Molecular Biology

FeatureArticle

Hot Start RT-PCR Results in Improved Performance of the Enhanced Avian RT-PCR System*

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Introduction

The reverse transcription-polymerase chain reaction (RT-PCR) is an almost ubiquitous technique in molecular biology. It is used to clone full-length genes, to determine the expression profiles of organisms under differing conditions, to generate sequencing templates without introns and a myriad of other applications. The RT reaction employs a reverse transcriptase, which uses a DNA-primed RNA template and dNTPs to generate a complementary DNA (cDNA) transcript. Subsequent amplification of this DNA using PCR can generate the sequence of interest in picomole quantities.

Sigma's thermally tolerant enhanced avian myoblastosis virus (eAMV) reverse transcriptase has been previously shown to transcribe RNA transcripts with stable secondary structures better than other commercially available reverse transcriptases. In addition, eAMV RT is also able to detect rare mRNA messages.[1] These results demonstrate that the reverse transcriptase in Sigma's RT-PCR kit performs very well when compared to other reverse transcriptases.

This report demonstrates how reverse transcriptase used in the RT reaction can effect later PCR results gained by adding an anti-*Taq* antibody. This antibody, termed JumpStart™, adds the advantages of Hot Start performance[2] to the AccuTaq™ LA DNA polymerase, and has improved the overall performance of the RT-PCR system. Adding this Hot Start mechanism has improved the length of read, sensitivity, and specificity of Sigma's RT-PCR kit.

Materials and Methods

All materials were supplied by Sigma-Aldrich Corporation (St. Louis, MO) unless otherwise stated. Moloney-Murine Leukemia Virus (M-MLV) RNase H-minus RT and AMV RNase H-minus RT were obtained from commercial sources.

RNA Templates

HEK 293 total RNA was isolated using GenElute™ Mammalian Total RNA Purification kit (Product Code: RTN-10). The resulting preparation was treated at room temperature with DNase I for fifteen minutes, then quenched with EDTA to yield a final EDTA concentration of 4.5 mM. The DNase I was ultimately heat-inactivated at 70 °C using a ten-minute incubation. Tobacco Mosaic Virus (TMV) RNA was obtained from a commercial source.

RT-PCR Conditions

Experiments were conducted using both one-step and two-step RT-PCR methods, as described in the RT-PCR technical bulletin (Bulletin No. MB-560). These conditions are described here in brief.

The one-step procedure was performed in a 50-µl reaction containing 1X PCR 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, 400 nM each of gene-specific primers, template RNA, 20 units of eAMV, and 2.5 units of AccuTaq™ LA or JumpStart™ AccuTaq™ LA. RT-PCR for HEK 293 total RNA was initiated with a 50 °C incubation for one hour (for RT), followed by a denaturation step of 94 °C for 3 minutes. This was immediately followed by 30 to 35 cycles of PCR consisting of a 30-second denaturation at 94 °C, a 30-second annealing at 55 °C and a 9-minute extension at 68 °C. A final extension period of ten minutes at 68 °C was applied in the last cycling step. TMV RNA RT-PCR was first subjected to 42 °C for one hour (for RT), followed by denaturation at 94 °C for 3 minutes, and a subsequent 30 to 35 cycles of two-step PCR. This thermocyling consisted of a denaturation at 94 °C for 30 seconds, followed by a 68 °C combined annealing and extension step totaling 90 seconds. A final extension period of five minutes at 68 °C was applied in the last cycling step.

The first of the two-step reactions 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), 1 mM each of dNTP, 20 units of RNase inhibitor, 3.5 µM anchored oligo$(dT)_{23}$, template RNA, and 20 units of eAMV at 42-50 °C for one hour. After RT, a 2-µl aliquot of the reaction was added to 48 µl of PCR master mix. The mix contained 1X AccuTaq™ buffer (50 mM Tris at pH 9.3, 15 mM ammonium sulfate, 2.5 mM $MgCl_2$, and 0.1% Tween 20), 500 µM each dNTP, 400 nM each of gene-specific primers, and 2.5 units of AccuTaq™ LA or JumpStart™ AccuTaq™ LA. PCR was performed using the conditions described in the PCR portion of the one-step RT-PCR for HEK 293 total RNA, previously detailed.

Results

RT-PCR length of read is improved by using a PCR Hot Start mechanism

A series of two-step RT-PCR experiments were executed to determine the effect of adding JumpStart™ antibody to the PCR reaction. A common RT reaction was performed using DNase I treated HEK 293 total RNA, eAMV RT and anchored oligo$(dT)_{23}$ primer as described in the Materials and Methods section. Each subsequent PCR consisted of a 2-µl aliquot (10%) from the pooled RT reactions in a final volume of 48 µl. PCR was performed as described in the Materials and Methods section using either AccuTaq™ LA or JumpStart™ AccuTaq™ LA with all other reaction components constant. Specific primers directed amplification of a 4.4 kb, 5.6 kb or 8.6 kb amplicon from the human

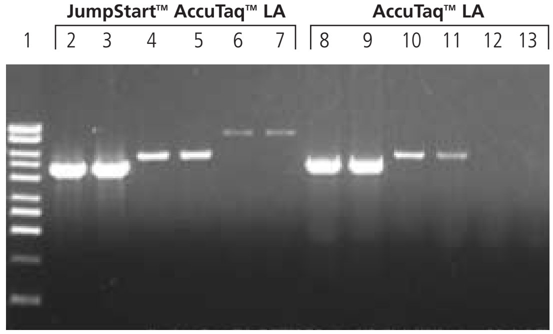

***Figure 1. RT-PCR length of read improved using PCR Hot Start mechanism. Lane 1:** 1 kb ladder; **Lanes 2-3, 8-9:** 4.4 kb amplicon; **Lanes 4-5, 10-11:** 5.6 kb amplicon and **Lanes 6-7, 12-13:** 8.6 kb amplicon.*

guanine nucleotide exchange factor gene.[3] Two-step RT-PCR using JumpStart™ AccuTaq™ LA produced amplicons of the expected sizes while two-step RT-PCR using AccuTaq™ LA was unable to produce the 8.6 kb amplicon (Figure 1).

eAMV is efficient in transcribing long targets in two-step RT-PCR

The effect of the reverse transcriptase source on RT-PCR was examined in a series of experiments in which the PCR reagents and conditions were kept constant. HEK 293 total RNA was reverse transcribed in a progression of twenty microliter reactions using oligo(dT) primers and reaction conditions in accordance with the RT supplier's recommendations. A 2-µl aliquot from each of these reactions was added to 48-µl PCR reactions containing JumpStart™ AccuTaq™ LA as the DNA polymerase. Three different mRNAs were reverse transcribed using oligo(dT) and amplified using four different specific primer pairs targeting a range of 3.5 kb to 8.9 kb sized amplicons.[4] eAMV and AMV RNase H-minus enzymes were able to reverse transcribe all of the transcripts, although the yield for the 6.8 kb amplicon was higher for eAMV. The M-MLV RNase H-minus enzyme was unable to reverse transcribe the 8.9 kb transcript (Figure 2).

Because the RT buffer for the M-MLV RNase H-minus enzyme has a lower $MgCl_2$ concentration than the buffers for other enzymes, it was a concern that the EDTA carried over from the RNA sample might have chelated a critical amount of the magnesium. This could make the enzyme less efficient. Additional $MgCl_2$ was added to the reaction mix to compensate, but did not effect the outcome of the experiment (data not shown).

eAMV routinely transcribes over 6.8 kb targets in a one-step RT-PCR format

Sigma's one-step RT-PCR system using eAMV and JumpStart™ AccuTaq™ LA was compared with an equivalent competitor's one-step RT-PCR kit. In this procedure, HEK 293 total RNA was reverse transcribed using specific primers for 3.5 kb, 6.8 kb and 8.9 kb targets, then subsequently amplified by PCR. The competitor's one-step RT-PCR system was used according to the manufacturers' recommendations, and included the conditions suggested for long RT-PCR products. Conditions for the Sigma one-step RT-PCR were as detailed in the Materials and Methods section. Sigma's one-step RT-PCR system amplified the 3.5 kb, 6.8 kb, and 8.9 kb reverse transcribed

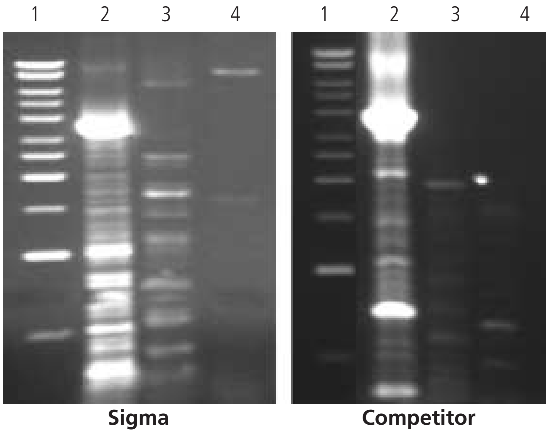

***Figure 3. Comparison between one-step and Hot Start RT-PCR systems on long targets. Lane 1:** 1 kb ladder, **Lane 2:** 3.5 kb Pol, **Lane 3:** 6.8 kb Pol, and **Lane 4:** 8.9 kb APC.*

cDNAs while the competitor's one-step RT-PCR system, which also includes a Hot Start PCR enzyme, was only able to amplify the 3.5 kb reverse transcribed cDNA (Figure 3).

The results shown in Figure 3 suggest that products of ≥ 6.8 kb are not generated in the competitor's system. In order to ascertain whether this is due to the reverse transcriptase blend or to the Hot Start DNA polymerase, a series of two-step RT-PCR experiments was completed.

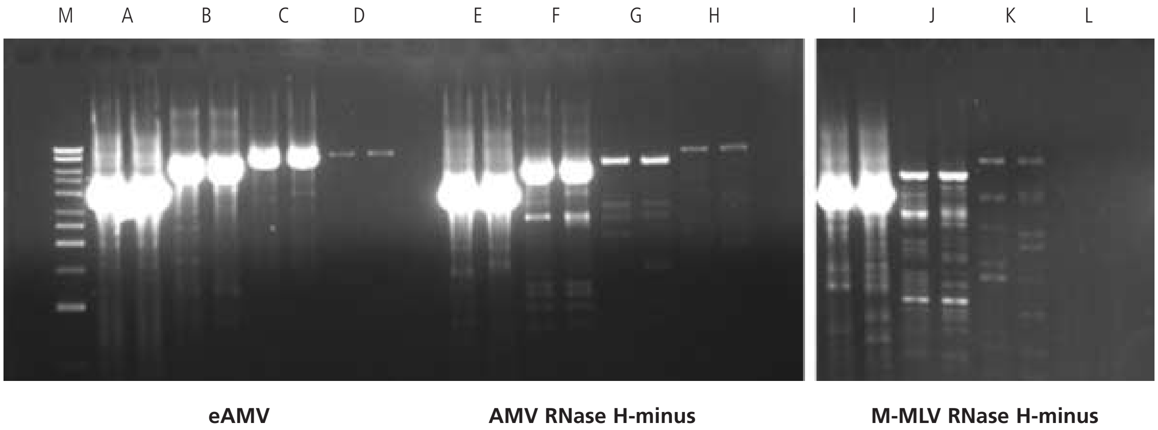

***Figure 2. Comparison of reverse transcriptase enzymes performing reverse transcription on long targets. Lanes A, E, I:** 3.5 kb Pol; **Lanes B, F, J:** 5.3 kb TSC-2; **Lanes C, G, K:** 6.8 kb Pol, and **Lanes D, H, L:** 8.9 kb APC.*

In these experiments, which were performed as previously described using a common set of PCR reaction conditions, the competitor's RT reaction was run side by side with the eAMV reactions. No PCR amplicon was visible in the competitor's reactions, although identical PCR conditions for both reverse transcription reactions were used (data not shown). These results suggest that the competitor's reverse transcriptase blend is unable to reverse transcribe RNA transcripts of 6.8 kb or greater.

RT-PCR sensitivity is improved by the addition of a PCR Hot Start mechanism

The addition of JumpStart™ antibody increased the sensitivity of the one-step RT-PCR system. These experiments were performed with TMV RNA[5] using primers directed toward a 440 bp amplicon, and carried out as described in the Materials and Methods section. RT-PCR with JumpStart ™ AccuTaq™ LA DNA polymerase showed a ten-fold increase in sensitivity when compared with one-step RT-PCR without a Hot Start mechanism (Figure 4).

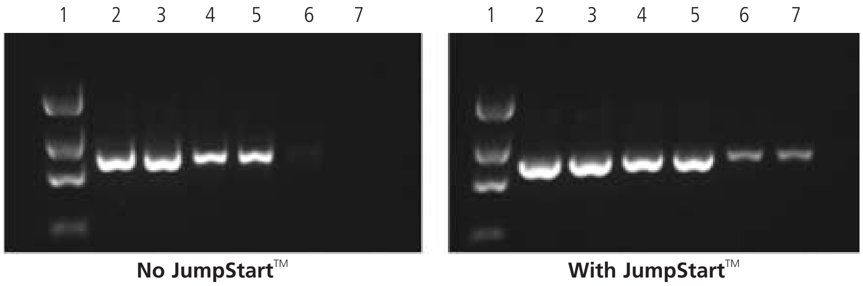

Figure 4. RT-PCR sensitivity improved using PCR Hot Start mechanism. Lane 1: *Low mass DNA ladder,* ***Lanes 2 & 3:*** *2 ng starting RNA,* ***Lanes 4 & 5:*** *200 pg starting RNA, and* ***Lanes 6 & 7:*** *20 pg starting RNA.*

RT-PCR specificity is improved by using a Hot Start PCR

The effect of an antibody PCR-mediated Hot Start or a manual RT Hot Start coupled with an antibody PCR-mediated Hot Start, was explored in a series of one-step RT-PCR format experiments. In one set of reactions a pair of specific primers targeted to a 5.3 kb transcript was applied in a one-step format using AccuTaq™ LA with or without JumpStart™. In another reaction, the same primers were used in a manual RT Hot Start in which eAMV was added only after the reverse transcription reaction temperature reached 50 °C for two minutes.

The results of these experiments are shown in Figure 5. The RT-PCR reaction without JumpStart™ did indeed produce the desired 5.3 kb amplicon, but with considerable mispriming and primer-dimer formation. The RT-PCR reactions with JumpStart™ AccuTaq™ LA showed less mispriming and greater yield of the expected 5.3 kb amplicon than a comparable reaction without antibody. The manual RT Hot Start reactions do not show a decrease in nonspecific RT-PCR products when compared with the JumpStart™ AccuTaq™ LA RT-PCR reactions without the manual RT Hot Start. The manual RT Hot Start reactions yielded less of the expected 5.3 kb amplicon when compared with the other RT-PCR reactions with or without JumpStart™ antibody (Figure 5).

Discussion

In this study, we have demonstrated that Hot Start PCR increases RT-PCR length of read, sensitivity, and specificity. There are several different ways to add a Hot Start mechanism to a DNA polymerase or PCR portion of a RT-PCR reaction. The DNA polymerase can be modified chemically by adding a reversible bond, causing the DNA polymerase to become inactive at room temperature and yet active at higher temperatures.[6] Mechanical methods, such as wax barriers, may be used to separate the RT reaction and the PCR reaction in a one-step RT-PCR reaction. In this technique, the wax barrier is not melted at the RT temperature but rather at the denaturation temperature of PCR, thus allowing the PCR components to become mixed with the RT components.[7] Manual addition of the DNA polymerase or RT once the RT-PCR reaction has reached a suitable temperature is another Hot Start option.[8] Finally anti-polymerase antibodies have also been shown to allow Hot Start PCR.[9]

Each of these methods has its potential drawbacks. Chemical modifications of the polymerase often require a long re-activation time at 95 °C to activate the enzyme, and can partially degrade components of the reaction mixture. Wax barrier techniques have been shown to increase sensitivity, but limit the use of thermally tolerant RTs. The manual Hot Start method brings a greater risk of contamination to the RT-PCR reactions, because the tubes have to be opened to add the enzyme at the elevated temperature. Specific primers need to be used if performing a manual RT Hot Start because oligo(dT) and random primers are not specific by design. Manual RT Hot Start also increases the risk for RNA degradation, and we speculate that this is why the yield of the 5.3 kb amplicon was less in the manual RT Hot Start reactions. Antibody-mediated Hot Start provides the greatest level of low temperature *Taq* inactivation, but requires adding one more component to the reaction mix.

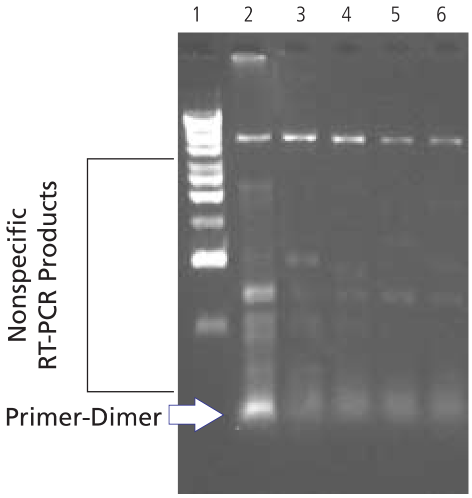

Figure 5. RT-PCR specificity improved using PCR Hot Start mechanism. Lane 1: *1 kb ladder,* ***Lane 2:*** *5.3 kb TSC-2 without JumpStart™,* ***Lanes 3 & 4:*** *5.3 kb TSC-2 with JumpStart™ without manual RT Hot Start, and* ***Lanes 5 & 6:*** *5.3 kb TSC-2 with JumpStart™ with manual RT Hot Start.*

Sigma's high fidelity PCR Hot Start enzyme, JumpStart™ AccuTaq™ LA DNA polymerase, provides a convenient, proven Hot Start method that inactivates Taq polymerase at temperatures below 70 °C. Adding JumpStart™ AccuTaq ™ LA to the RT-PCR has increased the length of read, sensitivity, and specificity of the reaction. The extension of non-specifically primed template is reduced, as is primer-dimer formation. This reduction of non-specific products and primer-dimer formation allows more of the PCR components to be dedicated to the specific RT-PCR product being amplified, resulting in increased yields of the desired product.

Conclusions

- Adding JumpStart™ to AccuTaq™ LA DNA polymerase improves RT-PCR length of read, sensitivity, and specificity.
- The improved RT-PCR kit maintains the ability to transcribe rare mRNA/or RNAs with difficult complex structures.
- Sigma's RT-PCR kit offers convenience and maximum flexibility, allowing one-step and two-step procedures.

Acknowledgments

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About the Authors

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ORDERING INFORMATION

Product Code	Product Name	Unit
HSRT20	Enhanced Avian HS RT-PCR-20 Kit	1 kit (20 reactions)
HSRT100	Enhanced Avian HS RT-PCR-100 Kit	1 kit (100 reactions)

RELATED PRODUCTS

Product Code	Product Name	Unit
A 4464	Enhanced Avian Reverse Transcriptase	500 units
		1000 units
D 5809	JumpStart™ AccuTaq™ LA DNA Polymermase	125 units
		500 units
		1500 units

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Enhanced Avian RT-PCR brochure (CBQ)

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