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Article *in* Research Journal of Pharmaceutical, Biological and Chemical Sciences · July 2016

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ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

High Integrity Total RNA Isolation from Human Peripheral Blood that is as Competitive to Commercialize Kits.

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ABSTRACT

A substantial problem when isolating total RNA from human whole blood is the susceptibility to degradation and inconsistent yield of RNA especially when isolation is performed from clinical samples. Here, we report an improved method for isolating high integrity of total RNA from human peripheral blood using guanidium thiocyanate-phenol-chloroform. We found that this cost effective method was able to consistently produced high yield and high integrity of total RNA with average RNA concentration > 2000 ng/mL of blood and RNA integrity number (RIN) > 8.0, as well as negligible genomic DNA contamination from human whole blood that was comparable to other commercial blood total RNA isolation kits. In addition, the present method is also suitable for blood sample that required up to 24 hr transportation time prior total RNA extraction, and able to obtain consistent result in real-time polymerase chain reaction (qPCR) downstream application.

Keywords: Total RNA isolation, Human peripheral blood, RNA integrity number, qPCR.

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ISSN: 0975-8585

INTRODUCTION

Peripheral blood offer several advantages for transcriptomic studies as it has an important role in mediating the interaction between different organs of an organism and the environment. Blood has been used as an important source for gene expression studies and may provide a deeper insight into immune response, diseases or stress metabolism [1, 2, 3]. However, a common issue faced during isolating total RNA from human whole blood is obtaining a consistently high integrity RNA and sufficient yield for subsequent process. The major concern is the effective preservation of RNA upon sample collection as it is extremely sensitive to endogenous and exogenous RNases. RNases can degrade the nucleic acid, compromised the gene expression profiles and a potential loss of transcripts immediately after sample collection.

Based on that, we have modified a conventional RNA isolation method previously reported by Chomczynski and Sacchi to a whole peripheral-based total RNA isolation approach [4]. Our improved isolation method for total RNA from human whole blood was able to obtain RNA with high yield and high RNA integrity number (RIN) value. Chomczynski and Sacchi [4] protocol was useful in extraction of RNA from cell culture but not from human whole blood as it contains red blood cells and the method did not address the contamination of gDNA that affects transcriptomic studies. Hence, red blood cell lysis and DNase-treatment was performed in this improved protocol. Moreover, the overnight precipitation of total RNA was performed to ensure maximum total RNA precipitation as compared to Chomczynski and Sacchi [4] protocol of 1 hr precipitation time.

MATERIALS AND METHODS

Sample Collection and Processing

Nine mL of blood was collected in BD Vacutainer® Plus Plastic K_2-EDTA tube (Becton and Dickinson, USA) from 4 healthy subjects and divided into 3 aliquots (3 mL each) for each respective individual. The aliquots were stored at 4°C at various time points (30 min, 6 hr, and 24 hr) before proceeding with total RNA extraction.

Total RNA Isolation from Human Whole Blood

The total RNA extraction protocol was mainly divided into three parts, including red blood cell lysis (Part A), phenol-chloroform extraction (Part B), as well as DNase-treatment and final recovery (Part C).

Part A: Red Blood Cell Lysis

Blood samples were treated with 10 mL of 1X red blood cell lysis buffer (RBCL) (contains 0.0155 M of ammonium chloride, 0.01 mM of ethylenediaminetetraacetic acid (EDTA), and 0.001 M of sodium bicarbonate), incubated at 4°C for 10 min and centrifuged at 600 xg for 10 min followed by decant. The pellet was mixed with 500 uL lysis solution A (contains 4 M of guanidium thiocyanate, 25 mM sodium citrate (pH 7.0), 50 mM Tris-hydrochloride (pH 7.6), and 10 mM EDTA) and 150 uL lysis solution B (contains 0.19 M glacial acetic acid and 0.61 M sodium acetate). The mixture was incubated and subjected to centrifugation at 12000 xg for 10 min at 4°C.

Part B: Phenol-Choloform Extraction

Supernatant was obtained from Part A and mixed with 500 uL of phenol-chloroform solution (pH 4.3 - 4.7) (Amresco, USA) and centrifuged at 12000 xg for 15 min at 4°C. The top aqueous layer was obtained and mixed with 100% isopropanol (v/v) followed by overnight incubation (~16 hr). The overnight mixture was centrifuged at 12000 xg for 15 min at 4°C. Pellet obtained was air-dried and eluded with 16 uL of diethylpyrocarbonate (DEPC)-treated water.

Part C: DNase-Treatment and Final Recovery

DNase-treatment was performed using RQ1 RNase-Free DNase Kit (Promega, USA) following manufacturer's instructions. Part B was repeated once but with final elution of 20 uL of DEPC-treated water was added, and kept at -80°C for subsequent analysis.

Total RNA Analysis and cDNA Synthesis

The yield and purity (A_{260}/A_{280} and A_{260}/A_{230}) of extracted total RNA were measured using Implen NanoPhotometer® (Implen Inc., Westlake Village, CA, USA). The RIN of the isolated total RNA was obtained using RNA 6000 Nano LabChip® on Agilent 2100 Bioanalyzer System (Agilent Technologies, Santa Clara, CA, USA) according to manufacturer's recommendations. One microgram of total RNA was reverse transcribed (RT) to cDNA using High-Capacity RNA to cDNA™ kit (Applied Biosystems, Forster City, CA, USA) according to manufacturer's instructions. Three total RNA samples were randomly selected and subjected to negative RT (-RT) control without the presence of reverse transcriptase.

Gene Expression Analysis

Gene expressions of all samples were performed in duplicates using StepOnePlus™ Real-Time PCR System using *TaqMan*® Fast Advanced Master Mix (Applied Biosystems, Forster City, CA, USA) according to manufacturer's instructions. Common reference gene [5] of pre-designed *TaqMan*® Assays of *β*-actin (*ACTB*) (Hs01060665_g1) (Applied Biosystems, Forster City, CA, USA) was used. Approximately 5 ng of synthesized total cDNA was used as the template. All amplifications were performed included a positive RT (+RT) extraction, and a no template control. -RT was also included for genomic DNA (gDNA) contamination evaluation for three randomly selected RNA sample.

Statistical Analysis

Results obtained were expressed as mean of quantitative cycle ± standard deviation (Cq ± S.D.). One-way analysis of variance (ANOVA) was performed using SPSS V17.0 (SPSS Inc, Chicago, Illinois, USA) to determine the influence of different storage gap time (30 min, 6 hr, and 24 hr) to mean of Cq yielded.

RESULTS AND DISCUSSION

Reliability and reproducibility of gene expression studies using RNA is highly affected by the integrity, purity and gDNA contamination of the isolated RNA from whole blood. Despite a number of commercial total RNA isolation kits from blood in the market are able to isolate high quality RNA, the cost of commercial kits is not affordable by some research laboratories especially in middle-income or low-income countries. Hence, this study demonstrated a cost effective total RNA isolation method from human peripheral blood with high yield and high RIN value that was comparable to commercial kits.

In this study, an additional step using RBCL buffer was performed to the previous method to lyse and eliminate red blood cells from the rest of the blood components, mainly white blood cells. Isolated white blood cells were then treated with lysis buffers in which the guanidium thiocyanate in the lysis solution A is an effective protein denaturant and can protect RNA from degradation [6, 7]. In addition, we found that by increasing the RNA precipitation time in 100% isopropanol (v/v) to about 16 hr or overnight yielded better quantity and integrity of total RNA when compared to at least 1 hr incubation time as suggested in the previous method [4].

All 12 blood samples with different storage time showed a consistently high yield and high integrity with average total RNA concentration of 2359 ng (S.D. ± 1604) per mL of blood and average RIN of 8.7 (S.D. ± 1.0), respectively, suggesting the reproducibility of the modified method in total RNA extraction from human whole blood (Table 1). Interestingly, our results revealed that the average RIN value of this study was comparable to; and some even greater than commercial blood total RNA isolation kits previously reported [8, 9, 10] (Table 2).

ISSN: 0975-8585

Table 1: Isolated total RNA quantity and quality of this study

Measurements	Overall	Range
Mean A_{260}/A_{280} (± S.D.)	1.671 (± 0.230)	1.218 - 1.952
Mean A_{260}/A_{230} (± S.D.)	2.152 (± 0.553)	1.508 - 3.320
Concentration of total RNA (ng/mL of blood) (± S.D.)	2359 (± 1604)	1154 - 5400
RIN (± S.D.)	8.7 (± 1.0)	6.3 - 10.0

Table 2: Mean of RIN value of current study and other commercial kits

Total RNA isolation kit (brand and company)	Present study	[8]	[9]	[10]
Current protocol	8.7	-	-	-
Tempus™ (Applied Biosystems, USA)	-	8.2	-	7.9
PAXgene™ (QIAGEN, USA)	-	8.7	7.7	7.9
LeukoLOCK™ (Ambion®, USA)	-	-	7.6	8.5
Trizol® LS (Invitrogen, USA)	-	-	6.2	-
RNeasy® (QIAGEN, USA)	-	-	6.9	-
RiboPure™ (Ambion, USA)	-	-	4.6	-

Taken into account the variability of blood count across different individuals, we have performed biological replicate across a total of four different individuals to evaluate the consistency of yield and integrity of total RNA using current modified method. Our results showed that the yield of total RNA varies across different subjects, which could be caused by individual's blood count variation (Table 1). However, the quality of total RNA isolated using this modified protocol was high and no indication of substantial degradation in all samples including those stored up to 24 hours at 4°C with RIN > 9.0 and distinctive peaks of 18S and 28S ribosomal RNA (Fig. 1). This finding is useful especially for study that requires a time period up to 24 hr to transport blood sample from sampling site to laboratory for processing and analysis. Our results also showed that RIN was independent to different storage time gaps prior total RNA isolation in one-way ANOVA test in this study ($p > 0.05$), indicating that blood sample stored from 30 min and up to 24 hr before RNA isolation did not affect the RIN of the total RNA extracted using this modified method.

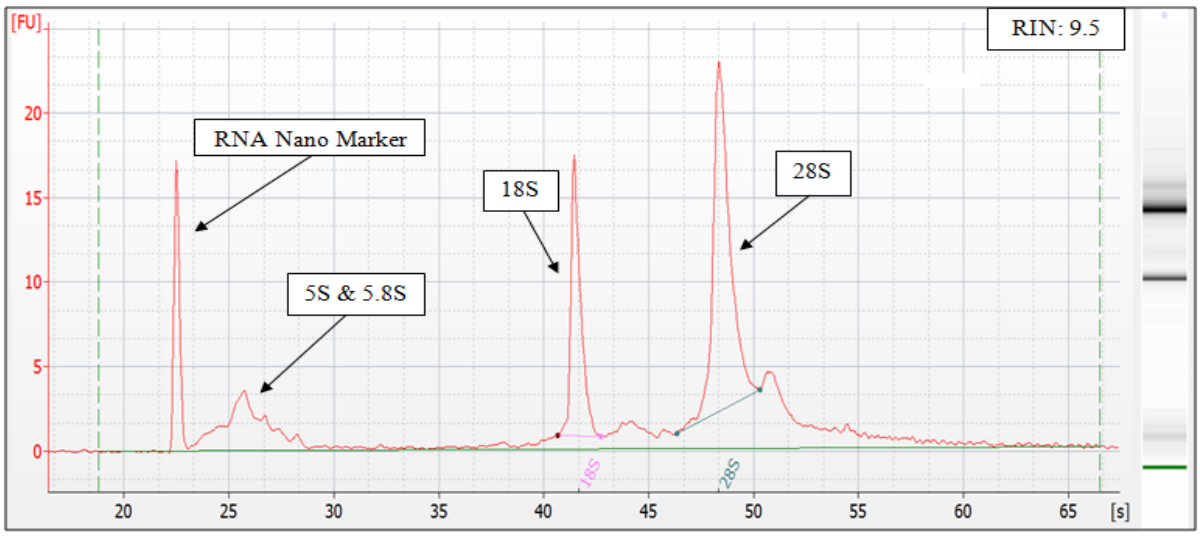

Figure 1: Intact high integrity of total RNA obtained from Agilent 2100 Bioanalyzer System. An example of the electropherogram and electrophoretic tracings from human peripheral blood extracted showed RIN of 9.5 after 24 h storage time gap

ISSN: 0975-8585

The total RNA isolated using this protocol was in good quality for subsequent downstream molecular analysis where the minimal recommended RIN value of higher than 5.0 is required for reliable qPCR applications was achieved [11,12]. Samples subjected to qPCR amplification of *β*-actin were successfully amplified for all 12 samples. Importantly, the Cq values of the amplification plot that were ranged from 21.18 to 21.53 indicating that *β*-actin expression does not exhibit large variation caused by the different storage time gap before total RNA isolation as depicted in one of the example (Fig.2). This total RNA isolation method is able to isolate high quality RNA and low variation in gene expression analysis but this study did not address a different target gene expression to evaluate the variation.

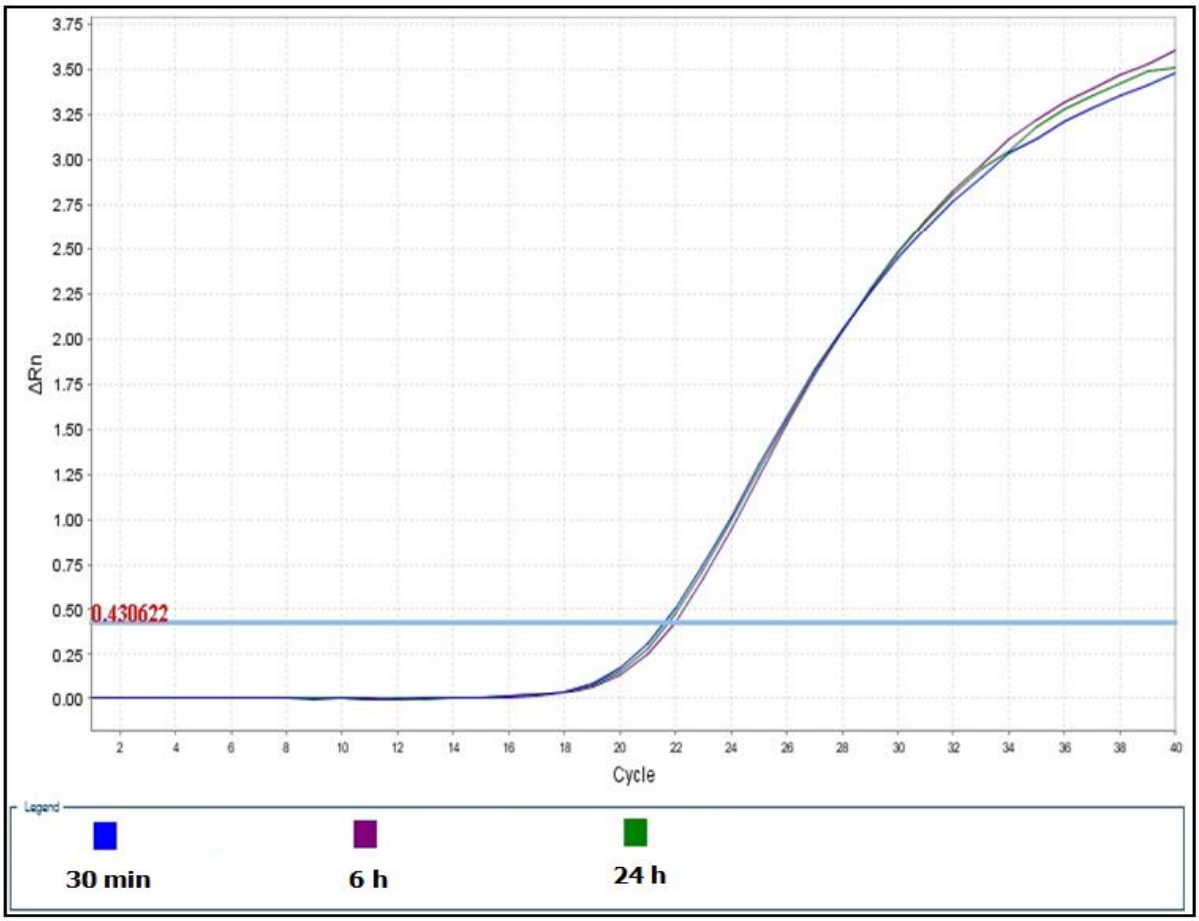

Figure 2: qPCR output of RNA isolated from human whole blood. The cDNA amplification of a representative sample for 30 min, 6 h, and 24 h were determined by the C_q values against the threshold value (0.430622) that was generated by the StepOnePlus™ PCR Real-Time System

The percentage of gDNA contamination in the total RNA samples isolated from this study was ≤ $1.0x10^{-3}$% (data not shown), indicating the method could eliminate a majority of gDNA contamination. The contamination of gDNA in RNA samples could cause erroneous results in microarray expression and qPCR applications such as absolute quantification of RNA [13,14]. gDNA contamination is unavoidable but can be minimized by treating RNA samples with DNase. Therefore, this modified protocol utilized DNase to treat our RNA sample when compared to previous method [4].

CONCLUSIONS

In summary, our method is cost effective and able to consistently isolate high quantity and high integrity total RNA from human peripheral blood, and is competitive to commercial blood total RNA extraction kits. This modified method is also useful for blood sample oblige a time period up to 24 hr for transportation to the laboratory for RNA extraction. RIN value of total RNA extracted using this method was high and eliminate gDNA contamination effectively which is suitable for subsequent molecular analysis such as qPCR.

ISSN: 0975-8585

ACKNOWLEDGEMENTS

We would like to thank all volunteers involved in this study.

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