT-PCR reactions were performed without the addition of AMV RT. Amplification of the newly synthesized first strand cDNA was performed for 40 cycles in a Perkin-Elmer Thermal Cycler 480. All amplification reactions were performed in the same thermal cycler to minimize experimental variation. Equivalent aliquots of each amplification reaction were separated on a 3% NuSieve®/1% agarose gel in 1X TAE buffer containing 0.5µg/ml ethidium bromide. The gels were electrophoresed for 3 hours at 100V and photographed. Lanes M, Promega's 100bp DNA Ladder (Cat.# G2101).

In contrast to the more complex total RNA experiment described above, all three RT-PCR systems showed approximately equivalent sensitivity (using <100 molecules of starting material) with an *in vitro*-synthesized IL-1alpha-transcript (Figure 3). In this experiment, the Promega Access RT-PCR System generated slightly more product than the *Tth* or the MuLV/*Taq* combination. The control reactions generated detectable PCR product from less than 10^4 molecules of RNA.

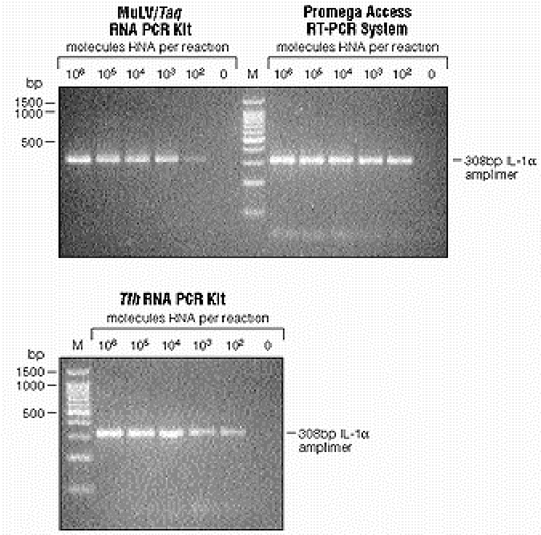
1. 

Figure 3. RT-PCR amplification of an *in vitro*-transcribed IL-1alpha RNA transcript. The IL-1alpha RNA is the control provided by Perkin-Elmer (15, 7). Serial 10-fold dilutions of the RNA were prepared in nuclease-free water. RT-PCR reactions containing the indicated amounts of RNA were performed using the MuLV/*Taq* PCR Kit (15), *Tth* RNA PCR Kit (7) or the Promega Access RT-PCR System (16) as described in the manufacturer's protocols (Figure 1) using the control oligoncleotide primers provided by Perkin-Elmer. Amplification of the newly synthesized first strand cDNA and gel analysis were performed as described in Figure 2. Lanes M, Promega's100bp DNA Ladder (Cat.# G2101).

Choice of enzyme systems

A number of factors should be considered when selecting the optimal system for RT-PCR. Important considerations include the optimal temperature for reverse transcription, the level of sensitivity re-quired, the downstream applications for the PCR product, and ease of use.

RNA transcripts exhibiting significant secondary structure must be denatured for efficient reverse transcription (18-23). Viral reverse transcriptases (e.g., MuLV and AMV) are inactivated at elevated temperatures; therefore, the first strand synthesis reactions must be performed at 37°-48°C. (The maximum recommended reaction temperature for MuLV is 42°C.) AMV RT, however, is active at 48°C in our optimized buffer conditions. Thus, the use of AMV RT eliminates many problems associated with secondary structure.

Thermostable DNA polymerases exhibiting intrinsic reverse transcriptase activity permit first strand cDNA synthesis to be performed at elevated temperatures (60°-70°C), which is advantageous for eliminating secondary structure. At the reduced temperatures required for random primer-initiated first strand synthesis (7), however, thermostable enzymes exhibit minimal activity and the AMV and MuLV reverse transcriptases are recommended instead.

Recent experimental evidence demonstrates that the viral reverse transciptases offer greater sensitivity than *Tth* DNA polymerase (17). Our experiments confirmed that AMV RT and MuLV RT were approximately 1,000-fold more sensitive than *Tth* DNA polymerase when amplifying limited amounts of target from total RNA (Figure 2). The *Tth* reverse transcriptase requirement for manganese also may result in reduced enzyme fidelity (11-13). Therefore, use of either MuLV or AMV reverse transcriptase is recommended when fidelity is a critical factor (e.g., when the amplified DNA is to be used for cloning) (7).

Single buffer systems, such as Promega's optimized AMV/*Tfl* reaction buffer and Perkin-Elmer's bicine buffer provide added convenience. They eliminate the requirement for opening the reaction vessel between steps, reducing the potential for sample contamination and the hands-on time required. Once the reaction cocktail is assembled, the thermal cycler can be programmed for the cDNA synthesis and DNA amplification steps.

Summary

The mixture of AMV RT and *Tth* DNA Polymerase provided in Promega's Access RT-PCR System gives sensitivity equivalent to or better than the combination of MuLV RT and *Taq* DNA polymerase. Both systems successfully generated a 540bp beta-actin PCR product from less than 10pg of total RNA. These data confirm previous reports (17): the viral reverse transcriptase-based systems were significantly more sensitive than the *Tth*-based system. The Promega Access RT-PCR System is optimized to permit reverse transcription and second strand cDNA synthesis/amplification reactions in a single tube with no intermediate additions to the reaction mix. This approach reduces the possibility of introducing contaminants and requires less hands-on time than other published protocols.

References

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Ordering Information

Product	Size	Cat.#
Access RT-PCR System	100 reactions	A1250
