

# Comparison of Six Different Pretreatment Methods for Blood RNA Extraction

Xiaopan Liu,<sup>1</sup> Qiyuan Li,<sup>1</sup> Xian Wang,<sup>1</sup> Xiaolin Zhou,<sup>1</sup> Qiuyan Liao,<sup>1</sup> Xuheng He,<sup>1</sup> Jiuling Zhang,<sup>1</sup> Jianbo Sun,<sup>1</sup> Jinhua Wu,<sup>1</sup> Le Cheng,<sup>1,2</sup> and Yong Zhang<sup>1</sup>

Human blood specimens serve as important research materials in the field of translational medicine research. The RNA extracted from blood, for example, represents the gene expression profiles of individuals or groups, and can be indicative of the pathological basis for human diseases. Meanwhile, the RNA quality may have severe impacts on the results of RNA studies. RNA is susceptible to many factors, such as the time of sample collection, transportation conditions, protectants, pretreatments, and extraction methods. In this study, six different pretreatment methods are evaluated for their effects on blood RNA extraction including the RNA yields and quality. Results show that most of these methods meet the basic requirements for RNA studies. While considering the simplicity of the procedure, the cost factor, and how to make full use of the samples, the proper method should be employed by researchers who have specific requirements for their research.

## Introduction

**H**UMAN BLOOD SPECIMENS are now routinely collected, processed and archived in many biobanks, due to the ease and low cost of collection, and the variety of available fractions (serum, plasma, white blood cells, and red blood cells). In addition, the blood samples contain various biomolecules (DNA, RNA, proteins, and metabolites) and thus are ideal biological materials for conducting multiple assays.<sup>1</sup>

Peripheral blood has been considered as a less invasive and valuable source of RNA in vertebrates. With the development of -omics technologies, blood RNA has been increasingly used in high-throughput applications such as gene expression profiling or transcriptome sequencing.<sup>2-4</sup> However, blood RNA is so vulnerable to degradation that many factors such as endogenous and exogenous RNases, pretreatment, preservation, or isolation procedures may have influences on the results of RNA studies to varying degrees. Thus care must be taken throughout the collection, processing, and storage of blood RNA, to ensure that substantial RNA of good quality can be obtained.<sup>1,5</sup>

Previous studies were carried out to compare the effects of different sampling techniques and extraction methods on RNA analysis,<sup>6,7</sup> but little work has addressed the pretreatment methods for blood RNA. In this study, six different pretreatment methods were compared to assess their

effects on RNA yield and quality. The goal was to determine the optimal pretreatment protocol for blood RNA extraction in biobanking practices.

## Materials and Methods

### *Informed consent*

The study protocol was approved by the BGI-IRB (BGI-Institutional Review Board). All donors gave their written consent for nontherapeutic use of their donated blood samples.

### *Blood collection and processing*

Peripheral blood samples were obtained from 11 healthy donors: five women and six men. The samples were collected into two kinds of tubes for individual donors: EDTA-coated vacuum tubes (INSEPACK) and PAXgene Blood RNA tubes (Becton-Dickinson). All tubes were inverted ten times, gently, immediately after blood collection. For EDTA-coated blood tubes, the blood samples were aliquoted into 1 mL per tube for the procedures that followed. For PAXgene tubes, the sample was treated according to manufacturer's instructions.

Six different blood pretreatment protocols were used in this study before total RNA extraction. The workflow is shown in Figure 1 (experiments from protocol A, B, C were finished within 3 hours).

<sup>1</sup>China National Genebank, BGI-Shenzhen, Shenzhen, China.

<sup>2</sup>BGI-Yunnan, Kunming, China.

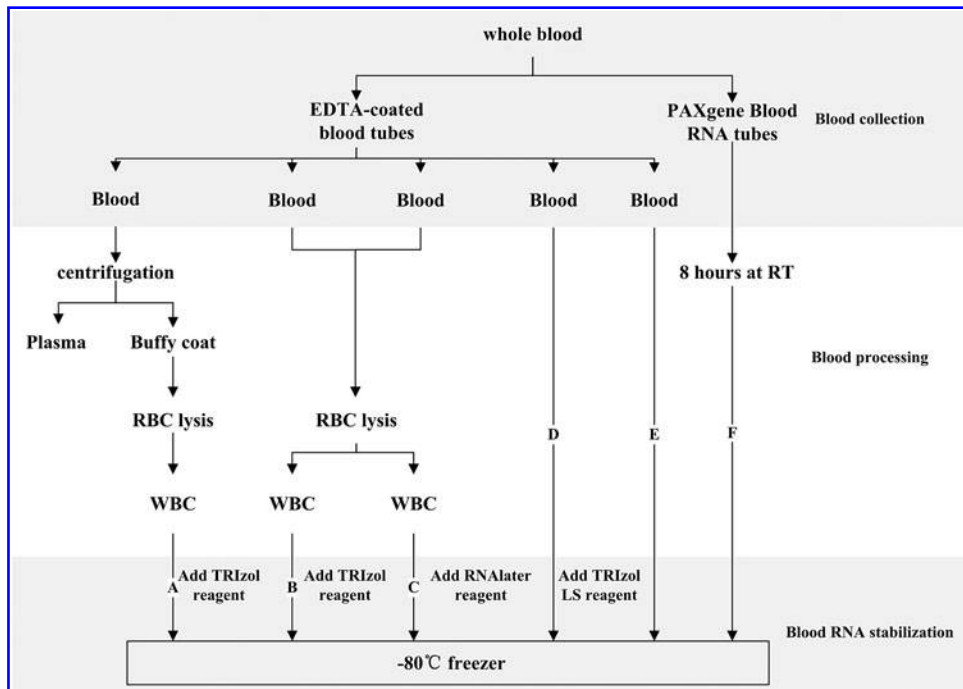


FIG. 1. Experiment workflow.

#### Protocol A: Buffy coat-TRIZol method

- Centrifuge the tube at 1600  $g$  for 10 min at 4°C.
- Remove the supernatant (plasma) by pipetting, add 1 mL RBC lysis buffer into the tube. Mix gently and incubate at room temperature for 10 min.
- Centrifuge the tube for 5 min at 10,000  $g$ , 4°C.
- Remove the supernatant, add 1 mL RBC lysis buffer to the tube, and mix it gently using a clean pipette tip.
- Centrifuge the tube at 10,000  $g$  for 5 min at 4°C.
- Remove the supernatant. Wash the pellet once with 1 mL PBS buffer, mix the sample gently and centrifuge at 10,000  $g$  for 5 min at 4°C. Discard the supernatant.
- Add 1 mL TRIzol reagent, use a pipette to mix uniformly, incubate for 15 min at room temperature (RT).
- Transfer the mixture of blood white cells and TRIzol reagent to a  $-80^{\circ}\text{C}$  freezer.

#### Protocol B: Blood-Trizol method

Add 1 mL RBC lysis buffer into the blood aliquot, mix gently and incubate at room temperature for 10 min. The following steps of the procedure are the same as step c to h in Protocol A.

#### Protocol C: Blood-RNAlater method

Add 1 mL RBC lysis buffer to the blood aliquot. Mix gently and incubate at room temperature for 10 min. The following steps of the procedure are the same from step c to f in Protocol A. Add 100  $\mu\text{L}$  PBS buffer, suspend the sediment from step f, add 10 volumes of RNAlater (Ambion) to stabilize the RNA (following the manufacturers' instructions). Then transfer the stabilized sample into a  $-80^{\circ}\text{C}$  freezer.

#### Protocol D: Blood-TRIzol LS method

Add 3 volumes of TRIzol LS reagent (Ambion) to the blood aliquot; lyse cells in the sample suspension by passing

the suspension several times through pipette, vortex until the lysates are totally mixed, allow for lysis for 10–30 min, at RT. Then transfer the stabilized sample into a  $-80^{\circ}\text{C}$  freezer.

#### Protocol E: freezing method

Put the tubes into a  $-80^{\circ}\text{C}$  freezer directly.

#### Protocol F: PAXgene Blood RNA tube

Blood in PAXgene Blood tubes is processed according to manufacturer's instructions, incubated at room temperature for 8 hours, and then transferred to a  $-80^{\circ}\text{C}$  freezer.

#### RNA extraction

RNA extraction methods are in accordance with the pretreatment protocols, respectively. Three methods were applied to RNA extraction: the TRIzol reagent single-step method,<sup>8</sup> TRIzol LS reagent single-step RNA isolation method,<sup>9</sup> and PAXgene Blood RNA kit.<sup>10</sup> TRIzol and TRIzol LS reagent are monophasic solutions of phenol and guanidine isothiocyanate and can maintain the integrity of the RNA while disrupting cells and dissolving cell components. The PAXgene Blood RNA kit method was performed according to the manufacturer's instructions.

The corresponding relation between the pretreatment protocol and RNA extraction methods is as shown in Table 1.

All operations were carried out in accordance to the manufacturer's instructions.

#### RNA quantitative and quality analysis

RNA purity was determined by NanoDrop (Thermo Scientific, ND-8000). RNA concentration and integrity were calculated by Agilent 2100 bioanalyzer (Agilent Technologies, G2939A) and accompanying software.

TABLE 1. RNA EXTRACTION METHODS

<i>pretreatment method</i>	<i>RNA extraction method</i>
Protocol A	TRIZol reagent single-step method
Protocol B	
protocol C	
Protocol D	TRIZol LS reagent single-step method
Protocol E	
Protocol F	PAXgene Blood RNA kit

Protocol A, Buffy coat-TRIZol method; Protocol B, RBC lysis-TRIZol method; Protocol C, RBC lysis-RNALater method; Protocol D, TRIZol LS method; Protocol E, storage at  $-80^{\circ}\text{C}$  directly without any treatment; Protocol F, PAXgene Blood RNA tube.

### Statistical analysis

Data processing and analysis were conducted using Prism GraphPad V5.0d software (GraphPad Software, CA). Results are presented as means  $\pm$  standard error of mean (SEM), with  $N=11$ . The significant difference was determined by the test and  $p$ value  $<0.05$  was regarded as significant difference.

### Results

To compare and validate the effectiveness of the six different pretreatment methods for blood RNA extraction, we evaluated the quantity and quality of isolated total RNA, including RNA yield, purity, and integrity (Table 2).

First, we determined the mean yields of total RNA extracted from the 11 samples. The mean yields of total RNA using six different blood pretreatment methods were as follows: Buffy coat-TRIZol,  $3.50 \pm 0.32 \mu\text{g/mL}$  (1.90–4.93), RBC lysis-TRIZol,  $3.29 \pm 0.30 \mu\text{g/mL}$  (1.90–4.73), RBC lysis-RNALater  $2.33 \pm 0.33 \mu\text{g/mL}$  (1.43–4.38), TRIZol LS reagent  $9.68 \pm 0.79 \mu\text{g/mL}$  (5.40–13.60),  $-80^{\circ}\text{C}$  without any treatment  $14.29 \pm 1.54 \mu\text{g/mL}$  (6.60–23.20), and PAXgene  $5.10 \pm 0.70 \mu\text{g/mL}$  (1.98–9.10). TRIZol LS reagent, PAXgene, and  $-80^{\circ}\text{C}$  without any treatment are the three methods which yielded highest total RNA.

Then we measured the RNA purity by measuring the ratios of  $A_{260}/A_{280}$  and  $A_{260}/A_{230}$ . The general rules are that the RNA samples with an  $A_{260}/A_{280}$  ratio in the range of 1.8–2.0, and  $A_{260}/A_{230}$  ratio greater than 2 are identified as high purity. No significant differences of ratio  $A_{260}/A_{280}$  ratio occurred among all of the pretreatment procedures. The  $A_{260}/A_{280}$  ratio was  $1.99 \pm 0.01$  with Buffy coat-TRIZol,

$1.99 \pm 0.01$  with RBC lysis-TRIZol,  $1.95 \pm 0.02$  with RBC lysis-RNALater,  $1.95 \pm 0.01$  with TRIZol LS reagent,  $2.05 \pm 0.01$  with PAXgene, and  $1.93 \pm 0.01$  with  $-80^{\circ}\text{C}$  without any treatment. All of the pretreatment protocols gave optimal  $A_{260}/A_{280}$  ratios. However, the ratios of the  $A_{260}/A_{230}$  for all extracted RNA samples were lower than 1.5. This could possibly be caused by the high salt/phenol concentration in TRIZol/TRIZol LS reagent, and high salt concentration in the PAXgene elution buffer.<sup>11</sup>

Finally we evaluated the RNA integrity by assessing the ratios of ribosomal 28S/18S RNA and RIN values. The optimal range for the ratio of 28S/18S is considered to be between 1.0~2.0. It can be indicative of contamination with genomic DNA or degradation of RNA if the 28S/18S ratio is greater than 2.0 or less than 1.0.<sup>12</sup> Here we demonstrated that the 28S/18S ratios for most of the pretreatment methods were within a reasonable range, except for the samples stored at  $-80^{\circ}\text{C}$  without any pretreatment, whose 28S/18S ratio was less than 1.0. The mean RIN values were  $8.20 \pm 0.16$  with buffy coat-TRIZol,  $8.01 \pm 0.16$  with RBC lysis-TRIZol,  $8.01 \pm 0.14$  with RBC lysis-RNALater,  $7.75 \pm 0.11$  with TRIZol LS reagent,  $7.76 \pm 0.10$  with PAXgene, and  $5.29 \pm 0.24$  with  $-80^{\circ}\text{C}$  without any pretreatment, respectively. Most of the RIN values were higher than 7.0 except for those stored at  $-80^{\circ}\text{C}$  without any pretreatment, indicating those pretreatment methods yield good integrity RNA.

### Discussion

With the advent of the big-data era, the preservation and access to specimens with high quality and in adequate amounts are critical for downstream systematic applications and analyses. How to achieve the goal of making full use of the specimens remains a very big challenge for researchers. The human blood specimen is an important and practical resource in the field of translational medicine research, and is often used to produce RNA, including total RNA from buffy coat and cell-free RNA. The aim of the present study was to compare five different blood pretreatment methods before freezing for total RNA extraction, and to evaluate those procedures in terms of RNA quality and quantity. For Protocol A, to make full use of the blood specimen, first we separated the buffy coat from whole blood for total RNA extraction. Other components such as plasma or red blood cells could then be used immediately or stored for long term. Protocol B (TRIZol) and Protocol D (TRIZol LS) used the

TABLE 2. RNA QUALITY CONTROLS (MEAN  $\pm$  SEM, N = 11)

<i>protocols</i>	<i>RNA yield (ug/mL blood)</i>	<i>RNA purity</i>		<i>RNA integrity</i>	
		<i>A260/280</i>	<i>A260/230</i>	<i>RIN value</i>	<i>28S/18S</i>
Protocol A	$3.50 \pm 0.32$	$1.99 \pm 0.01$	$1.35 \pm 0.16$	$8.20 \pm 0.16$	$1.44 \pm 0.07$
Protocol B	$3.29 \pm 0.30$	$1.99 \pm 0.01$	$1.40 \pm 0.12$	$8.01 \pm 0.16$	$1.55 \pm 0.07$
Protocol C	$2.33 \pm 0.33$	$1.95 \pm 0.02$	$1.10 \pm 0.16$	$8.01 \pm 0.14$	$1.14 \pm 0.05$
Protocol D	$9.68 \pm 0.79^*$	$1.95 \pm 0.01$	$1.30 \pm 0.06$	$7.75 \pm 0.11$	$1.31 \pm 0.09$
Protocol E	$14.29 \pm 1.54^*$	$1.93 \pm 0.01$	$1.26 \pm 0.08$	$5.29 \pm 0.44$	$0.75 \pm 0.14$
Protocol F	$5.10 \pm 0.70^{\text{a}}$	$2.05 \pm 0.01$	$1.30 \pm 0.16$	$7.76 \pm 0.10$	$1.41 \pm 0.11$

\*RNA is extracted from 0.25 mL blood, <sup>a</sup>RNA is extracted from 0.5 mL blood. The remainder are from 1 mL blood.

Protocol A, Buffy coat-TRIZol method; Protocol B, RBC lysis-TRIZol method; Protocol C, RBC lysis-RNALater method; Protocol D, TRIZol LS method; Protocol E, storage at  $-80^{\circ}\text{C}$  directly without any treatment; Protocol F, PAXgene Blood RNA tube.

whole blood for RNA isolation. TRIzol and TRIzol LS are both mono-phasic solutions of phenol and guanidine isothiocyanate, and RNA can be isolated by a single step. They are also used for specimens' preservation. RNeasy (Protocol C) is an aqueous, nontoxic tissue storage reagent that can stabilize and protect cellular RNA *in situ* in unfrozen specimens, and can also be used for the processing and preservation of white blood cells, including short-term storage or transportation at room temperature and long-term preservation at  $-80^{\circ}\text{C}$ .<sup>13</sup> The PAXgene Blood RNA tube (Protocol F) is a kind of blood collection tube that could stabilize intracellular RNA immediately upon blood collection. Some researchers choose to freeze the blood specimen directly at  $-80^{\circ}\text{C}$  (Protocol E) without any additives such stabilizers, to rule out the possibilities of toxicity during long-term storage and interference with other possible future assays.<sup>14</sup>

The results demonstrated that most of the isolated RNA can meet the requirements for most experiments. The data showed that RNA could be used for highly demanding gene array assays with RIN value requirements of more than 7, and may be suitable for quantitative PCR applications and gene expression profiling with RIN value requirements between 4 and 7 ([www.asterand.com/Asterand/human\\_tissues/Asterand\\_RIN.pdf](http://www.asterand.com/Asterand/human_tissues/Asterand_RIN.pdf)). The RNA extracted by Protocol A produced the highest mean RIN value (7.30–9.00), in which the plasma and the buffy coat were separated in the first step. The plasma can be preserved for further research. Protocol B, Protocol C, Protocol D, and Protocol F all yielded highly intact RNA. As for the yield, Protocol C involved adding RNeasy reagent to stabilize RNA during the RNA extraction process, and it is necessary to remove the RNeasy from the solution with extra procedures. It is possible that those procedures caused extra losses, resulting in a relatively low RNA yield (1.43–4.38). In Protocol D (TRIzol LS) and Protocol E (freezing at  $-80^{\circ}\text{C}$  without any processing), RNA was extracted from whole blood directly, thus a lot of cell-free RNA may be included, resulting in a higher yield. The freeze-thaw cycle may affect the quality of RNA, a difference for this method compared with other pretreatment methods. The RNA extracted by Protocol E without any RNA pretreatment produced poor integrity (mean RIN value 5.29). Protocol E is widely applied in China currently. Jin-Hee Kim and his co-workers<sup>6</sup> compared different RNA extraction methods from frozen blood samples including TRIzol reagent, PAXgene Blood RNA kit, and the NucleoSpin blood RNA kit. The results showed that the average RIN values of the RNA were still below 7. The explanation for the relatively low  $A_{260}/A_{230}$  ratios could be that most of reagents contain high salt concentration during the pretreatment and the extraction processes, which can lead to high absorption at 230 nm and decrease the  $A_{260}/A_{230}$  ratio. In addition, the main material costs of six protocols are also listed in supplementary material (Supplementary Table S1; supplementary material is available online at [www.liebertonline.com/bio](http://www.liebertonline.com/bio)), which can give researchers more references for their research.

In summary, the present study demonstrated that: 1) the pretreatment method of separation of the buffy coat for RNA extraction (Protocol A) yields the highest quality of total RNA (high intactness and purity), and allows for full preservation and utilization the whole blood specimen as well; 2) TRIzol LS pretreatment specimens (Protocol D)

produced the highest yield RNA with satisfactory quality. This method simplifies the specimen pretreatment procedure, but the whole blood specimen can only be used for RNA extraction. The RNA quality was also high when extracted from the whole blood samples pretreated by TRIzol (Protocol B). This method yielded lower quantity RNA but with lower cost than TRIzol LS; 3) The commercial kit PAXgene Blood RNA (Protocol F) tube is a good choice for those who cannot process the blood specimens in a timely way after collection. This method allows the specimens to be stored or transported at room temperature for a while, but can only be used for RNA extraction and might be too costly. 4) The whole blood samples treated by RNeasy produced high quality RNA, and the potential advantages of this method, including transport of pretreated blood samples at ambient temperature, could be further investigated in the future.

### Acknowledgments

We want to express our sincere thanks to our colleagues in the China National Genebank for their selfless help.

### Author Disclosure Statement

No competing financial interests exist. This research was supported by Special Fund for BGI-Yunnan's High Throughput Sequencing Platform (P.R. China, Yunnan Provincial Science and Technology Department, 2013 DA008), National Key Technology Research and Development Program (P.R. China, 2012 BAK11B06-4), The Research of High Throughput Sequencing Technology in the Application of Prevention and Control of the Mediterranean Anemia High-risk Groups (P.R. China, Yunnan Province, 2014FC003), The Cultivation of Backup Young and Middle-aged Academic Technology Leaders in Yunnan Province (P.R. China, Yunnan Province, 2014HB053) and The Application of Genomics Technology in Notoginseng Breeding (P.R. China, Yunnan Province, 2014BC005).

### References

1. Elliott P, Peakman TC. The UK Biobank sample handling and storage protocol for the collection, processing and archiving of human blood and urine. *Int J Epidemiol* 2008;37:234–244.
2. Debey S, Schoenbeck U, Hellmich M, et al. Comparison of different isolation techniques prior gene expression profiling of blood derived cells: Impact on physiological responses, on overall expression and the role of different cell types. *Pharmacogenomics J* 2004;4:193–207.
3. Hebels DG, Georgiadis P, Leun HC, et al. Performance in omics analyses of blood samples in long-term storage: Opportunities for the exploitation of existing biobanks in environmental health research. *Environ Health Perspect* 2013;121:480–487.
4. Schwochow D, Serieys LE, Wayne RK, Thalmann O. Efficient recovery of whole blood RNA—A comparison of commercial RNA extraction protocols for high-throughput applications in wildlife species. *BMC Biotechnol* 2012;12:33.
5. Kang JE, Hwang SH, Lee JH, Park do Y, Kim HH. Effects of RBC removal and TRIzol of peripheral blood samples on RNA stability. *Clin Chim Acta* 2011;412:1883–1885.
6. Kim JH, Jin HO, Park JA, Chang YH, Hong YJ, Lee JK. Comparison of three different kits for extraction of high-quality RNA from frozen blood. *Springerplus* 2014;3:76.

7. Remakova M, Skoda M, Faustova M, Vencovsky J, Novota P. Validation of RNA extraction procedures focused on micro RNA expression analysis. *Folia Biol (Praha)* 2013; 59:47–50.
8. Chomczynski P, Sacchi N. The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: Twenty-something years on. *Nat Protoc* 2006;1: 581–585.
9. Chadderton T, Wilson C, Bewick M, Gluck S. Evaluation of three rapid RNA extraction reagents: Relevance for use in RT-PCR's and measurement of low level gene expression in clinical samples. *Cell Mol Biol (Noisy-le-grand)*, 1997;43:1227–1234.
10. Chai V, Vassilakos A, Lee Y, Wright JA, Young AH. Optimization of the PAXgene blood RNA extraction system for gene expression analysis of clinical samples. *J Clin Lab Anal* 2005;19:182–188.
11. Wilfinger WW, K. Mackey K, Chomczynski P. Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. *Biotechniques* 1997;22:474–476, 478–481.
12. Le Page C, Kobel M, De Ladurantaye M, et al. Specimen quality evaluation in Canadian biobanks participating in the COEUR repository. *Biopreserv Biobank* 2013;11:83–93.
13. Weber DG, Casjeno S, Rozunek P, et al. Assessment of mRNA and microRNA stabilization in peripheral human blood for multicenter studies and biobanks. *Biomark Insights* 2010;5:95–102.
14. Peakman TC, Elliott P. The UK Biobank sample handling and storage validation studies. *Int J Epidemiol* 2008; 37:2–6.

Address correspondence to:

*Dr. Le Cheng*

*E-mail: chengle@genomics.cn*

or

*Dr. Yong Zhang*

*E-mail: zhangy@genomics.cn*

*China National Genebank*

*BGI-Shenzhen*

*Beishan Industrial Zone*

*Yantian District*

*Shenzhen 518083*

*China*