

Rapid Isolation of RNA from Small Quantities of Human Whole Blood for use in RT-PCR Analysis



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Isolation of RNA from whole blood and analysis by RT-PCR is a powerful technique that is useful for a variety of applications. We have used Promega's RNAgents[®] Total RNA Isolation System, the PolyAtract[®] System 1000, and a modified freeze-thaw technique to develop RNA isolation protocols that use small amounts (less than 100µl) of human whole blood as starting material. The RNA samples obtained were analyzed using the Access RT-PCR System^(a) to specifically detect the presence of an endogenous human RNA species. These techniques enable the rapid and efficient isolation of RNA from whole blood and could readily be scaled-up to a high-throughput format.

^(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 system is recommended for persons that either have a license to perform PCR or are not required to obtain a license.

Introduction

Coupled reverse transcription and PCR amplification (RT-PCR) is an extremely sensitive and versatile technique that can be utilized for a variety of applications. In this technique, total or selected RNA [i.e., poly(A)⁺] is used as a substrate for detecting specific transcripts, to estimate relative expression levels of a gene of interest or to amplify cDNA products for use in applications such as cloning or *in vitro* translation. Promega's Access RT-PCR System facilitates experiments by combining avian myeloblastosis virus (AMV) reverse transcriptase and *Thermus flavus* (*Tfl*) DNA polymerase with an optimized reaction buffer system that enables both the synthesis and amplification of cDNA products in a single reaction (1,2).

The use of RT-PCR in a high-throughput format requires the rapid isolation of RNA from a sample source. Although peripheral blood is a readily accessible tissue source, whole blood contains extremely high concentrations of serum proteins and derivatives of hemoglobin that can interfere with efficient RNA extraction, and subsequent amplification (3,4). Techniques routinely used to purify RNA from blood require relatively large quantities of starting material to isolate peripheral blood mononuclear cells prior to RNA extraction. This is time-consuming and not appropriate for high-throughput screening of relatively small amounts of blood. In this update, we describe protocols that use Promega's RNAgents[®] Total RNA Isolation System and PolyAtract[®] System 1000 to isolate RNA from human whole blood. These two techniques are compared to a third protocol in which white blood cells are prepared for RT-PCR using a freeze-thaw technique that avoids RNA isolation. Although all three procedures generate RNA that can be used directly in RT-PCR analysis, the techniques differ in their sensitivity and ease of use.

RT-PCR with RNA isolated from human blood using the RNAgents[®] Total RNA Isolation System

The RNAgents[®] Total RNA Isolation System uses a rapid, solution-based extraction procedure. This system utilizes a combination of powerful denaturants to quickly inactivate endogenous RNase in the sample, followed by organic extraction and alcohol precipitation to further purify and concentrate the RNA. This system was adapted to isolate total RNA from human blood. Peripheral blood was collected by human venipuncture into standard heparin-coated tubes and an aliquot (100µl) placed in an RNase-free 1.5ml microcentrifuge tube. Total RNA was extracted using the protocol outlined in [Figure 1A](#). The inclusion of RNase-free glycogen assured quantitative precipitation of small quantities of RNA.

Purified RNA was resuspended in 100µl of RNase-free water (equivalent to the original volume of blood) and aliquots were used in RT-PCR using the Access RT-PCR System. The beta-actin primers used were designed to allow discrimination between the 511bp product obtained from RT-PCR amplification of the mRNA and potential products obtained from amplification of contaminating genomic DNA. Amplification of a 511bp product specific to endogenous beta-actin mRNA could be detected using RNA isolated from the equivalent of 10µl of blood ([Figure 2](#)). RT-PCR analysis of a serial dilution of the final RNA sample indicated that a specific signal could be obtained using the equivalent of 0.625µl of whole blood. Comparison of the amount of amplification product generated from the equivalent of 10µl to that generated from the equivalent of 2.5µl and 0.625µl revealed some inhibition of amplification at the 10µl level. This may be due to increasing amounts of cell-derived inhibitory agents or an excess of template. An optional extraction with *n*-butanol and precipitation with alcohol after final resuspension of the isolated RNA in water has been reported to yield more consistent RT-PCR results using blood as a sample source (5), but had little effect in these experiments (data not shown).

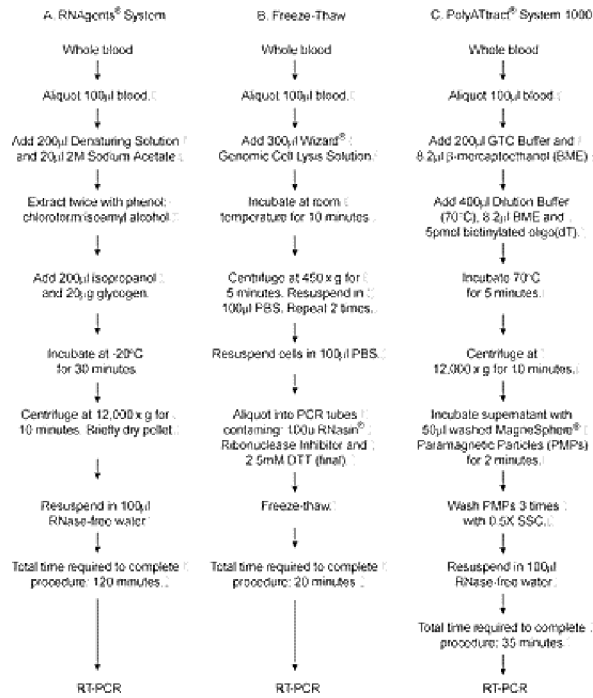


Figure 1. Outline of the RNA isolation procedures. Panel A: Isolation of RNA from whole blood using the RNAgent's[®] Total RNA Isolation System. **Panel B:** Preparation of RNA from whole blood using a freeze-thaw procedure. **Panel C:** Isolation of RNA from whole blood using the PolyAtract[®] System 1000.

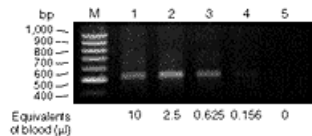


Figure 2. RT-PCR amplification of human beta-actin from total RNA isolated from human blood. Total RNA was isolated from 100 μ l of human blood using Promega's RNAgent's[®] Total RNA Isolation System as outlined in [Figure 1A](#). The final RNA pellet was resuspended in 100 μ l of nuclease-free water, 10 μ l was removed and serial 4-fold dilutions of the RNA prepared. RT-PCR reactions using amounts of RNA equivalent to the indicated volume of starting blood and primers complementary to the human beta-actin transcript were performed using the Promega Access RT-PCR System as described (1). Reaction products were resolved on a 1.5% agarose gel and visualized by staining with ethidium bromide. Lane M contains Promega's 100bp DNA Ladder (Cat. #G2101).

RT-PCR with RNA isolated from human blood using a freeze-thaw protocol

A protocol for preparing cells for RT-PCR without purification of RNA has been reported previously (6). In this technique, target cells are lysed by rapid freeze-thaw in the presence of DTT and Animal Injectable Grade Recombinant RNasin[®] Ribonuclease Inhibitor^(b). The ribonuclease inhibitor protects the endogenous RNA from degradation while reverse transcription proceeds. [Figure 1B](#) illustrates a modification of this protocol that adapts it for use with whole blood. In this procedure, which is a variation on the protocol for isolating genomic DNA from blood samples using the Promega Wizard[®] Genomic DNA Purification Kit, reticulocytes present in whole blood are preferentially lysed and mRNA-containing leucocytes are collected by centrifugation. The leucocytes are briefly washed and aliquots are placed into PCR compatible tubes that contain Animal Injectable Grade Recombinant RNasin[®] Ribonuclease Inhibitor and DTT. A single freeze-thaw cycle is used to lyse the cells, and RT-PCR is performed directly in the reaction tubes.

^(b)U.S. Pat. No. 5,552,302 has been issued to Promega Corporation for the methods and compositions for production of human recombinant placental ribonuclease inhibitor (PRI). Inhibitors of Angiogenin, which comprises a segment of human PRI, is the subject of U.S. Pat. Nos. 4,966,964, 5,019,556 and 5,266,687 assigned to the President and Fellows Harvard College and exclusively licensed to Promega Corporation.

As shown in [Figure 3](#), the production of the 511bp amplified beta-actin product was completely dependent on the inclusion of RNasin[®] Ribonuclease Inhibitor in the reaction tube during the freeze-thaw cycle. The sensitivity of this technique was similar to that obtained using purified RNA; a specific amplification product could be detected when the equivalent of 0.156 μ l of whole blood was used in the RT-PCR amplification. The extreme simplicity and high-throughput capacity of this procedure may make it an appropriate alternative to traditional RNA isolation techniques for use in some RT-PCR applications. However, the presence of increasing amounts of cell-derived

inhibitory agents at higher cell concentrations (Figure 3, lane 2) and the potential for increased background due to genomic DNA contamination may interfere with the detection of rare mRNAs.

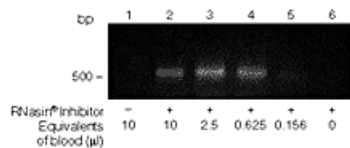


Figure 3. RT-PCR amplification of human beta-actin from cells prepared by freeze-thaw. Samples were prepared for analysis in the absence (lane 1) or presence (lanes 2-6) of Animal Injectable Grade Recombinant RNasin® Ribonuclease Inhibitor (Cat.# N2312) as outlined in Figure 1B. Serial 4-fold dilutions of the RNA were prepared in nuclease-free water. RT-PCR reactions containing amounts of RNA equivalent to the indicated volume of starting blood and primers complementary to the human beta-actin transcript were performed using the Promega Access RT-PCR System as described (1). The reaction products were analyzed as described in Figure 2. The position of the 500bp marker is indicated to the left of the figure.

RT-PCR with RNA isolated from human blood using the PolyAtract® System 1000

Promega's PolyAtract® System 1000 utilizes the high affinity of biotin for streptavidin to quickly purify mRNA directly from tissue using magnetic-capture technology. This system avoids organic extraction and precipitation steps and is therefore more amenable to high-throughput analysis of small amounts of blood. Peripheral blood was collected by human venipuncture into standard heparin-coated tubes. An aliquot of blood (100µl) was placed in an RNase-free 1.5ml microcentrifuge tube and total RNA was extracted using the protocol outlined in Figure 1C. In the final step of the procedure, the streptavidin-paramagnetic particles (PMPs) were resuspended in 100µl of RNase-free water (an amount equivalent to the original volume of blood).

Aliquots of the resuspended mRNA were used in RT-PCR amplification. The expected beta-actin amplification product was obtained using an amount of mRNA equivalent to that in 0.625µl of whole blood (Figure 4). Amplification using the equivalent of 10µl of blood exhibited none of the inhibition seen with the other preparation methods. To test the ability of this system to isolate mRNA from smaller volumes we used 10µl of the PMPs to purify mRNA from 10µl of blood and obtained results identical to those shown in Figure 4, verifying that working volumes can be reduced significantly (data not shown). These results show that the PolyAtract® System 1000 offers several significant advantages including ease of use, lack of organic extraction, and potential for high-throughput RT-PCR analysis using the PolyAtract® Series 9600™ Multi-Magnet^(c) (Cat.# Z3811) (7).

^(c)U.S. Pat. No. 5,567,326 has been issued to Promega Corporation for a multi-sample magnetic separation device.

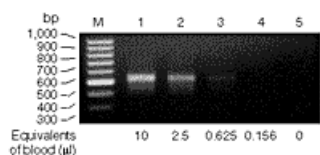


Figure 4. RT-PCR amplification of human beta-actin from mRNA isolated from human blood. mRNA was isolated from human blood using Promega's PolyAtract® System 1000 RNA Isolation System as outlined in Figure 1C. Serial 4-fold dilutions of the RNA were prepared in nuclease-free water. RT-PCR reactions that contained amounts of RNA equivalent to the indicated volume of starting blood and primers complementary to the human beta-actin transcript were performed using the Promega Access RT-PCR System as described (1). RNA samples used for RT-PCR were obtained from the final water resuspension of the Streptavidin MagneSphere® Paramagnetic Particles without magnetic capture, as the particles had no detrimental effect on the RT-PCR reaction. The reaction products were analyzed as described in Figure 2. Lane M contains Promega's 100bp DNA Ladder.

Isolation of nucleic acids from serum samples

In addition to its utility in isolating RNA from whole blood/serum samples, the RNagents® Total RNA Isolation System can be used for the simultaneous isolation of DNA and RNA from a single serum sample. This is advantageous for a variety of research applications, such as the simultaneous isolation of nucleic acids from samples that contain blood-borne pathogens of unknown genetic content. To demonstrate how RNA and small, closed circular DNA can be simultaneously extracted from a serum sample, the following experiment was performed. An aliquot (100µl) of human serum was incubated with Proteinase K to digest endogenous proteins (Figure 5) and a small amount (either 1×10^6 or 1×10^3 copies) of pGEM^(d)-7Zf(+) Vector was added to the serum sample. The sample was processed using the RNagents® RNA Isolation System protocol as described in Figure 1, with the following exception.

The organic phase of the first extraction was back-extracted with a buffer that adjusted the pH to 8.0 and increased the ionic strength of

the aqueous phase, and the nucleic acids present in the resulting aqueous phase were recovered by isopropanol precipitation.

DNA recovery was monitored using the M13 forward and reverse primers and Promega's Access RT-PCR System to amplify a 233bp fragment of the pGEM[®]-7Zf(+) Vector ([Figure 5](#)). This fragment was easily amplified from the DNA fraction recovered from the serum samples supplemented with the vector (lanes 3 and 4). Visual comparison of the amount of amplification product produced by the reactions containing the recovered DNA to that produced in a reaction to which 1×10^6 copies of pGEM[®]-7Zf(+) Vector were added directly to the amplification reaction suggests that recovery of the vector DNA was almost quantitative (compare lanes 2 and 3). An amplification reaction containing no added DNA yielded no detectable amplification products (lane 1).

^(d) U.S. Pat. No. 4,766,072 has been issued to Promega Corporation for transcription vectors having two different bacteriophage RNA polymerase promoter sequences separated by a series of unique restriction sites into which foreign DNA can be inserted.

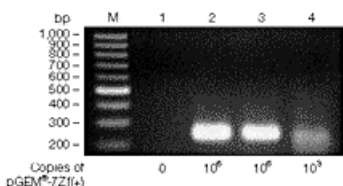


Figure 5. Isolation of plasmid DNA from human serum samples using Promega's RNAgents[®] RNA Isolation System. Human serum (100µl) was treated for two hours with Proteinase K (100µg) at 50°C. Samples were then supplemented with 1×10^6 copies (lane 3) or 1×10^3 copies (lane 4) of pGEM[®]-7Zf(+) Vector and were processed as described in [Figure 1A](#), with the following modifications. After the phenol:chloroform:isoamyl alcohol extraction step used to extract RNA and leave DNA in the organic phase, the organic phase was back-extracted with 200µl of aqueous neutralization solution [0.5M Tris-HCl (pH 8.0), 0.1M NaCl]. Nucleic acid in the aqueous phase was recovered by adding 1µg of glycogen and 200µl of isopropanol, incubating at 20°C for 0.5 hour and centrifuging at 12-14,000 x g for 10 minutes. Recovery of nucleic acid was monitored by amplification of a fragment of the vector using the M13 forward and reverse primers and the Promega Access RT-PCR system as described (1). Amplification reactions were also performed with no added DNA (lane 1) or 1×10^6 copies of pGEM[®]-7Zf(+) (lane 2) added directly to the reactions for comparison. The reaction products were analyzed as described in [Figure 2](#).

Summary

Whole blood is a valuable tissue source for RT-PCR methodologies used in a variety of research applications. We describe three methods that can be used to prepare RNA from whole blood for RT-PCR analysis, and have used Promega's Access RT-PCR System to detect an endogenous RNA species present in the preparations. The three protocols, which differ in their basic strategy, enable researchers to utilize the methods that best suit their laboratory and research needs. These methods enable quick and simple RNA preparation that can be completed in 20-120 minutes, and are appropriate for high-throughput analysis of both RNA and DNA species present in small numbers of white blood cells, serum or whole blood samples.

References

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7. Brisco, P. *et al.* (1995) *Promega Notes* **52**, 8.

Ordering Information

Product	Size	Cat.#
RNAgents [®] Total RNA Isolation System		Z5110
PolyAtract [®] System 1000 with Magnetic Stand		Z5420
PolyAtract [®] System 1000 without Magnetic Stand		Z5400
Wizard [®] Genomic	100 isolations	A1120

DNA Purification Kit	500 isolations	A1125
Access RT-PCR System	100 reactions	A1250
	500 reactions	A1280
Animal Injectable Grade Recombinant RNasin [®] Ribonuclease Inhibitor	10,000 units	N2312

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