Sigma's New Enhanced Avian RT-PCR Kit

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Introduction

Reverse transcriptase-PCR (RT-PCR) is a powerful tool for gene expression analysis at the RNA level, primarily due to its sensitivity, speed, ease of use, and versatility. In a typical RT-PCR procedure, a reverse transcriptase is used to generate a complementary DNA (cDNA) copy of RNA molecules, followed by polymerase chain reaction (PCR) to amplify the cDNA. Since the PCR process part is usually robust and generic, performance of a given RT-PCR system is mainly determined by the activity of the reverse transcriptase used.

There are several limiting factors in the current RT-PCR technology. One of those limitations is the uncertainty of transcribing rare mRNA[1] and/or RNAs with difficult complex structures. This is the primary reason for RT-PCR failure.

To address this issue, we have developed a new and improved RT-PCR system, which has superior performance in detecting rare and difficult complex RNA transcripts. The major feature of this system is an enhanced avian myeloblastosis virus (eAMV) enzyme that has a high processivity and tolerance for elevated temperature. These features enable eAMV to transcribe low-abundance RNA and/or RNAs with difficult complex secondary structures more efficiently than all other commercially available reverse transcriptases, and therefore, increase overall success of expression analysis based on RT-PCR.

In this report, we compare eAMV and other commercially available reverse transcriptases with emphasis on sensitivity for low abundance mRNA and for RNA with complex secondary structure.

Materials and Methods

All materials were supplied by Sigma Chemical (St. Louis, MO) unless otherwise stated.

RNA templates

Three sources of RNA used in this study were: total and poly(A)$^+$ RNA from human HeLa cells, total RNA from mouse submaxillary gland, and RNA from tobacco mosaic virus (TMV). The HeLa cell poly(A)$^+$ RNA and TMV RNA were obtained from commercial sources, whereas the mouse submaxillary RNA was isolated using TRI Reagent® (Product Code: T 9424).

RT-PCR conditions

Most experiments were conducted using the standard two tube-two step method. In this protocol, RT was performed in a 20 µl reaction containing 1x RT buffer (50 mM Tris-HCl, pH 8.3, 40 mM KCl, 8 mM $MgCl_2$, 1 mM DTT), 0.5 mM each of dNTP, 3.5 µM anchored oligo$(dT)_{23}$ or 1 µM gene specific primers, and 5 units of eAMV, at 42 – 50 °C for 45 minutes. After RT, a 2 µl aliquot of the reaction was added to 48 µl of PCR master mix. The mix contained 1x PCR buffer (50 mM Tris at pH 9.3, 15 mM ammonium sulfate, 2.5 mM $MgCl_2$, and 0.1% Tween® 20), 200 µM each of dNTP, 400 nM each of gene specific primers, and 2.5 units of AccuTaq™ LA (Product Code: D 8045) or *Taq* polymerase (Product Code: D 1806). PCR was performed using the following profile: 94 °C for 3 minutes; 30 to 35 cycles at 94 °C, 45 seconds; 62 °C, 45 seconds; 68 °C, 2-5 minutes (depending on amplicon size); and a final extension at 68 °C for 7 minutes.

In the one tube-one step procedure, all components for RT and PCR were assembled in 50 µl reactions containing 1x buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 3 mM $MgCl_2$, 200 µM each of dNTP, 20 units of RNase inhibitor, 1 µM each of gene specific primers, template RNA, 5 units of eAMV, and 2.5 units of AccuTaq LA. RT-PCR was conducted using the following profile: 42-50 °C for 45 minutes (for RT), 94 °C for 3 minutes, then 30 to 35 cycles at 94 °C for 45 seconds; 62 °C for 45 seconds; 68 °C for 2-5 minutes (depending on amplicon size); and a final extension at 68 °C for 5 minutes.

In the one tube-two step procedure, the RT reaction was performed as in the two tube-two step protocol. Accordingly, 30 µl of 1x PCR master mix (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1 µM each of gene specific primers) was added to the entire RT reaction and PCR was performed using the same profile as in the two tube-two step protocol.

Results and Discussion

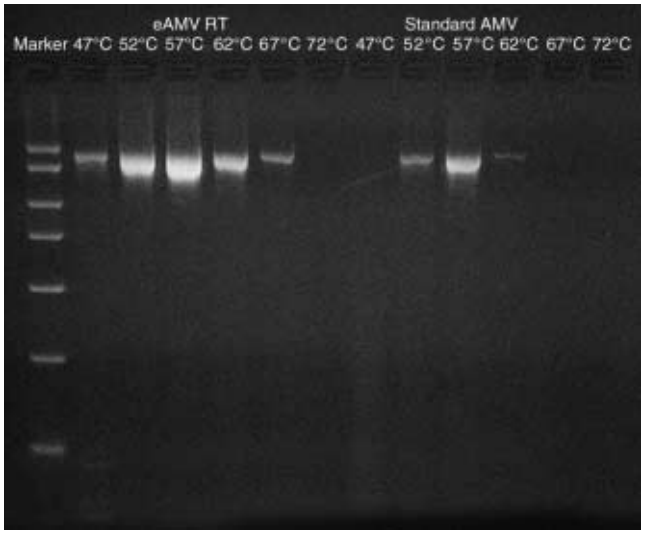
Comparison of eAMV and standard AMV using TMV targets

In the initial experiment, a 1.7 kb fragment with extensive secondary structure from tobacco mosaic virus (TMV) RNA was chosen for comparative study.[2] The RT primer used to reverse transcribe this region is located within a stable stem loop structure (Figure 1), requiring high processivity and elevated temperature during extension.

As expected, both eAMV and standard AMV failed to produce any RT-PCR product at 42 °C (data not shown), even though the TMV RNA was pre-incubated with the RT primer at 75 °C for 10 minutes to denature secondary structures before performing RT. At 47 °C and 52 °C, eAMV subsequently produced an increased quantity of PCR product compared with standard AMV, possibly indicating that this enzyme is more processive than the other enzymes tested. At these temperatures, the stem loop structure may not be completely denatured. Thus the efficiency of priming and the speed of reverse transcription may be critical for generating enough adequate cDNA. At even higher temperatures, eAMV performed consistently better, whereas the standard AMV failed, indicating the standard AMV may fail at a faster rate at high temperatures than eAMV (Figure 1).

Comparison of enzymes performing reverse transcription of messages with complex secondary structure

To examine the ability of eAMV to transcribe complex RNA, a target region from the 3' untranslated region (UTR) of mouse epidermal growth factor (EGF) message was chosen for comparative study. Based on a previous report, several reverse transcriptases, including Moloney murine leukaemia virus (M-MLV), RNase H-minus



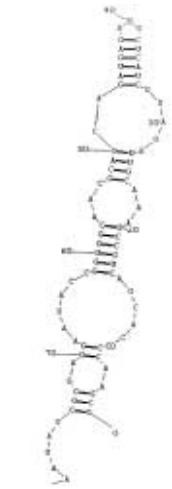


Figure 1. Comparison between eAMV and standard AMV at elevated temperatures. *RT-PCR was conducted on a 1.7kb TMV transcript with complex secondary structure. The primer used for RT is located within a stable stem-loop region (positions 39-59), making RT at elevated temperatures a prerequisite for successful assays.*

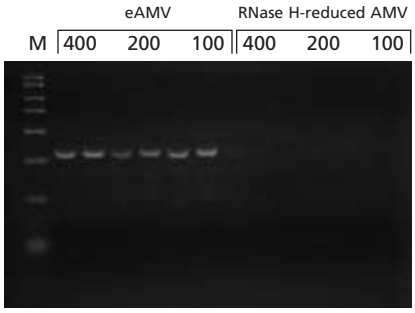


Figure 3. Comparison between eAMV and RNase H-reduced AMV. *RT-PCR was performed on mouse submaxillary RNA diluted serially, two-fold, in duplicate from 400 to 100 ng. Target is the 3' untranslated region of EGF mRNA (384 bp product). All RT reactions were performed at 50°C for 50 minutes. M is same as in Figure 2.*

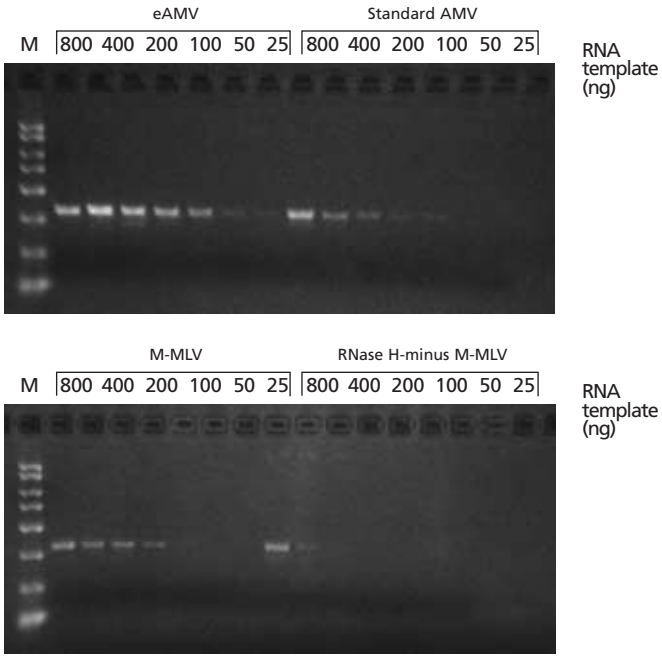


Figure 2. Comparison between eAMV and other reverse transcriptases. *RT-PCR was performed on mouse submaxillary RNA. RNA was serially diluted, two-fold, from 800 to 12.5 ng. All RT reactions were conducted at 42°C using anchored oligo dT23 primers. PCR of a 348 bp fragment from the epidermal growth factor (EGF) 3' UTR was performed using Taq DNA polymerase. Reverse transcription was performed according to manufacturers' recommendations. M = Sigma Product Code P9577; 50-2000 base pairs.*

M-MLV, and *r*Tth, could not efficiently transcribe this region of EGF mRNA, possibly because the mRNA possesses substantial secondary structure.[3] In this study, total RNA from mouse submaxillary gland was reverse transcribed at 42 °C using anchored oligo$(dT)_{23}$ followed by PCR amplification of a 348 bp fragment (Figure 2). Since the PCR primers used were directed at the upstream region of the 3' UTR, reverse transcription must extend through the entire region (~ 600 bp from the oligo-(dT) priming site). As shown in figure Figures 2 and 3, eAMV performed the best, producing a visible RT-PCR product from only 12.5 ng of template RNA. Standard AMV and M-MLV produced visible amplification products with 100 ng of template, whereas RNase H-minus M-MLV and RNase H-reduced AMV required 400 ng of RNA in order to generate amplification products.

Comparison of enzymes performing reverse transcription of low abundance messages

In order to obtain additional data on relative RT sensitivity, further studies were performed with the low abundance housekeeping gene, phospholipase A_2. When eAMV, was compared with RNase H-minus M-MLV and RNase H-reduced AMV, it exhibited greater sensitivity for the low abundance mRNA from phospholipase A_2 (Figure 4). To indicate that the systems were working correctly, RT-PCR was performed on a medium abundance message for the HPRT gene.

Comparison of various RT-PCR procedures on sensitivity

It is well known that different varied combinations of RT and PCR steps have significant impact on the outcome of the assay. In practice, there are three formats for RT-PCR systems: one tube-one step, one tube-two step, and two tube-two step protocols (Figure 5). The one-tube-one step protocol provides convenience, sensitivity, and improved

contamination control.[4,5] However, it is difficult to optimize both RT and PCR because all reagents are mixed before starting RT. The one tube-two step protocol requires performing RT first, then adding PCR mix to the entire RT reaction. This protocol allows researchers to optimize both RT and PCR conditions separately, and thus potentially achieve the maximum sensitivity. In the two tube - two step procedure, cDNA synthesis is performed first, and then an aliquot of the RT reaction is added to a new tube containing PCR reagents. This procedure is useful when analyses of multiple transcripts from the same cDNA pool are necessary, such as with differential display-PCR. However, the sensitivity of the assay may be compromised since only part of the RT reaction is used for PCR. The eAMV RT-PCR system works well in all of these formats. The researcher should recognize there are compromises in sensitivity and convenience associated with each procedure.

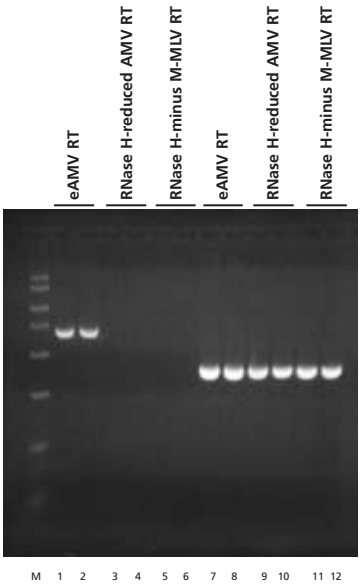


Figure 4. Sensitivity of eAMV on low abundance mRNA. *RT-PCR was performed on human phospholipase A_2 (low abundance) message (706 bp product; lanes 1-6) and on human HPRT (medium abundance) message (467 bp product; lanes 7-12). RT was conducted at 42°C using anchored oligo (dT)23. PCR was performed according to manufacturer's recommendations. M is same as in Figure 2.*

Conclusions

The new eAMV RT-PCR system demonstrates an improved ability to transcribe rare and complex RNA transcripts. This technique increases the overall success of expression analysis using RT-PCR technology. The system is flexible and adaptable to one-step and two-step formats.

References

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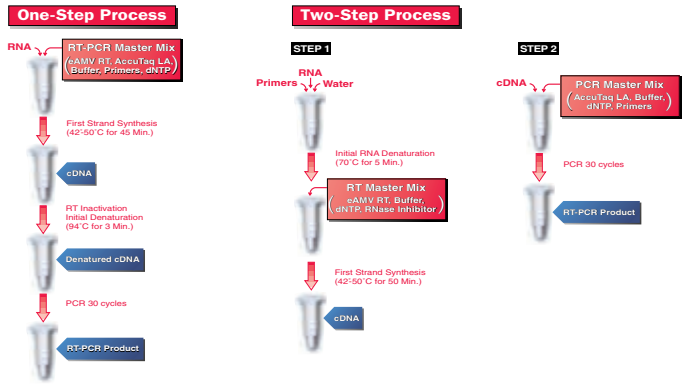

Figure 5. A schematic representation of the typical assay formats used to perform RT-PCR.

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ORDERING INFORMATION

Product Code	Product Name	Unit	Price
RTPCR-20	Enhanced Avian RT-PCR kit	1 kit (20 reactions)	$73.90
RTPCR-100	Enhanced Avian RT-PCR kit	1 kit (100 reactions)	$316.70

SUPPORTING LITERATURE

RT-PCR Flyer (CBQ), PCR Brochure (BKR)