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Short communication

Effects of RBC removal and TRIzol of peripheral blood samples on RNA stability

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ABSTRACT

Background: Purification of mRNA from stored specimens is very important because results from RT-PCR and microarray analyses are largely affected by the quality of mRNA. Moreover, many preanalytical factors during collection, processing, and storage may affect mRNA quality and the expression of peripheral blood mononuclear cells (PBMC). In this study, we evaluate the effects of RBC removal techniques and TRIzol on RNA quality in blood samples.
Methods: We obtained EDTA-blood samples from 50 adult volunteers, and made 10 pools of buffy coats for comparison between protocols and also evaluated RNA quality of clinical samples in biobank. Use of TRIzol and RBC removal (RBC lysis or cell separation) were evaluated their effect on the quality of mRNA from the stored blood samples.
Results: RNA integrity with TRIzol was significantly better than that without TRIzol (RIN 4.5 vs. 9.2, respectively; $P = 0.002$). The change in RIN of the PBMC separation method was equivalent to that of the RBC lysis method. After 12 months, *IL6* mRNA expression from stored clinical samples in cell separation/TRIzol was stable.
Conclusions: The blood samples frozen in TRIzol after RBC removal preserved RNA quality well. PBMC/TRIzol preservation for storage of blood samples could be a simple protocol for rapid, low-cost biobanking.

1. Introduction

Whole blood stored in biobanks is increasingly considered a valuable source of RNA for gene expression profiling [1]. However, results from RNA studies are largely affected by the quality of mRNA. Many pre-analytical factors such as the time of collection, sampling tubes, preservatives, transportation condition, storage condition and purification methods affect the quality of mRNA [2]. Although introduction of blood collection systems such as the PAXgene Blood RNA tubes (PreAnalytiX, Quiagen, Hilden, Germany) have significantly improved the quality of RNA isolated from blood samples [3], PAXgene system might be too costly to be used for long-term storage of all cohort samples. In UK Biobank, they are currently not including reagents in the blood samples that actively prevent degradation of RNA due to interfering with future assay and the cost of collecting and processing[2]. TRIzol reagent (Invitrogen, Carlsbad, CA, USA) is widely used for the isolation RNA and often for preservation, by placing fresh samples into TRIzol for long term storage. However, there are few studies which have evaluated the effect of long-term TRIzol storage on blood samples in the aspect of RNA integrity.

In this study, we investigated the suitability of TRIzol-based storage for isolation of RNA from long-term stored blood samples. We also evaluated the effect of RBC removal techniques (RBC lysis and cell separation) on RNA recovery from storage.

2. Methods

For comparison, EDTA-blood samples were collected from 50 healthy adult volunteers between January and May 2010. The separated buffy coats was pooled from every 5 samples and made 10 pools, which made several aliquots used to compare protocols. From clinical samples in the Pusan National Hospital Biobank, we randomly selected 15 clinical samples for long-term (12 months) RNA quality which were processed and stored in TRIzol after cell separation.

The study was composed of three experiments in Fig. 1; the effect of TRIzol (frozen with TRIzol vs. without TRIzol), the effect of RBC removal techniques on RNA degradation, and the prospective effect

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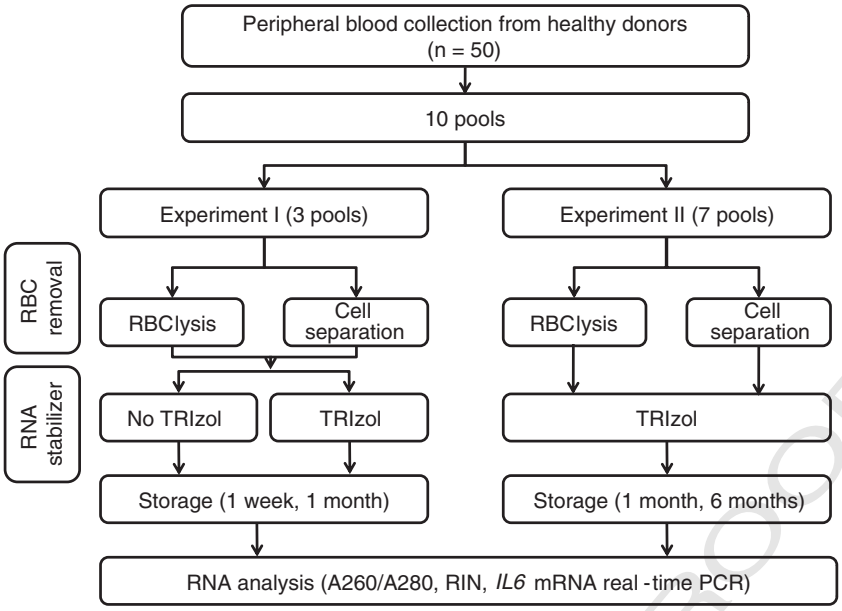

Fig. 1. Flow chart of the two experimental methods used to evaluate RNA stability.

on clinical samples. First, three pools were used to compare paired aliquots frozen without TRIzol (Protocol A— No TRIzol/frozen) and paired aliquots frozen in TRIzol (Protocol B— TRIzol/frozen) (Fig. 1). RBC lysis with lysing solution (hypotonic buffer) or mononuclear cell separation was performed in each pool before being frozen. Second, seven of the ten pools were used to compare the effect of RBC lysis and mononuclear cell separation before freezing in TRIzol (Fig. 1). Before the aliquots of each pool were frozen in TRIzol, we removed RBCs by using RBC lysing solution (Protocol C— RBC lysis/TRIzol), and separated peripheral blood mononuclear cells (PBMC) using the Ficoll–Paque solution (Sigma, St. Louis, MO, USA) (Protocol D— PBMC/TRIzol). Third, fifteen stored clinical samples of thyroid cancer according to Protocol D— PBMC/TRIzol were analyzed for RNA quality after 12 months.

2.1. RNA isolation and quality assessment

RNA purity and concentration were determined by the absorbance at 260 nm (A260) and 280 nm (A280) using NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA). RNA integrity was determined by RNA integrity number (RIN; Agilent 2100 RIN Beta Version Software). All measurements were duplicated.

2.2. RNA expression analysis

Interleukin 6 [*IL6*] mRNA was also measured by real-time RT-PCR. The primer used for *IL6* was the Hs_IL6_1_SG QuantiTect Primer Assay, QT00083720 (Qiagen, Hilden, Germany), and for β-actin (*ACTB*) was Hs_ACTB_1_SG QuantiTect Primer Assay, QT00095431 (Qiagen, Hilden, Germany). The Ct of the target genes (*IL6*) was normalized to C_T of *ACTB*. The fold change of mRNA was calculated by $\Delta\Delta C_T$ method [4].

3. Results

Median and range of RNA yield, purity and integrity according to stabilization protocols were showed in Table 1. RNA integrity after storage with TRIzol was significantly better than without TRIzol (paired *t*-test, $P = 0.002$, Fig. 3). Shown in Fig. 2 are typical results of the integrity of the RNA for stored in each protocol. RNA yield, A260/A280 ratio and RIN of individual clinical samples ($n = 15$) from storage by Protocol D— PBMC/TRIzol for 12 months were 65.8 μg/μL (26.5–168.5 μL), 1.7 (1.6–1.9) and 9.2 (8.8–10.0), respectively.

The fold changes of *IL6* mRNA after 6 months storage by Protocol C— RBC lysis/TRIzol and Protocol D— PBMC/TRIzol were 2.19 ± 2.43 and 1.18 ± 1.64. There was no statistically significant change between RBC removal techniques (paired *t*-test, $P = 0.319$). For clinical samples stored by Protocol D— PBMC/TRIzol, the fold changes of IL6 mRNA expression after 12 months were 0.81 ± 0.78.

4. Discussion and conclusions

The current study demonstrated that RNA removal and TRIzol storage of whole blood samples before isolation of RNA do not significantly affect the yield and quality of the RNA extracted. Unpreserved mRNA of whole blood is well known to be readily degraded [3]. The PAXgene system is suitable for large-scale gene expression profiling, particularly where immediate sample processing is not always practical. Although the PAXgene apparatus is good for RNA stability, its use is not

Table 1
Median and range of RNA yield, purity and integrity according to stabilization protocols.

Protocols	RNA yield (ng/uL)	RNA purity (A260/A280)	RNA integrity (RIN)
Protocol A— No TRIzol/frozen (1 month)	275.5 (212.5–539.0)	1.2 (0–2.3)	4.5 (2.6–6.5)
Protocol B— TRIzol/frozen (1 month)	131.8 (70.5–264.5)	1.6 (1.3–2.0)	9.2 (8.6–9.8)
Protocol C— RBC lysis/TRIzol (6 months)	200.0 (118.5–323.5)	1.9 (1.8–2.0)	8.8 (7.2–9.3)
Protocol D— PBMC/TRIzol (6 months)	146.5 (109.0–386.0)	2.0 (1.9–2.0)	9.4 (9.2–9.8)

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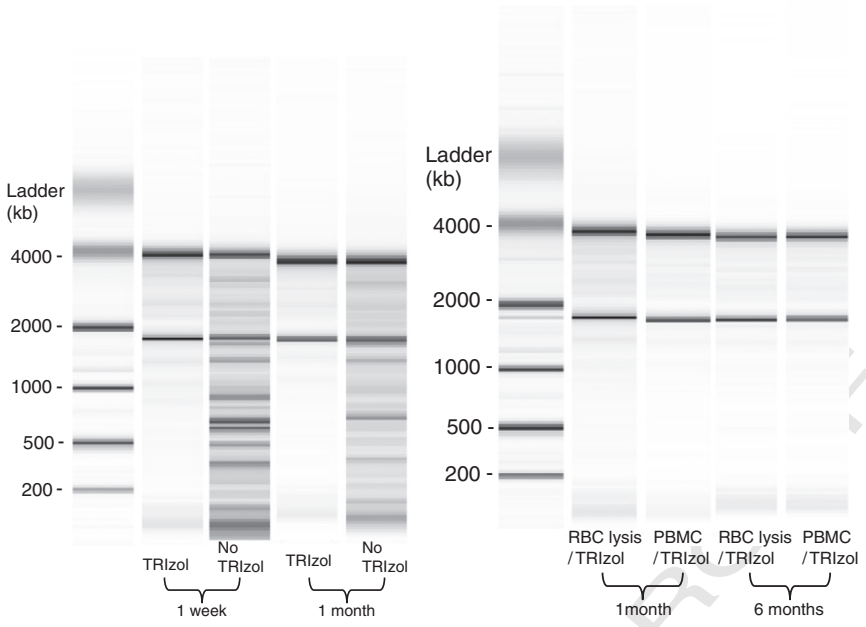

Fig. 2. Total RNA integrity for samples from whole blood that was stored according to protocols.

cost-effective unless most of the samples stored in PAXgene system are used.

In our study, after RBC lysis or isolation with PBMCs, the samples were stored in TRIzol at −70 °C. Adding TRIzol to RBC lysed or PBMC isolated samples preserves the integrity of RNA that has been extracted from frozen long-term storage blood samples. Because blood contains a variety of cell types, the PBMC which deplete other cell types and collect relative homogenous cell population such as T or B lymphocytes would be better than RBC lysis before storage in terms of the clinical utility of mRNA expression [1].

TRIzol, a monophasic solution of phenol and guanidine isothiocyanate, was developed for obtaining high-quality total RNA at a high yield from samples that have previously been in long term storage [5]. Expression of inflammatory cytokine genes is known to be affected by long-term storage, or by a change in the sample environment after phlebotomy [6]. Thus, minimizing changes in gene expression during blood sampling and storage has very important implications for clinical studies on cytokine production and the cellular immune response in blood. In this study, we compared the effects of RBC removal techniques on the change of expression of the *IL6* gene, a well-known cytokine gene family. There was no statistical difference in the *IL6* mRNA expression isolated from samples frozen in TRIzol regardless of RBC removal techniques (RBC lysis or PBMC) and the *IL6* mRNA expression did not significantly change over the storage period. Pahl et al. reported that *IL6* mRNA increased up to 30-fold without stabilizer [6]. However, we did not perform the microarray for gene expression profiling nor evaluate the mRNA changes of many cytokines.

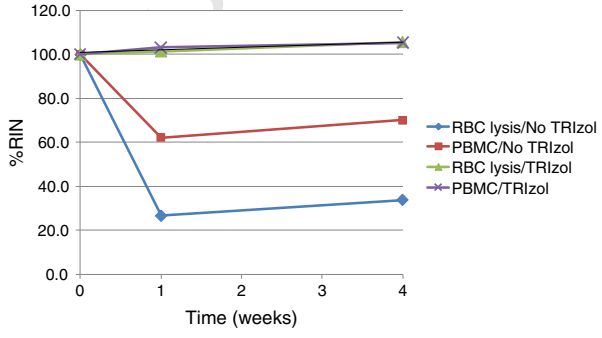

Fig. 3. Changes of RIN of total RNA from blood stored in TRIzol or without TRIzol at 1 week and 4 weeks.

In conclusion, the blood samples frozen in TRIzol after RBC removal preserved RNA quality well. PBMC/TRIzol preservation of whole blood for storage could be a simple protocol for rapid, low-cost biobanking without specialized collection system, such as the PAXgene Blood RNA System.

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