Isolation of True Total RNA using the Tempus™ Blood RNA Tube and Norgen's Total RNA Purification Kit

B. Lam, PhD[1], C. Dobbin, PhD[1], Y. Haj-Ahmad, Ph.D[1,2]
[1]Norgen Biotek Corporation, Thorold, Ontario, Canada
[2]Centre for Biotechnology. Brock University, St. Catharines, Ontario, Canada

INTRODUCTION

Many of today's gene expression studies that use real-time PCR or microarrays require high-quality RNA purified from whole blood. This RNA must be highly pure, because accuracy and sensitivity are critical for interpretation of results. RNA purification from whole blood is a formidable challenge. Blood contains high levels of protein, genomic DNA and RNases. The copy numbers of individual mRNA species in whole blood can change more than 1000-fold during storage or transport at room temperature. This is due to both rapid RNA degradation and by induced expression of certain genes after the blood is drawn. Such changes in the RNA expression profile make reliable studies of gene expression impossible. In addition, the presence of multiple PCR inhibitors (heme, immunoglobulin G, and lactoferrin) plague existing extraction procedures and downstream assays. Blood samples collected with the anticoagulant heparin are also known to inhibit PCR. Contaminating gDNA may affect the accuracy of RT-PCR, and other associated procedures. Sample preparation of RNA from blood must deal with these factors, and provide material that is highly pure, free from contaminating gDNA and inhibitors of PCR or reverse transcription.

Whole blood is routinely collected into various anticoagulants, including salts of citrate, heparin, and EDTA. These additives inhibit clotting but do little to maintain RNA quality. Whole blood RNA collection systems are an attractive approach in the clinical setting because of their ability to stabilize nucleic acids immediately upon collection. One such system, PAXgene (PreAnalytiX, Hombrechtikon, Switzerland), has been shown to prevent RNA degradation in anti-coagulated whole blood when stored at room temperature or 4°C for periods up to 5 and 90 days, respectively. Similarly, other commercially available whole blood collection tubes, such as the ZR Whole Blood collection tubes (Zymo Research, Orange, CA) and the Vacuette EDTA and Nucleic Acid Stabilization Tubes (Greiner Bio-one, GmbH), contain RNA stabilization agents for delayed processing during blood storage and transport. Applied Biosystems Tempus Blood RNA tubes are designed for the collection and stabilization of whole blood for RNA isolation when real-time RT-PCR, microarrays or other high quality RNA experiments will be performed. Each Tempus Blood RNA Tube contains 6 mL of Stabilizing Reagent. When the blood is drawn into the tube and mixed with this reagent, lysis occurs almost immediately. The stabilizing reagent inactivates cellular RNases and selectively precipitates RNA, while the genomic DNA (gDNA) and proteins remain in solution. RNA stabilization occurs during the initial interaction of the blood with the stabilizing reagent. In contrast, other suppliers require a two hour incubation in the blood tube in order to achieve RNA stabilization, during which degradation may occur. Blood drawn into the Tempus Blood RNA Tube is stable for up to five days at room temperature, or minimally seven days at 4°C.

Norgen's Total RNA Purification Kit provides an innovative and rapid method for the isolation and purification of total RNA, including microRNA, from blood samples. The procedure is based on spin column chromatography using Norgen's proprietary resin as the separation matrix. The purified RNA can be used in a number of downstream applications including real-time, reverse transcription PCR, Northern blotting and expression arrays.

In this application note, Norgen's Total RNA Purification Kit is used to isolate total RNA from hamster blood samples collected in Applied Biosystem's Tempus™ RNA Blood Tubes. True total RNA is successfully isolated from the samples without the use of organic extraction or added steps. The downstream application of RT-qPCR is performed indicating the purity and biological activity of the purified RNA.

MATERIALS AND METHODS

Blood Collection. For the collection of blood, 3mL of hamster blood was drawn directly into Tempus™ Blood RNA Tubes (a kind gift from Dr. Youn Kwak, Baylor Health) according to the laboratory's standard procedures.

3430 Schmon Parkway
Thorold, ON Canada L2V 4Y6
Phone: 905-227-8848 • Fax: 905-227-1061
Toll-Free (North America): 1-866-667-4362
www.norgenbiotek.com

Immediately after filling each, the tubes were shaken vigorously for 20 seconds. The stabilized blood was then stored at 4°C for 3 days prior to RNA extraction.

RNA Isolation using Norgen's columns. RNA was isolated using Norgen's Total RNA Purification Kit with a few modifications. Briefly, the preserved blood sample was poured from the Tempus™ tube into a new 50 mL conical tube. The volume of the sample was then adjusted to 12 mL by the addition of RNase-free 1X Phosphate Buffered Saline (PBS). The tube was then closed tightly and mixed by vortexing vigorously for 30 seconds. The tube was centrifuged at 4°C at 3,000 x g (~4500 rpm) on a Beckman JB-6 swing bucket centrifuge for 30 minutes. The supernatant was then carefully poured off and the tube inverted on power towel for 2 minutes to dry. Lysis solution (600 µL) was added to the tube and mixed by vortexing 30 seconds to resuspend the pellet. To this was then added 300 µL of 95-100% ethanol and the tubes again vortexed to mix. Up to 600 µL of the lysate was then transferred to a spin column assembly. The tube was centrifuged at 14,000 rpm for 1 minute and the flow-through discarded. This step was repeated until all lysate had been applied to the column. The column was washed 3 times using 400 µL of Wash Solution. An additional 2 minute spin was performed to thoroughly dry the resin. The total RNA was then eluted in 50 µL of Elution Solution by centrifuging at 2,000 rpm for 2 minutes, followed by a 1 minute spin at 14,000 rpm.

Agarose Gel Electrophoresis. Agarose gel electrophoresis was used to visualize the purified blood RNA. From the 50 µL elutions, 5 µL of the RNA was routinely run on a 1X MOPS, 1.2% Formaldehyde-Agarose gel at 150 V and visualized using ethidium bromide staining.

Capillary Electrophoresis. From the 50 µL elution, 1 µL of the purified RNAs was loaded onto an Agilent® RNA Nano 6000 chip and resolved on an Agilent® 2100 BioAnalyzer according to the manufacturer's instruction.

Real-time Polymerase Chain Reaction. The purified blood RNA was then used as the template in real-time, reverse-transcription PCR using primers specific for miR-21 5S rRNA or beta-Actin. Briefly, 1µg of RNA was used as the template for reverse transcription (RT) in a 20µL RT reaction using Invitrogen's Superscript III system with miR-21 Stem-loop (3), or 5S rRNA reverse primers or Oligo dT. For real-time PCR cDNA (3µL of RT reaction as template) was added to the Bio-Rad IQ SYBR Green Mastermix using 0.3 µM of primers specific for miR-21, 5S rRNA or beta-Actin. PCR was performed in a three-step real-time PCR program (95°C 15 sec. 58°C for 30 sec, 72°C for 45 sec for 40 cycles).

RESULTS AND DISCUSSION

Total RNA was isolated in duplicate from 3 mL hamster blood samples collected into Tempus™ RNA Blood Tubes using Norgen's Total RNA Purification kit as per the provided protocol (**Figure 1**). The entire protocol was completed in 20 minutes. Once the RNAs were isolated, they were run on a 1X MOPS, 1.2% Formaldehyde-Agarose gel for visual inspection.

The Formaldehyde-Agarose gel in **Figure 2** demonstrated that the total RNA purified by Norgen's protocol was of a high quality, with no signs of degradation of the major rRNA species. More importantly, Norgen's kit truly isolated total RNA of all sizes, including microRNAs < 200 nucleotides (**Figure 3**). This is of particular significance as most small regulatory RNAs are in the size range of 15 to 30 nucleotides. Thus, Norgen's kit offers a quick, non-organic-based method to isolate high quality small RNAs that are less than 200 nucleotides in size.

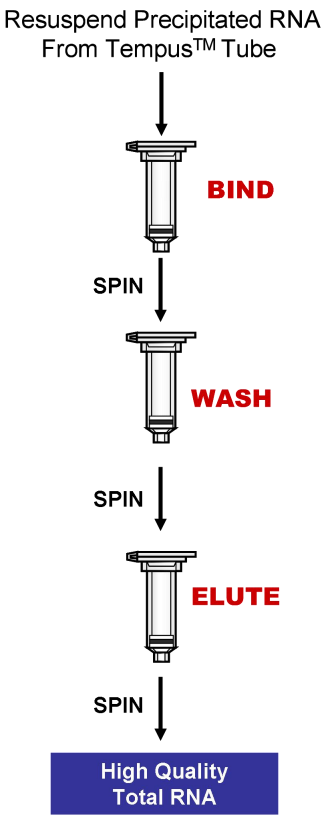

Figure 1. Procedure flowchart for the purification of total RNA using Norgen's Total RNA Purification Kit.

3430 Schmon Parkway
Thorold, ON Canada L2V 4Y6
Phone: 905-227-8848 • Fax: 905-227-1061
Toll-Free (North America): 1-866-667-4362
www.norgenbiotek.com

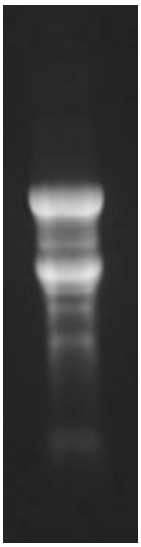

Figure 2. Total RNA isolation from whole hamster blood. Samples of purified RNA were run on a 1X MOPS, 1.2% Formaldehyde-Agarose gel. The major 18S and 28S rRNA bands can be observed on the gel.

The quality of RNA isolated by Norgen's Total RNA Purification Kit was further demonstrated by capillary gel electrophoresis (**Figure 3**). Total RNA was resolved on an Agilent® Lab-on-a-Chip. Panel A in Figure 3 is an electropherogram of total RNA isolated from hamster blood cells using Norgen's Total RNA Purification Kit. All RNA species, including microRNA, 18S rRNA and 28S rRNA can be observed. A high yield of RNA (1146 ng/µL) was obtained which was of very high quality (RIN = 9.0).

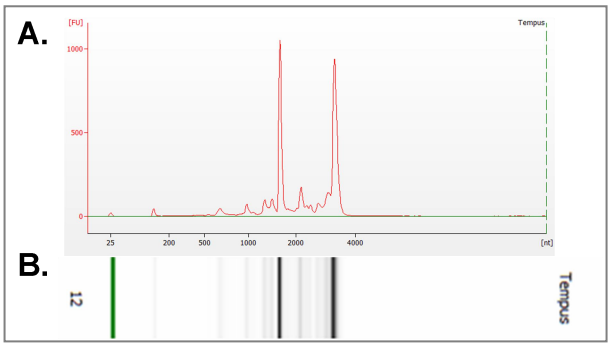

Figure 3. Resolution of Hamster Blood RNA on the Agilent® BioAnalyzer. Panel A shows the electropherogram from the Agilent Bioanalyzer. The y axis represents fluorescence units and the x axis represents the runtime (s). The bands of the 18S and 28S rRNA fragments are clearly visible and the RNA integrity number score is 9.0. Panel B demonstrates the gel banding pattern of the RNA species purified from hamster blood. Abundant blood transcripts such as the globin mRNAs constitute the additional bands to the two major rRNA bands.

In order to assess the biological activity of the purified RNAs, RT-qPCR was performed. Unlike regular RT-PCR, the amplification and detection of small RNA molecules such as microRNA requires the addition of an adaptor. One of the commonly used protocols involves the addition of a stem-loop to the microRNA (3). This method was used here and **Figure 4** shows the amplification of the *miR-21* transcript from small RNAs isolated from the hamster blood using Norgen's Total RNA Purification Kit. Similarly, the 5S rRNA which is commonly used as a loading control for miRNA RT-PCR was amplified from the purified small RNA (**Figure 4**). This suggested that the small RNA isolated was of a high purity and had retained it's biological activity. This is a significant competitive advantage of Norgen's kit over the equivalent isolation product from a competitor, as this silica column-based kit does not allow for the isolation of the small RNA species.

In addition to the successful isolation of the microRNA, the large RNA (beta-actin) present in the sample was successfully isolated and was amplified in an RT-PCR reaction (**Figure 4**). The RT-PCR was successful for all species of RNA assayed using Total RNA as template

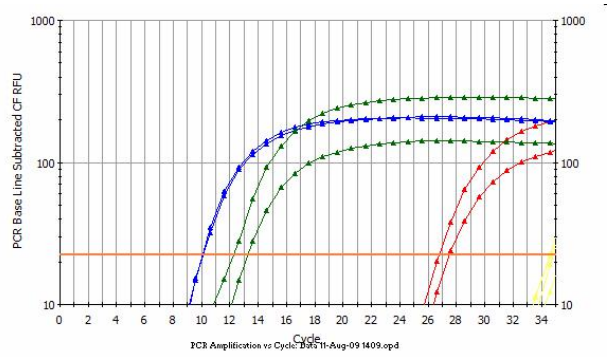

Figure 4. RT-PCR results for RNAs isolated from hamster blood using Norgen's Total RNA Purification Kit. All sizes of RNA were successfully amplified including large RNA (Beta-Actin = green), small RNA, such as the 5S rRNA (blue) and microRNA, *miR-21* (red). No template control is shown in yellow.

CONCLUSIONS

Norgen's Total RNA Purification Kit provides a very attractive method for the isolation of RNA from blood samples. Large amounts of total RNA were successfully recovered from Applied Biosystems Tempus™ Blood RNA Tubes when using the current protocol developed by the manufacturer. The RNA subsequently isolated from the preserved samples is of a high quality and can be used directly in sensitive downstream diagnostic assays such as

3430 Schmon Parkway
Thorold, ON Canada L2V 4Y6
Phone: 905-227-8848 • Fax: 905-227-1061
Toll-Free (North America): 1-866-667-4362
www.norgenbiotek.com

RT-qPCR. According to Applied Biosystems, microRNAs are preserved in the Tempus™ Blood RNA Tubes, however they could not be recovered when using an Applied Biosystems-supplied RNA Purification Kit. Based on the results demonstrated here, it appears that Norgen's Total RNA Purification Kit can successfully isolate RNA of all sizes from the Tempus™ Blood RNA Tubes. These RNA species are of high quality and yield and are suitable for use in sensitive downstream assays such as RT-qPCR.

REFERENCES

1. Bartel, D.P. 2004. MicroRNAs: Genomics, Biogenesis, Mechanisms and Function. Cell. 116: 281-297.

2. Nilsen, T.W. 2007. Mechanisms of microRNA-mediated gene regulation in animal cells. Trends in Genetics. **23**: 243-249.

3. Chen, C., D. A. Ridzon, A. J. Broomer et al. 2005. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res. 33(20): e179.

3430 Schmon Parkway
Thorold, ON Canada L2V 4Y6

Phone: 905-227-8848 • Fax: 905-227-1061
Toll-Free (North America): 1-866-667-4362
www.norgenbiotek.com