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Comparison of two RNA isolation methods for determination of SOD1 and SOD2 gene expression in human blood and mononuclear cells

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In the current study, two RNA isolation techniques were compared and their abilities to produce high-quality RNA were evaluated. mRNA expression profiles of SOD1 (Cu/Zn superoxide dismutase) and SOD2 (Mn superoxide dismutase) genes were measured by real-time PCR. From a pool of fresh human citrate-whole blood and ten healthy individuals, RNA was isolated with the TRIzol™ extraction method (TRI) and with the ABI PRISM™ 6100 Nucleic AcidPrepStation (ABI). The concentration and purity of RNA extracts were determined spectrophotometrically. RNA integrity was evaluated by electrophoresis on a 1% agarose gel. PCR was performed on a 7500 Real-Time PCR System. The student's *t*-test was applied to compare normally distributed variables. Both protocols gave similar RNA quantities when adjusted to the initial blood volume. Relative quantification values obtained from the TRI method for SOD1 were significantly higher ($p<0.01$) and for SOD2 were significantly lower ($p<0.05$) as compared to those obtained from the ABI method, respectively. Coefficients of variation (CV) for gene expression parameters in SOD1 and SOD2 analyses were lower when the TRI method was used. The TRI method was generally more consistent in yielding pure RNA in comparison to the ABI and better reproducibility in gene expression analyses was apparent.

Keywords: AbiPrism™, blood, PBMC, real-time PCR, SOD, TRIzol™

Introduction

Whole blood is a readily available source of human tissue for gene expression analyses and a valuable clinical sample for diagnosis and prediction of diseases[1,2]. Genetic information primarily resides in white blood cells and in reticulocytes, which comprise 0.1% and 5% of the total blood cells, respectively[3]. The main obstacles during RNA isolation from whole blood are exposure to ribonucleases, high protein and DNA content. It is very important to prepare RNA samples of a high quality and satisfactory quantity for real-time polymerase chain reaction (PCR) application[4], which is one of the most sensitive, accurate and reproducible methods for quantification of messenger RNA (mRNA)[5]. RNA may be rapidly degraded *ex vivo* by endogenous and exogenous nucleases, therefore it is highly recommended that the blood is immediately and adequately stabilized upon sampling or processed for RNA isolation[6,7].

Besides the usual biological factors (age, gender and other aspects of human condition), technical factors including method of blood collection, sample handling, RNA stabilisation, storage, isolation and purification steps can greatly influence the results of gene expression profiling[1,2,8-10]. Furthermore, various manipulation steps during RNA processing can represent a possible source of cross contamination.

Protocols for total RNA isolation from blood vary from laboratory to laboratory. Accordingly, each laboratory needs to establish its own method. There are many different ways to extract total RNA from biological material[2,9,10]. In the present study, authors evaluated the extraction performance of two established RNA extraction protocols: (a) a commonly used manual extraction method using TRIzol™ RNA isolation reagents (TRI) (Invitrogen Life Technologies, USA) from peripheral blood mononuclear cells (PBMC); and (b) a semi-automated vacuum extraction system on an ABI Prism™ 6100 Nucleic Acid PrepStation™ (ABI) (Applied Biosystems, USA) from fresh human whole blood, focusing on two parameters, yield and purity of RNA. Real-time PCR was employed to determine the effect of these extraction methods on mRNA gene expression patterns of two isoenzymes that play a central role in the anti-oxidative defence system,

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SOD1 (Cu/Zn superoxide dismutase or superoxide dismutase isoenzyme 1) and SOD2 (Mn superoxide dismutase or superoxide dismutase isoenzyme 2)[11].

Materials and Methods

Blood Sample Collection

Venous blood was drawn from the antecubital vein after overnight fasting from 10 individuals (5 males and 5 females) matched by age (31.3±0.9 years old). The time of blood sampling was 9.00 AM. All the individuals were found to be healthy on the basis of routine blood examinations and they were not taking any drugs including over-the-counter medication or nutritional supplements. They were non-smokers and provided their written informed consent for participation in the study after receiving a full explanation of the study procedures. The institutional review committee of the Faculty of Pharmacy, University of Belgrade approved the study protocol.

The blood taken from every study participant was kept in two sodium citrate-coated 5 mL tubes. Both tubes were used for RNA extraction employing two different extraction protocols—one for RNA extraction with TRI extraction reagents and the second for RNA extraction on the ABI. Extracted RNA was stored at −80°C. Using the above approach, it was able to compare the two RNA extraction protocols in all the participants. In addition, bloods from 5 citrate vacutainer tubes drawn from one healthy female donor were pooled. The pool was divided into ten parts; five for RNA extraction using TRI and five for RNA extraction using ABI within 15 min of venipuncture. The samples from the pool were used to estimate the coefficient of variance (CV) and to determine the effects of RNA isolation methods on gene expression.

In order to attenuate variables, such as, age, gender, time of blood sampling and health status, which might influence gene expression profiling[1,8], all analyses were performed on replicates from a single blood pool from the same individual and then confirmed in a homogenous group of healthy non-smoking adults matched by age, gender and social habits and not under any medical treatment.

RNA Isolation Protocols

TRI Extraction

PBMCs were isolated using Ficoll-Paque™ PLUS (GE Healthcare, USA) immediately after collection of blood using a modified protocol designed to minimise sample processing time, maximise the consistency of processing between samples and to avoid contamination with erythrocytes. Modifications consisted of slightly changed velocities and times of centrifugation compared to the manufacturer's instructions. Citrate whole blood was centrifuged 10 min at 750 relative centrifugal force (rcf) for plasma separation. Plasma, lymphocytes' layer and some erythrocytes in total volume of 2 mL were transferred on Ficoll-Paque™ PLUS gel and separated by 20 min centrifugation at 1000 rcf. After buffy coat removal, lymphocytes' pellet was washed out twice for 5 min at 750 rcf using phosphate buffered saline (PBS). After isolation, the PBMCs pellet was suspended in 1mL of TRIzol™ reagent. The TRI extraction protocol was also slightly modified. The time for RNA extraction with chloroform at +4°C was extended to 15 min compared to the suggested 3 min to allow better separation of RNA from proteins. RNA was precipitated with the same volume of isopropanol (500-600 µL) as the volume of aqueous phase from the chloroform extract. After addition of isopropanol, the tubes were incubated at room temperature for 30 min (the Invitrogen protocol suggests only 10 min) for better precipitation and higher yield of RNA. The RNA pellet was visible on the side of each tube and was dissolved in 50 µL of PCR grade water. Before experimental set up, all these slightly modified steps were evaluated and found better than the originally proposed.

ABI Extraction

Five blood lysates from the pooled sample and lysates from all the 10 participants were prepared as follows. 750 µL of whole fresh blood was diluted with 750 µL of PBS and then mixed with 1500 µL 2× nucleic acid purification lysis solution (P/N 4305895). Lysates were frozen at −80°C for 1 d prior to extraction. The maximum quantity of blood lysates (3000 µL) was purified on the instrument employing the Ultra-low gDNA protocol using absolute RNA wash solution (P/N 4305545). Extraction steps were performed according to the manufacturer's recommendations and 150 µL of RNA solutions were collected[12].

RNA Quality and Quantity Assessment

RNA from the pooled and individual samples were quantified by absorbance spectrometry at 260 nm and expressed as µg per mL. The ratios at 260 nm and 280 nm (A260/280) as well as 260 nm and 230 nm (A260/230) were used to assess the protein and organic purity of the RNA solutions, respectively. RNA integrity was evaluated by electrophoresis. A similar amount (400 ng) of each RNA sample was run on a 1% native agarose gel[13]. Horizontal submarine

electrophoresis was performed at 140 V (170 mA) for 15 min using a EPS 601 power supply (Amersham Pharmacia Biotech Inc., USA). The 18S and 28S ribosomal RNA (rRNA) bands were visualised with ethidium bromide on a MacroVue UV-25 Transilluminator (Hoefer Inc., USA) at 306 nm.

All extracted RNAs were then stored at −80°C prior to reverse transcription (RT) reactions.

RT and Real–Time PCR Analyses

To determine the performance of isolated RNA in a real-time PCR setting, aliquots of pooled and individual RNAs were amplified using specific primers corresponding to regions of human SOD1, SOD2 and β-actin. RT and real-time PCR experiments were carried out on the 7500 Real-Time PCR System (Applied Biosystems, USA) using TaqMan® reagent-based chemistry.

RT was performed with MultiScribe™ reverse transcriptase, random primers and RNase inhibitor using high-capacity cDNA reverse transcription kit (Applied Biosystems, USA) following the manufacturer's instructions. RNA was added in 2× RT master mix and each reaction mixture was incubated with the following three-step thermal protocol: At 25°C for 10 min, at 37°C for 120 min and at 85°C for 5 sec. 500 ng of RNA was transcribed into single stranded cDNA in a 20 µL reaction volume. TaqMan® control total human RNA (P/N 4307281; Applied Biosystems, USA), used as a calibrator in the relative gene expression method, served as a positive control. Synthesised cDNAs were kept at −20°C.

The real-time quantitative assessments were performed using TaqMan® 5′-nuclease gene expression assays (Applied Biosystems, USA) for SOD1 (Hs00533490_m1) and SOD2 (Hs00167309_m1) genes. In order to correct sample-to-sample variation, human β-actin (Hs99999903_m1) was used as a housekeeping gene. Gene expression measurements were performed in triplicate for each cDNA sample. 5 µL of cDNA was amplified in a 25 µL reaction volume. Every experimental plate included one reaction for each gene with PCR grade water as a negative control for cross contamination. Amplification cycling was carried out in a thermal cycler under the following conditions: AmpErase®UNG activation for 2 min at 50°C, followed by AmpliTaq Gold® DNA polymerase activation for 10 min at 95°C and then 40 cycles of denaturation for 15 sec at 95°C, followed by annealing/extension for 1 min at 60°C. Negative controls for RT (no reverse transcriptase) and for real-time PCR (no cDNA) were included in the experiments.

Cycle threshold (Ct) values were obtained for each sample and were normalised for each plate by subtracting the mean Ct value for gene of interest (SOD1 or SOD2) and Ct value for β-actin, which gave the ΔCt value. The normalised values were compared to control values (calibrator) to assess the relative expression level (RQ) of a given mRNA using the following formula $RQ=2^{-\Delta\Delta Ct}$, where ΔΔCt was calculated as the difference between $\Delta Ct_{(SOD1 \text{ or } SOD2),\ \beta\text{-actin}}$ and $\Delta Ct_{calibrator,\ \beta\text{-actin}}$. The fold change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ relative quantitation method[14]. The results were analysed in real-time mode using SDS Version 1.4.0.25 software. Baseline fluorescence levels and thresholds were determined automatically by this software and manually by the user[15]. In manual settings (MS) normalised background fluorescence of cycles 3-11 was for β-actin and 3-13 for SOD1 and SOD2. In automatic setting (AS) mode normalised background fluorescence was 3-15 cycles for all genes. All the reagents and supplies were sterile, nuclease free and molecular biology grade.

Statistical Analysis

Distribution of variables was normal according to the Kolmogorov-Smirnov test. For the evaluation of data from RNA isolation methods, the Student's *t*-test was used. The Student's paired *t*-test was applied to detect differences between two isolation methods in ΔCt and RQ of selected genes. Results are reported as the mean±standard deviation (SD). Different isolation techniques and values obtained from gene expression analyses were tested for imprecision (data shown as CV, %). Differences in means or magnitude of a varying quantity between the methods were also calculated as the root mean square (RMS). Two-tailed P values less than 0.05 were considered statistically significant. All the analyses were conducted using the MedCalc® (Mariakerke, Belgium) Version 11.4.4.0 and Microsoft® Office Excel 2007.

Results

The TRI isolation method gave significantly higher ($p<0.001$) RNA concentrations as compared to the ABI method (Table 1). However, when RNA yields were adjusted with respect to the starting blood volumes used, which were 2 mL for TRI and 0.75 mL for ABI (see Material and Methods), no significant differences in RNA quantity were found. Protein-free RNAs were obtained with both the methods as A260/280 ratios were found to be higher than 1.8;

Table 1—Characteristics of RNA obtained from the pooled sample (n=5)

	TPI		ABI	
	Mean±SD	CV (%)	Mean±SD	CV (%)
RNA conc., µg/mL[a]	102.08±8.83	8.65	34.16±2.34[b]	6.85
A260/280 ratio	1.98±0.05	2.52	1.91±0.08	4.19
A260/230 ratio	2.03±0.08	3.94	0.53±0.09[b]	16.98

[a]Concentrations were determined by absorbances measured at 260 nm.
Values are presented as mean±SD.
[b]p<0.001, P value for Student *t*-test.
TRI=TRIzol™ RNA isolation reagents; ABI=ABI Prism™ 6100 Nucleic Acid PrepStation™; CV=Coefficient of variation.

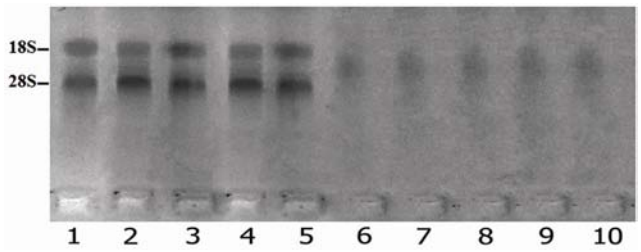

Fig. 1—Electrophoretic bands of rRNA isolated with different methods on a 1% agarose gel: Lanes 1-5, rRNA bands represent total RNA isolated by TRI method; lanes 6-10, rRNA bands represent total RNA isolated by ABI method. [TRI=TRIzol™ isolation reagents method; ABI=ABI Prism™ 6100 Nucleic Acid PrepStation. All RNAs were obtained from the same pooled sample.]

comparatively, TRI-processed samples yielded a higher purity of RNA as having significantly higher A260/230 ratios. No phenolic, sugar or alcoholic contamination was reported as A260/230 ratio was greater than 1.9 in TRI samples. The CVs for RNA concentrations as well as for the absorbance ratios from both the isolation methods were less than 10%, except for A260/230 of the RNA isolated by the ABI platform (Table 1). The TRI isolation method gave high quality RNA as indicated by visible rRNA bands of 28S and 18S on native agarose gels (Fig. 1). The presence of these bands was persuasive evidence of the integrity of the samples, which meant that samples had not been degraded[13]. These rRNA bands were within the expected ratio[13] and no fragments were observed that could represent genomic DNA (gDNA). However, no rRNA bands were discernible in ABI-prepared samples suggesting RNA degradation (Fig. 1).

The ΔCt and RQ values obtained from the pooled RNA samples isolated with the two different techniques were compared. The SDS software sets the baseline and threshold automatically and permits the user to set them manually[15]. First, the differences for mean ΔCt and RQ were made between AS and MS set up in each method. In both TRI and ABI isolation methods, highly significant differences were observed for ΔCt (p<0.001) between AS and MS set up for both the genes. However, no significant differences were observed for RQ values (Table 2). Second, the ΔCt

Table 2—Comparison of data obtained from the SDS software for SOD1 and SOD2 gene expression analysis from the pooled sample (n=5)

	TRI-AS	TRI-MS	P	ABI-AS	ABI-MS	P
SOD1						
ΔCt	4.86±0.30 (6.17)	10.1±0.31 (3.05)	<0.001	5.87±0.51[a]** (8.67)	10.67±0.45 (4.32)	<0.001
RQ	0.30±0.06 (20.77)	0.25±0.05 (19.53)	0.189	0.11±0.04[a]** (34.85)	0.14±0.04[b]** (29.62)	0.427
SOD2						
ΔCt	3.61±0.38 (10.54)	8.98±0.26 (2.94)	<0.001	2.42±0.60[a]** (24.57)	7.85±1.03[b]* (48.08)	<0.001
RQ	2.08±0.54 (26.05)	1.73±0.30 (17.36)	0.243	5.22±1.88[a]** (35.95)	5.11±2.41[b]* (47.12)	0.944

Values are presented as mean±SD (CV, %).
[a]Statistically significant difference between TRI-AS and ABI-AS by Student *t*-test.
[b]Statistically significant difference between TRI-MS and ABI-MS by Student *t*-test.
*p<0.05; ** p<0.01.
TRI-AS=TRIzol™ isolation reagents-automatic setting mode; TRI-MS=TRIzol™ isolation reagents-manual setting mode; ABI-AS=ABI Prism™ 6100 Nucleic Acid PrepStation™ isolation method-automatic setting mode; ABI-MS=ABI Prism™ 6100 Nucleic Acid PrepStation™ isolation method-manual setting mode; CV=Coefficient of variation; Ct=Cycle threshold; $\Delta Ct=Ct_{\text{gene of interest}}-Ct_{\beta\text{-actin}}$; $\Delta\Delta Ct=\Delta Ct_{\text{gene of interest, }\beta\text{-actin}}-\Delta Ct_{\text{calibrator, }\beta\text{-actin}}$; RQ (relative expression level)$=2^{-\Delta\Delta Ct}$.TaqMan® control total human RNA was used as calibrator.

and RQ mean values for AS mode in TRI method were compared for corresponding values of the same mode in ABI method. In a similar manner, values for MS mode were also compared in both the methods. The SOD1 RQ values obtained by the TRI method were significantly higher than those obtained by the ABI method in both AS and MS modes ($p<0.01$). In contrast, the SOD2 RQ values for both AS and MS modes ($p<0.01$ & $p<0.05$, respectively) obtained by the TRI method were significantly lower than those obtained by the ABI method (Table 2).

The CVs (%) for ΔCt and RQ obtained in both the modes (AS & MS) from the TRI isolation protocol were lower as compared to those obtained from the ABI isolation protocol for SOD1 gene. Similar trends were also observed for SOD2 gene. The lowest CVs were noticed for the data obtained in TRI-MS in case of both the genes; while the highest CVs were observed for the data obtained in ABI-AS for SOD1 gene and in AMI-MS for SOD2 gene (Table 2).

To test gene expression parameters between the two methods, whole blood was collected from 10 healthy individuals and RNA was isolated using the same methods. The TRI method gave significantly higher RNA concentrations (100.8±46.68 µg/mL) compared with the ABI method (37.7±10.14 µg/mL) ($p=0.007$). However, when adjusted for blood collection starting volumes, which were 2 mL for TRI and 0.75 mL for ABI, RNA yields were similar and no significant differences were observed in RNA yields. Both methods gave protein-free RNA solutions based on satisfactory A260/280 ratios. RNA specimens obtained with the TRI method had a statistically higher organic-purity based on the A260/230 ratio ($p<0.001$). Gel electrophoresis of the TRI samples indicated 2 bright 28S and 18S rRNA bands with a ratio of intensities of approximately 2:1. Samples from the ABI were not visible on the gel (data not presented).

With respect to the gene expression analysis results obtained from the pool, the two RNA extraction methods were compared only using MS mode in the group of healthy subjects. All the calculated parameters for both genes showed statistically significant differences between the two methods (Table 3). The RQ values for SOD1 gene was significantly higher ($p=0.002$) in TRI-RNA samples (0.23±0.09) compared to ABI-RNA samples (0.09±0.03). However, the converse was true in case of SOD2 gene ($p=0.002$). Further, no significant differences were observed in the calculated parameters between males and females when they were compared within each RNA extraction method (data not presented).

Table 3—SOD1 and SOD2 gene expression parameters obtained from healthy individuals (n=10; 5 females and 5 males)

	SOD1			SOD2		
	TRI-MS	ABI-MS	P	TRI-MS	ABI-MS	P
ΔCt	10.56±0.77	11.22±0.32	0.024	10.13±0.77	6.83±0.50	<0.001
RQ	0.23±0.09	0.09±0.03	0.002	1.63±0.61	9.83±5.72	0.002

Values are presented as mean±SD; p for Student's paired *t*-test.
TRI=MS TRIzol™ isolation reagents-manual setting; ABI-MS=ABI Prism™ 6100 Nucleic Acid PrepStation™ isolation method-manual setting; Ct=Cycle threshold; $\Delta Ct = Ct_{\text{gene of interest}} - Ct_{\beta\text{-actin}}$; $\Delta\Delta Ct = \Delta Ct_{\text{gene of interest, } \beta\text{-actin}} - \Delta Ct_{\text{calibrator, } \beta\text{-actin}}$; RQ (relative expression level)$=2^{-\Delta\Delta Ct}$.
TaqMan® control total human RNA was used as calibrator.

All the parameters were also calculated according to the AS mode for both methods (data not presented) and used for RMS estimation. To quantify the differences in means between both RNA extraction methods in AS and then in MS modes, RMS was calculated. The RMS of ΔCt for TRI-MS and ABI-MS were less compared to those for TRI-AS and ABI-AS for both SOD1 and SOD2 (data not presented). The most important one was the 15% lower RMS for RQ (TRI-MS & ABI-MS; 0.17) compared with the RMS for RQ (TRI-AS & ABI-AS; 0.2) in SOD1 analysis. However, no differences in RMS (9) for RQ were obtained in SOD2 analysis.

Discussion

To define a reliable method that allows us to isolate high-quality RNA from whole blood and PBMCs for real-time PCR analysis, we evaluated the ABI semi-automated workstation and a manual extraction TRI method. To date there has not been any report about the comparison between the two methods of RNA extraction concerning yield, purity of RNA and results of gene expression analyses of SODs' isoenzymes. The present study describes the results of an evaluation focusing mainly on the yield and purity of RNA and its performance in real-time PCR. Both methods gave adequate RNA amounts as expected[10]. Spectrophotometric parameters and electrophoretic profiles indicated impurity and degradation of the RNA in pooled and healthy donor samples from the ABI (Table 1; Fig. 1). Despite the extent of RNA degradation and presence of organic contaminants in the ABI samples, amplification of SOD1, SOD2 and β-actin was hardly affected (Tables 2 & 3). Possible explanations might be that the amplified portion of the target RNAs still remained intact in the extracted

samples and only small regions were amplified for each gene (less than 200 base pairs)[4]. Furthermore, *in situ* stability of rRNA differs from mRNA. Complete degradation of rRNAs can occur, while mRNAs remain intact[16]. The positive results in the real-time PCR analysis were not surprising for the partially degraded ABI-RNA samples as TaqMan® gene expression inventoried assays possess high sensitivity[17].

The principal discrepancy between the two techniques was the inclusion of RNA from granulocyte and erythrocyte fractions in the ABI samples. It is well-known that different RNA isolation methods and RNA from various blood cells may lead to diverse gene expression data[18,19]. SOD1 and SOD2 gene expression was different in PBMCs and in whole blood (Tables 2 & 3). SOD1 was more highly expressed in PBMCs in comparison to whole blood in both the samples of pooled blood and group of healthy subjects ($p<0.01$). However, SOD2 was more highly expressed in whole blood in comparison to PBMCs in both the samples of pooled blood and group of healthy subjects ($p<0.01$ & $p<0.05$, respectively). Khymenets *et al*[19] compared the gene expression of the same gene in RNA samples isolated using four different procedures from PBMCs and white blood cells. They assumed that granulocytes contributed to the higher levels of SOD2 expression in white blood cells. In our study, besides granulocytes, reticulocyte RNA from whole blood could also has impact on SOD2 gene expression, but obviously not on SOD1 gene expression (Tables 2 & 3). A relatively high proportion of globin mRNA from reticulocytes present in the total RNA extracted from whole blood did not reduce the efficiency neither increase noise nor reduce sensitivity of real-time PCR analysis when compared with a leukocyte isolation protocol. Shou and colleagues[20] determined the Applied Biosystems 6100 semi-automated workstation's ability to isolate RNA from human and rat whole blood. They demonstrated that an adequate amount of high quality RNA could be obtained from whole blood. They also found minimal hemoglobin interference in samples prepared from whole blood. In addition, TaqMan™ gene expression assays were found highly specific for regions' of SOD1, SOD2 and β-actin genes[17]. Therefore we can infer that globin mRNA did not interfere with SOD1, SOD2 and β-actin gene expression profiles in whole blood in our samples. An Affymetrics technical note[21] reports a study where direct comparisons were performed between commonly used blood isolation and cell separation protocols concerning quality of gene expression profiling data used in GeneChip® microarrays. They showed greater variability (presented as CV, %) in probe sets within samples prepared from whole blood than from PBMCs. Besides variation in the extraction techniques regarding quantity and quality of RNAs (Table 1), our results revealed variation in gene expression data calculated by software in samples obtained from the same pool. Smaller CVs were found in the MS mode than in the AS mode for all gene expression parameters (Table 2). In the ABI isolation method, variations in the ΔCt and RQ for SOD1 were higher in the AS mode compared with the MS mode. The opposite was found for variations in SOD2 gene expression. Much higher CVs were detected for parameters in the MS mode than in the AS mode. Taken together, both genes and both methods, the smallest CVs were obtained with the TRI-MS mode analysis. When comparing the two methods' RMS in paired settings TRI-AS *vs* ABI-AS and TRI-MS *vs* ABI-MS, the smallest was obtained for the second pair for both SOD1 and the same for SOD2 analysis.

Thus, our results demonstrated that the RNA isolation method employing commonly used TRIzol® isolation reagents resulted in much smaller variability in gene expression data than that from the ABI platform, especially when the baseline and threshold MS mode was used. The use of PBMCs and the TRI isolation method revealed consistently better reproducibility in gene expression analysis in healthy subjects. Both the methods characterised herein still need to be tested in solid tissues.

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