

Using the Access RT-PCR System: Reaction Parameters That Affect Efficient Amplification



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The analysis of RNA using amplification-based methodologies is a fundamental technique used in many research and diagnostic applications. While the isolation of high-quality RNA is fundamental to the success of RT-PCR^(a), a variety of reaction parameters can profoundly affect efficient amplification of target sequences. This article demonstrates how titration of several amplification reaction components can affect amplification of endogenous RNA targets using the Access RT-PCR System^(a).

INTRODUCTION

Coupled reverse transcription and PCR (RT-PCR) amplification is an extremely sensitive and versatile technique that can be utilized for many applications. In this technique, RNA is isolated and reverse transcribed to cDNA that is subsequently amplified by PCR, a procedure that previously required two separate reaction steps. RT-PCR has enabled a variety of research and diagnostic methodologies for detection of specific transcripts, for estimation of the relative expression levels of a gene of interest or to amplify cDNA products for use in applications such as cloning and in vitro translation. Promega's Access RT-PCR System facilitates RT-PCR by combining Avian Myeloblastosis Virus Reverse Transcriptase (AMV RT) and *Thermus flavus* (*Tfl*) DNA Polymerase^(a) with an optimized reaction buffer system that enables both the synthesis and amplification of cDNA products in a single-tube, single-step reaction (1,2).

In addition to the convenience of a one-tube reaction format, Promega's Access RT-PCR System has been designed for robust performance. Even with a robust system it is important to understand the many factors that can affect RT-PCR results. Inappropriate sample handling, processing or storage prior to or during RNA isolation can lead to compromised RNA integrity and decreased performance in any RNA analysis technique (3,4). In addition, a number of amplification-specific reaction parameters can greatly affect amplification results, including primer choice, cycling parameters, reagent concentrations and the presence of inhibitory agents in the amplification reaction. This report demonstrates that varying concentrations of key RT-PCR components can influence the outcome of RT-PCR and hence the production of desired amplification products. Other parameters that can adversely affect amplification, such as incomplete mixing of reaction components and the introduction of genomic DNA or ethanol into RT-PCR, are also examined.

TITRATION OF REACTION COMPONENTS

Mouse total RNA was isolated using Promega's SV Total RNA Isolation System^(b) (Cat.# Z3100) and used as an amplification target in these experiments. Amplification reactions using an IL-1 β primer pair (5) and reaction conditions described in the legend to Figure 1 gave rise to products of approximately 600bp (major band) and 400bp (minor band). Total RNA input was positively correlated with amplification signal; no signal was detected in the absence of input RNA (lane 5). Based on these results for target RNA and oligonucleotide primers, five nanograms of total RNA was used in all subsequent experiments. The amplification conditions used generated an approximately 1.2kb product from mouse genomic DNA (lane 4) but did not amplify human genomic DNA (data not shown).

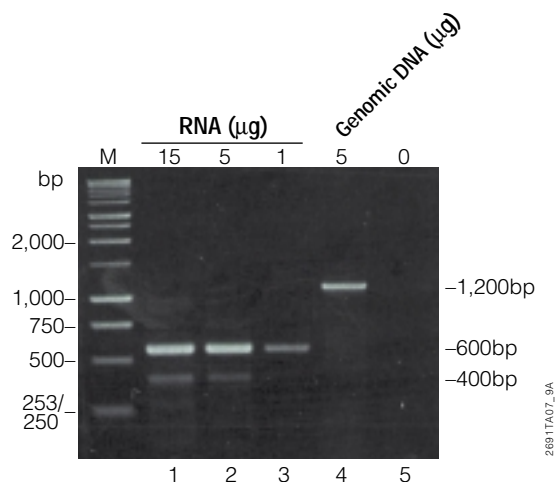


Figure 1. Amplification of mouse RNA and DNA using the Access RT-PCR System. Total RNA was isolated using Promega's SV Total RNA Isolation System (Cat.# Z3100) and amplified using the Access RT-PCR System, following the protocol provided with the system. A specific IL-1 β primer pair (5) was used for amplification in Figures 1–8. For all figures, magnesium concentration was 1mM (except as noted in Figure 3) and dNTP concentration was 200 μ M (except as noted in Figure 4). Reverse transcription was performed for 45 minutes at 48°C. The cDNA amplification program consisted of an initial denaturation step (94°C for 2 minutes) followed by 35 amplification cycles of denaturation (94°C for 30 seconds), annealing (60°C for 1 minute) and extension (68°C for 7 minutes) and a soak at 4°C. Equivalent amounts of each amplification reaction were analyzed by 1.5% agarose gel electrophoresis for all figures. Lane M, Promega's 1kb DNA Ladder (Cat.# G5711) was used in Figures 1–5. In Figure 1, lanes 1–3, mouse total RNA (15, 5 and 1ng, respectively) was amplified; lane 4, 5ng of mouse genomic DNA was amplified; lane 5, no-template control.

OLIGONUCLEOTIDE PRIMERS

Oligonucleotide primers, usually 15–30 nucleotides in length and homologous to regions flanking the target sequence, are used to specifically direct the polymerase to the target nucleic acids for amplification. The final concentration of each primer in a standard amplification reaction is usually 50pmol (1 μ M). However, the final primer concentration should be optimized for each template/primer pair combination to maximize specific signal while minimizing undesirable or nonspecific amplification. Figure 2 demonstrates how the final concentration of primers in RT-PCR can affect amplification. While an increase in amplification product is observed when up to 100pmol of oligonucleotides are used, higher oligonucleotide concentrations led to diminished RT-PCR performance (Figure 2). For a discussion of other considerations related to primer design, including base composition and calculation of oligonucleotide melting temperature (T_m), see reference 6.

MAGNESIUM CONCENTRATION

Divalent cations in the form of magnesium are a requirement for the enzymatic activity of AMV RT and *Tfi* DNA Polymerase present in the Access RT-PCR System. While typical magnesium concentrations of 1.0–3.0mM are required for maximal amplification, the optimal magnesium concentration should be determined empirically for each template and primer pair. Figure 3 demonstrates the considerable effect that suboptimal magnesium concentrations can have on RT-PCR. The inclusion of 2mM magnesium sulfate in the amplification reaction led to a marked decrease in signal intensity, and higher concentrations completely eliminated the specific product and led to an accumulation of shortened amplification products. The sensitivity of the amplification reaction to suboptimal magnesium concentrations may be different for other targets, depending on the nature and abundance of the target as well as the concentration of other reaction components.

dNTP CONCENTRATION

Deoxyribonucleotides (dNTPs) are a required reactant for AMV RT and *Tfi* DNA Polymerase-mediated amplification. While a final dNTP concentration of 200 μ M each is commonly used in amplification protocols, titration of dNTPs was performed to determine how the concentration of this reagent affected RT-PCR. As shown in Figure 4, an increase in amplification signal was detected when 200 μ M dNTPs were included in the reaction. However, increasing the final concentration of dNTPs to 600 μ M led to a significant decrease in amplification efficiency. dNTP concentrations above 600 μ M led to a complete loss of signal, presumably due to dilution of magnesium ions. Therefore, the final dNTP concentration in RT-PCR should be maintained at a maximum of 200 μ M.

The Access RT-PCR System uses 5 units each of AMV-RT and *Tfi* DNA Polymerase for cDNA synthesis and amplification, respectively. Figure 5 demonstrates the effect of varying the AMV-RT and *Tfi* DNA Polymerase concentrations on RT-PCR. While the addition of reduced amounts of AMV-RT had little effect on amplification, the addition of excess AMV-RT inhibited amplification to the extent that products were not detectable. Maximal signal was obtained when 5 units of *Tfi* DNA Polymerase was used in the reaction. The addition of both lesser and greater amounts of the polymerase resulted in suboptimal amplification of the target. These results are consistent with previously published data showing inhibitory effects of reverse transcriptases on *Taq* DNA Polymerase^(a) (7,8).

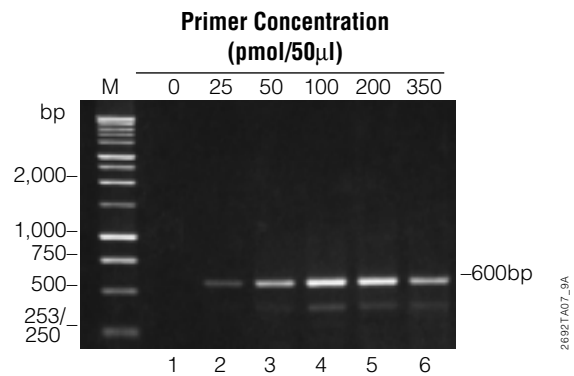


Figure 2. Titration of amplification primers (0–350pmol each) in RT-PCR. Total mouse RNA was amplified as in Figure 1 except the final concentration of oligonucleotide primers was altered. Lanes 1–6, IL-1 β primers were used at the final concentrations indicated.

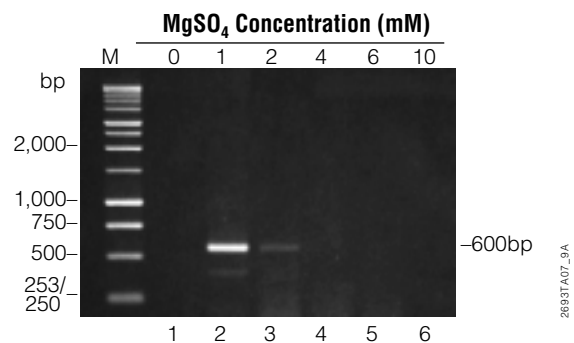


Figure 3. Titration of magnesium sulfate (0–10mM final) in RT-PCR. Total mouse RNA was amplified as in Figure 1, except the final concentration of magnesium sulfate was varied. For lanes 1–6, the indicated final concentrations of magnesium sulfate were included in the reaction.

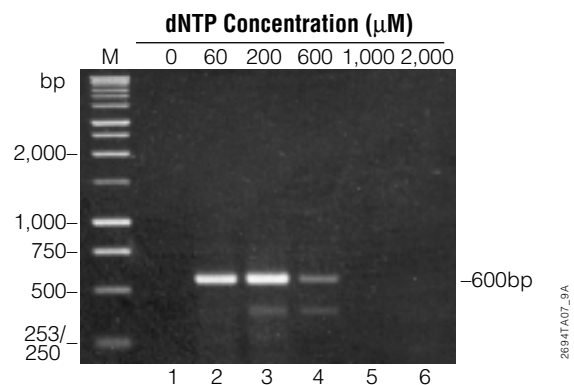


Figure 4. Titration of dNTP concentration (0–2mM final) in RT-PCR. Total mouse RNA was amplified as in Figure 1 except the final concentration of dNTPs was altered. Lanes 1–6, the indicated final concentration of dNTPs was included in the reaction.

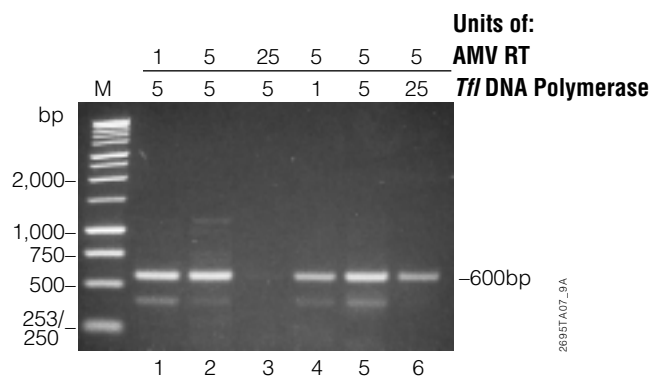


Figure 5. Titration of enzyme concentration in RT-PCR. Total mouse RNA was amplified as in Figure 1 except the units of the enzymes were altered. Lanes 1–6, the indicated final units of AMV Reverse Transcriptase and *T7* DNA Polymerase were included in the reaction.



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Ordering Information

Product	Size	Cat. #
Access RT-PCR System	100 reactions	A1250
	500 reactions	A1280
Access RT-PCR Introductory System	20 reactions	A1260

Related Products

Product	Size	Cat. #
AMV Reverse Transcriptase	300 units	M5101
	1,000 units	M5108
	600 units	M9004
<i>T7</i> DNA Polymerase	100 units	M1941
	1,000 units	M1945
<i>Taq</i> DNA Polymerase in Buffer B	100 units	M1661
	500 units	M1665
	2,500 units	M1668
SV Total RNA Isolation System	50 preps	Z3100
SV Total RNA Isolation System, Trial Size	10 preps	Z3101

CONCLUSIONS

Component concentrations of the Access RT-PCR System were titrated to show how each affected amplification of an RNA target from mouse total RNA. The Access RT-PCR System provides robust performance over a wide range of reaction parameters, but reactant conditions such as the final magnesium and primer concentration should be optimized for each primer and template combination. The addition of increased amounts of components such as dNTPs and enzyme should be avoided, as this can lead to suboptimal amplification. We recommend starting with a high-quality RNA sample that does not contain contaminants such as ethanol and genomic DNA (data not shown; see **Note** below).

Note: For the full version of this article, which includes the effects of contamination by ethanol and genomic DNA on RT-PCR, please visit: www.promega.com/enotes/.

REFERENCES

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^(a)The PCR process is covered by patents issued and applicable in certain countries. Promega does not encourage or support the unauthorized or unlicensed use of the PCR process. Use of this product is recommended for persons that either have a license to perform PCR or are not required to obtain a license.

^(b)Patent Pending.