Clinica Chimica Acta 413 (2012) 779–786

Contents lists available at SciVerse ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

# Implementation of a proficiency testing for the assessment of the preanalytical phase of blood samples used for RNA based analysis

Kalle Günther [a,g,1], Francesca Malentacchi [b,g,1], Paolo Verderio [c,g], Sara Pizzamiglio [c,g], Chiara Maura Ciniselli [c,g], Ales Tichopad [d,e,g], Mikael Kubista [d,f,g], Ralf Wyrich [a,g], Mario Pazzagli [b,g,*], Stefania Gelmini [b,g]

[a] QIAGEN GmbH, Hilden, Germany
[b] University of Florence (UNIFI), Clinical Biochemistry Unit, Department of Clinical Physiopathology, Italy
[c] Fondazione IRCCS, Istituto Nazionale dei Tumori, Milan, Italy
[d] Institute of Biotechnology, Academy of Sciences of the Czech Republic, Prague, Czech Republic
[e] Charles University, Faculty of Medicine in Pilsen, Pilsen, Czech Republic
[f] TATAA Biocenter AB, Gothenburg, Sweden
[g] The SPIDIA Consortium

ARTICLE INFO

*Article history:*
Received 19 October 2011
Received in revised form 10 January 2012
Accepted 15 January 2012
Available online 21 January 2012

*Keywords:*
Blood RNA stabilization
Gene expression
Profiling
Ring trial
Multicenter study
Standardization

ABSTRACT

*Background:* Although important improvements of downstream molecular *in vitro* diagnostics assays based on RNA from blood were made, the pre-analytical workflow is still poorly defined.
*Methods:* We performed a multicenter study within the EU-granted SPIDIA project to investigate blood collection and shipping influence on the following RNA quality parameters: yield, purity, integrity, RT-qPCR interference and IL1B, IL8, FOS and GAPDH gene expression. Two models were designed: Exp A. Ten laboratories collected blood from an own donor into two different tubes (with or without stabilizer) and extracted RNA at two different times; Exp B. Blood was drawn from a single donor and shipped to ten laboratories in two different tubes (with or without stabilizer) for RNA extraction.
*Results:* In both models and collection tubes, reliable results were obtained for purity, yield, GAPDH expression, and interferences. A substantial variation in RIN (Exp A) and in transcription levels of IL1B, IL8 and FOS (Exp B) was observed for blood collected in tube without stabilizer tubes. Overall the variability was higher among data obtained from unstabilized blood samples.
*Conclusions:* We defined the experimental setup for a larger ring trial throughout Europe. The chosen downstream analyses verified their potential, serving as adequate markers to test the quality of blood RNA.


## 1. Introduction

Molecular *in vitro* diagnostics will play an important role in future health care practice and gene expression profiling promises to provide insight into normal biological and pathological processes with the hope of predicting disease outcome and indicating individualized courses of therapy. In this field, significant improvements of downstream assays and data analysis (analytical process) have been made during the last years. In contrast, the influence of the preanalytical steps, such as sample collection and stabilization, has been highly underestimated [1–3].

The recently published guidelines on qPCR measurements [4] stress the need to document the quality assessment of RNA templates prior the analysis. This assessment should include data on RNA quality, yield, integrity, and the absence of PCR inhibitors. This also underlines that RNA degrades markedly *in vivo*, owing to the natural regulation of mRNAs in response to environmental stimuli. In fact, for blood RNA analysis, expression profile studies have shown that

*Abbreviations:* n, Total number; IQR, Interquartile range; RT-qPCR, Reverse Transcriptase Quantitative Polymerase Chain Reaction; qPCR, Quantitative Polymerase Chain Reaction; IL1B, Interleukin-1β gene; IL8, Interleukin-8 gene; FOS, c-fos gene; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase gene; KD, Kintetics Distance; PT, Proficiency Testing; $C_q$, Quantification Cycle in qPCR and RT-qPCR; PAXgene, PAXgene® Blood RNA tube; EDTA, $K_2$-Ethylenediaminetetraacetic acid Vacutainer® tube; Exp A, Experimental model A; Exp B, Experimental model B.

* Corresponding author at: Department of Clinical Physiopathology, Viale Pieraccini 6, 50139 Florence, Italy. Fax: +39 0554271442.
*E-mail address:* m.pazzagli@dfc.unifi.it (M. Pazzagli).
*URLs:* http://www.spidia.eu (K. Günther), http://www.spidia.eu (F. Malentacchi), http://www.spidia.eu (P. Verderio), http://www.spidia.eu (S. Pizzamiglio), http://www.spidia.eu (C.M. Ciniselli), http://www.spidia.eu (A. Tichopad), http://www.spidia.eu (M. Kubista), http://www.spidia.eu (R. Wyrich), http://www.spidia.eu (M. Pazzagli), http://www.spidia.eu (S. Gelmini).
[1] These authors contributed equally to this manuscript.

0009-8981/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.cca.2012.01.015

significant changes can occur after blood collection during transport and storage [5,6]. This source of degradation is beyond the control of the researchers and one of its manifestations is that even high-quality RNA samples can show differential deregulation and degradation of individual mRNAs. The use of a system to calculate an RNA integrity number or an RNA quality indicator number is considered important, however, these numbers relate to rRNA quality and cannot be expected to be an absolute measure of RNA quality [4]. The complexity of this situation requires further research in order to optimize the entire procedure for RNA analysis.

SPIDIA (www.spidia.eu) is a four-year large-scale integrated project funded by the European Commission that works on the standardization and improvement of pre-analytical procedures for *in vitro* diagnostics in order to close the gap between the more elaborated analytical procedures and the less standardized pre-analytical processes. For SPIDIA a key aim is to develop evidence-based guidelines and quality assurance schemes for collection, transport and processing of blood samples for RNA-based analyses.

To approach this goal a large proficiency testing (PT) program among routine laboratories in Europe is planned to evaluate current sample technologies for RNA-based analyses from blood. In preparation for the main ring trial, a preliminary study involving a limited number of laboratories was pursued to validate testing protocols, logistics and schemes. This study is based on two different experimental models. The first model was designed to evaluate the role of the pre-analytical variables from blood collection and storage to RNA extraction. In this case the participating laboratories collected blood samples from individual donors using both stabilized (PAXgene® Blood RNA Tube, PreAnalytiX) and unstabilized ($K_2$EDTA, BD) collection tubes.

The second model was designed to evaluate the influence of blood/RNA shipment conditions and RNA extraction procedures. In this case the participating laboratories received from SPIDIA the same blood sample collected in stabilized (PAXgene) and unstabilized (EDTA) blood collection tubes. A pre-extracted RNA sample was also included in the kit to evaluate the effect of the Blood RNA Tube shipment. The extracted RNA samples from the two experimental models were then sent to SPIDIA reference laboratory for analysis.

In both models, the influence of a regular EDTA blood collection tube was compared to a RNA stabilizing blood collection tube, which is intended to preserve the gene Blood RNA Tube expression profile at the point of blood collection. The PAXgene Blood RNA Tube was chosen for this purpose as it contains an additive that immediately stabilizes the *in vivo* gene transcription profile by lysing the cells and complexing the nucleic acids and it was already used in several studies for transcript analysis from blood samples [7–11]. The RNA quality parameters used in this study include spectrophotometric evaluation of purity and yield, and the RNA integrity number (RIN). In order to monitor the RNA stability, these parameters were complemented by measuring also the transcript levels of selected genes. Several genes are known to be induced or repressed by *ex vivo* blood handling, which can be used to monitor changes occurring during the pre-analytical steps. In particular, it has been reported that measurements of the expression of FOS, IL1B, and IL8 under many conditions can be used to monitor changes in expression levels in clinical samples of human whole blood and bone marrow samples [5,12–14].

An additional quality parameter investigated in this study was the matrix effect. Where samples are obtained with different pre-analytics, the effect of heterogeneous interfering substances may confound the test objective. Interfering substances may be exogenous (*e.g.* ethanol or phenol) or residual endogenous compounds (*e.g.* biological decay products) [4]. Employing RT-qPCR, amplification compatibility can be validated by analyzing the amplification trajectory of each individual sample and comparing it with defined references [15]. This method utilizes the raw amplification data as obtained by the qPCR software. This panel of RNA quality parameters has been employed in both experimental models proposed in this study.

## 2. Materials and methods

### 2.1. Study setup, patients, samples and shipment

Ten laboratories, routinely working with human blood in the field of gene expression analysis were invited to participate in this study, performing both the experimental models A and B.

#### 2.1.1. Experimental model A (Exp A): Evaluation of the pre-analytical variables from blood collection and storage to RNA extraction

Participant laboratories collected blood in 4 tubes. Blood was drawn from healthy volunteers in responsibility of the individual participating laboratory after informed consent. All laboratories ensured the approval by their local ethics committee. Peripheral human whole blood was collected into two PAXgene Blood RNA Tubes (Tubes 1 and 2) and two EDTA-containing tubes (Tubes 3 and 4). RNA from first tube per set of duplicates (Tubes 1 and 3) was prepared immediately after blood collection (Tube 3, EDTA) or after 2 h incubation at room temperature (Tube 1, PAXgene). Second blood replicates (Tubes 2 and 4) were prepared reflecting the laboratory's guidance of maximum blood sample storage before analysis. The time points were chosen to represent the operational procedures of sample transport between hospitals (blood collection) and laboratories (RNA isolation and analysis) (Fig. 1).

Extracted RNA samples (RNA 1, RNA 2, RNA 3 and RNA 4) were sent on dry-ice to the SPIDIA UNIFI laboratory for the RNA quality analysis.

#### 2.1.2. Experimental model B (Exp B): Evaluation of the pre-analytical variables from blood/RNA shipment and storage to RNA extraction

Peripheral human whole blood was collected from one healthy donor after informed consent at the SPIDIA QIAGEN laboratory into

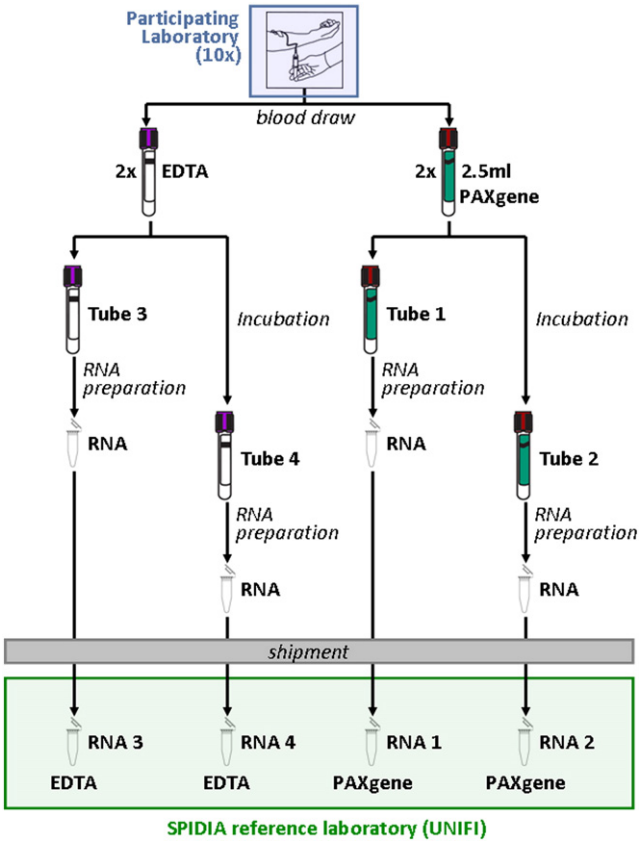


**Fig. 1.** General workflow of the Exp A study.

EDTA tubes (Vacutainer® PLUS $K_2$EDTA 10.8 mg, 6 ml, ref cat. no. 367864, BD) and aliquots of 2.5 ml blood were immediately transferred into 18 PAXgene Blood RNA Tubes (cat. no. 76216, PreAnalytiX). Eight of the 18 PAXgene Blood RNA Tubes were incubated at room temperature for 2 h followed by RNA isolation in the SPIDIA QIAGEN laboratory, as described below. This RNA was pooled and mixed, and RNA quality and yield were analyzed. This RNA serves as reference RNA (REFpool RNA) for the downstream analyses performed at SPIDIA UNIFI laboratory and also as the reference for the participating laboratories (REF RNA) to monitor the influence of the shipment on the quality of the isolated RNA (Fig. 2).

Each laboratory received one PAXgene Blood RNA Tube (Tube A, 2.5 ml of blood), and one EDTA tube (Tube B, 6 ml of blood), sent at ambient temperature with overnight delivery, as well as the REF RNA, shipped on dry ice. The laboratories extracted RNA (RNA A and RNA B) from the two shipped blood samples on the day of arrival or, at the latest, two days after arrival. REF RNA, RNA A and RNA B were then sent on dry ice to the SPIDIA UNIFI laboratory for the RNA quality analysis.

At SPIDIA UNIFI laboratory, an alpha-numeric code was associated to each participant laboratory, randomly, this association was recorded in a database. RNA sample tubes (RNA A, RNA B, RNA 1, RNA 2, RNA 3 and RNA 4) were properly defined. The samples were analyzed in blind.

### 2.2. RNA preparation from blood

RNA was prepared from blood samples by different procedures depending on the type and the experimental model. From blood sampled in PAXgene Blood RNA Tubes (Sample A; REF RNA and REFpool RNA), RNA was prepared according to the manual protocol of the PAXgene Blood RNA Kit (cat. no. 762174 or 762156, PreAnalytiX). From blood sampled in EDTA tubes, RNA was prepared by the participating laboratories using their standard protocol. The participating laboratories have reported to the organizers all the details related to the procedure in use in the individual laboratory's guidance (extraction method, time intervals, and storage conditions). These data are summarized in the Supplemental Data (Supplemental Table 1 A, B, C).

### 2.3. RNA yield and purity by spectrophotometric measurement

RNA was quantified from RNA aliquots by NanoDrop® 1000 UV spectrophotometer (NanoDrop Technologies) that was properly zeroed using the proper elution buffer or RNase-free water or PAXgene Blood RNA elution buffer BR5.

Spectrophotometric measurements were performed at 260, 280, and 320 nm. Absorption at 320 nm was used to subtract background absorption. RNA purity was calculated as absorbance ratio $R = (A_{260} - A_{320})/(A_{280} - A_{320})$ and RNA total yield as $Q = (A_{260} - A_{320}) \times 40 \times$ dilution factor $\times$ elution volume/extracted blood volume (ng/μl). Alternatively, when the reading at 320 nm was not reported by the participants, the R value was calculated as $R = A_{260}/A_{280}$ and RNA total yield as $Q = A_{260} \times 40 \times$ dilution factor $\times$ elution volume/extracted blood volume (ng/μl).

### 2.4. RNA integrity analysis

The integrity of RNA was assessed by capillary gel electrophoresis by analyzing 1 μl aliquot using RNA 6000 Nano reagents and chips (n. 5067-1511, Agilent Technologies) on a Bioanalyzer 2100 (n. G2938C, Agilent Technologies). The RIN calculation software algorithm (version B02.02.S1238, Agilent Technologies) was applied to the fluorescence profiles after separation of RNA by capillary electrophoresis, and RIN values were calculated on a scale of 1–10 (low to high RNA integrity) for each sample.

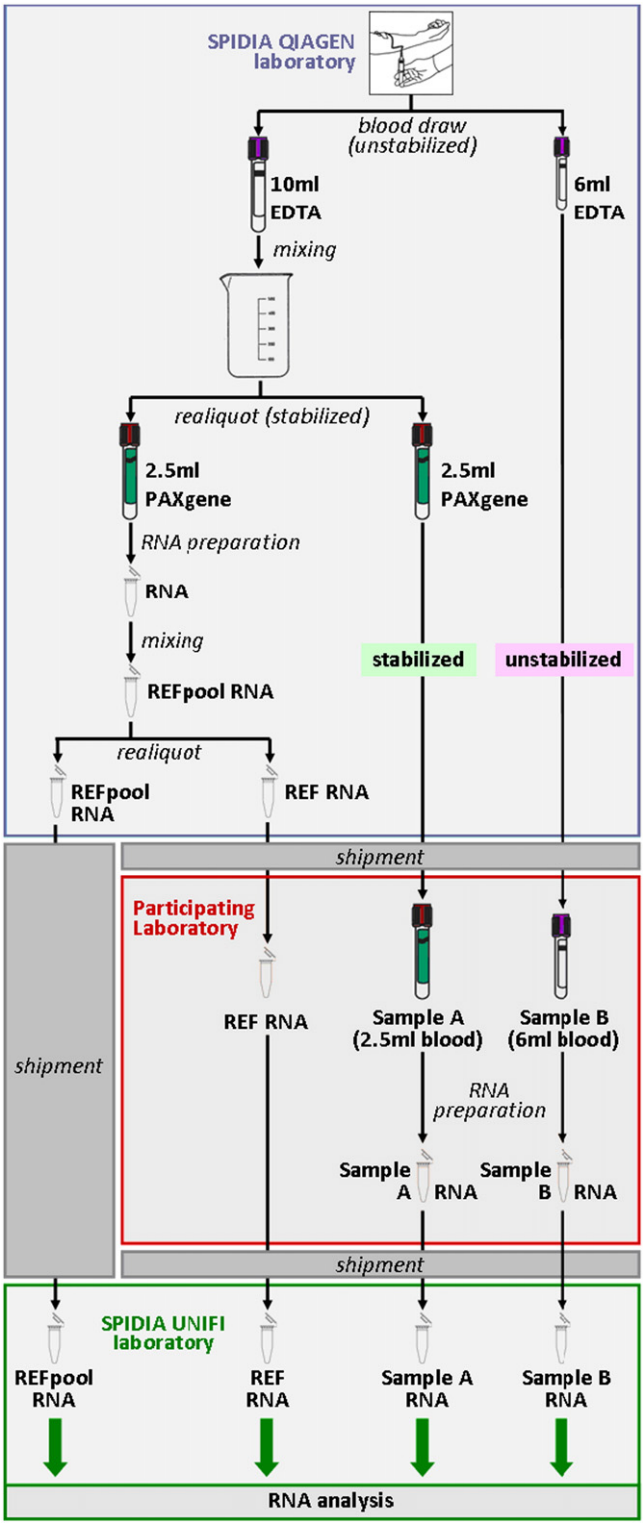


**Fig. 2.** General workflow of the Exp B study.

### 2.5. Gene expression analysis by RT-qPCR

Primers and probes for GAPDH (Pre-Developed TaqMan® Assay Reagents, P.N. 4326317E), IL1B, IL8 and FOS (TaqMan Gene Expression Assay; Hs00174097_m1, Hs99999034_m1 and Hs00170630_m1, respectively) were from Life Technologies. Total RNA (400 ng) was reverse transcribed using a TaqMan Reverse Transcription Reagents kit (n. N808-0234, Life Technologies). Reverse transcription was performed in a final volume of 80 μl containing 500 mM KCl, 0.1 mM EDTA,

100 mM Tris–HCl (pH 8.3), 5.5 mM $MgCl_2$, 500 μM of each dNTP, 2.5 μM random hexamers, 0.4 U/μl RNase inhibitor, and 1.25 U/μl Multiscribe Reverse Transcriptase. The reverse transcription reaction was performed at 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 3 min by 2720 Thermal cycler (Life Technologies). Gene expression was measured by qPCR. For each sample 12.5 ng of cDNA was added to 10 μl of PCR mix containing a primer set and 1× Universal PCR Master Mix (n. 4318157, Life Technologies). The samples were then subjected to 40 cycles of amplification at 95 °C for 15 s and 60 °C for 60 s in the ABI PRISM® 7900 Sequence Detector (Life Technologies). The amount of each target gene was evaluated against a standard curve. Each standard was obtained by cloning a cDNA fragment of the specific gene (FOS, GAPDH, IL1B, and IL8) into the plasmid pCR®2.1–TOPO® (n. 45-0641, Life Technologies) following user manual's instruction. Each standard curve was generated by plotting the mean Cq of the technical replicates *versus* the logarithm of the known starting concentration [16]. Samples and standards were measured in qPCR triplicates. The gene expression results are reported as $\log_{10}$ copies/μg RNA.

### 2.6. Kinetics analysis

The objective of the kinetics analysis was to evaluate the amplification compatibility as an additional RNA quality indicator of the tested samples. The analysis was conducted using a reference methodology as described [15]. Amplification response curves of individual samples were used to calculate the kinetics distance (KD) to a defined reference set of samples. The reference set consisted of all the RNA samples from all the participating laboratories. The KDs of the three qPCR technical replicates of each sample were averaged. The KD can be interpreted as a metric reflecting PCR interference due to unknown factors that adversely affect the PCR. RNA extracted from Tubes A and B were compared with the reference set, and the KDs of each group are presented in box-whisker plots, applying the boxplot rule for outlier exclusion: $Q1-(1.5\times IQR)$ and $Q3+(1.5\times IQR)$ with Q1 and Q3 indicating the 25th and 75th percentiles, respectively, and IQR indicating the interquartile range (*i.e.* 75th–25th percentile).

### 2.7. Statistical analysis

For the Exp A data, statistical analysis was focused on purity and yield values generated by the SPIDIA UNIFI laboratory and on the expression of the four considered genes. These measurements obtained for RNA 1 and RNA 3 were compared with those of RNA 2 and RNA 4, respectively by using the Kolmogorov–Smirnov test [17]. Furthermore, the differences between the measurements obtained for RNAs within collecting tube (*i.e.* RNA 1–RNA 2 for stabilized and RNA 3–RNA 4 for unstabilized blood) were represented by box-plots.

For the Exp B data, statistical analysis of the measurements was performed on REFpool RNA, REF RNA, RNA A and RNA B. The presence of a potential shipment effect was assessed by evaluating the values of the RNA quality parameters of REFpool RNA with those of REF RNAs received from the ten participating laboratories. These comparisons were performed by resorting to non parametric approaches [17].

## 3. Results

Due to the different experimental designs investigated in this study, the Results section has been dived in two parts.

### 3.1. Experimental Model A

#### 3.1.1. RNA extraction protocols, temperature and time storage conditions

Data on the RNA extraction protocols for blood collected in EDTA tubes (Tube 3 and Tube 4), storage temperature and time used by participating laboratories are reported in the Supplemental Table 1 A, B, C.

#### 3.1.2. RNA purity and yield and RIN values

In Supplemental Table 2 we report the descriptive statistics for the purity (R) and the RNA total yield (Q). For both measurements variability was comparable (IQR) within the RNA extracted from the same collection tube. Overall, for both purity and yield values the distribution of RNAs 1 and 2 as well as that of RNAs 3 and 4 were not significantly different (Kolmogorov–Smirnov test p-values higher than 0.05). Fig. 3 (Panel A) shows that the median of the differences between purity values of RNA 1 and RNA 2 was close to 0 as well as that of RNA 3 and RNA 4. On the other hand, a wider range was observed for the difference between purity obtained from unstabilized blood (RNA 3–RNA 4). A similar distribution was observed for the RNA total yield differences reported in Fig. 3 Panel B.

The RIN values of RNAs 1, 2, 3 and 4 are shown in Supplemental Table 3. RIN values were obtained from all laboratories for RNA 1 (range 7.1–9.6) and RNA 2 (range 5.9–8.8). For RNA 3 (range 1.1–9.5) and RNA 4 (range 1.1–9.4), RIN values from two laboratories were undetectable.

As emerged from Supplemental Table 3, for RNAs 1, 2 and 3 a median RIN value greater than 8.0 was observed, whereas for RNA 4, a lower median RIN value (3.1) was observed and only 2 out of seven labs had a RIN value greater than 8.0.

#### 3.1.3. RNA expression data

Overall, for each considered gene, the expression distributions in RNAs 1 and 2 as well as those in RNAs 3 and 4 were not significantly different (Kolmogorov–Smirnov test p-values higher than 0.05; Supplemental Table 4). The difference between the expression of the four genes in RNA 1 and RNA 2 (stabilized blood) and in RNA 3 and RNA 4 (unstabilized blood) is reported in Fig. 4 (Panel A, GAPDH; Panel B, IL1B; Panel C, IL8 and Panel D, FOS). For all genes, the median difference between RNA 1 and RNA 2 was close to zero. In contrast, the median difference between RNA 3 and RNA 4 was slightly greater than zero for all genes suggesting a decrease of their time-dependent expression. A better stability of RNA 1 and RNA 2 in comparison to RNA 3 and RNA 4 is also confirmed by the lower range of differences.

### 3.2. Experimental Model B

#### 3.2.1. RNA extraction protocols, temperature and time storage conditions

Data on the RNA extraction protocols for blood collected in EDTA tube (Tube B), temperature and time storage conditions used by participating laboratories are reported in the Supplemental Table 1 A, B, C.

#### 3.2.2. Effect of RNA shipment on RNA quality parameters in REFpool RNA and REF RNA samples

The values of REFpool RNA and of REF RNA parameters are reported in Table 1. By considering the quality parameters investigated, the Sign Test confirmed that their distribution in the REFpool RNA and in the REF RNA was not statistically significant different ($\alpha$ level of 0.05). Hence, we conclude that there is no significant shipment effect on RNA quality when the extracted RNA is shipped on dry ice.

#### 3.2.3. RNA purity and yield and RIN values in RNA A and RNA B samples

The variability of purity (R) and RNA total yield (Q) was higher in RNA B (unstabilized blood) than in RNA A (stabilized blood) (Supplemental Table 2). Also, the median value of the RNA purity and yield was higher in RNA A than in RNA B ([Q]: 3.1 *vs.* 1.7 ng/μl, [R]: 2.2 *vs.* 1.8, respectively).

The analysis of the RIN values (Supplemental Table 3) showed that all the RNAs A (range: 7.3–9.8; one RNA A sample was undetectable) and most of the RNAs B (range: 1.2–9.9) showed high RNA integrity (RIN > 7), except for two unstabilized samples (RNA B). The median

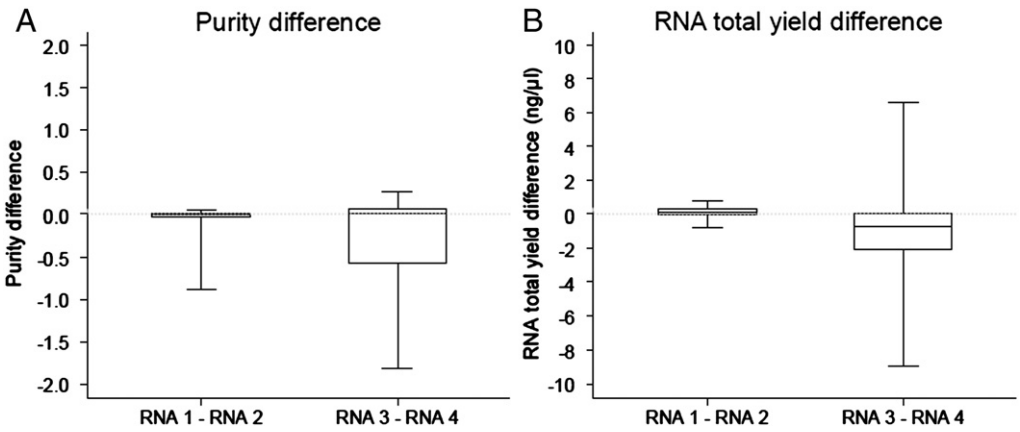


**Fig. 3.** Exp A. RNA purity and total yield. Panel A: difference in terms of purity of the RNA extracted by the participating laboratories in stabilized ($R_{RNA\ 1}$–$R_{RNA\ 2}$) or unstabilized ($R_{RNA\ 3}$–$R_{RNA\ 4}$) blood samples. Panel B: difference in terms of yield of the RNA extracted by the participating laboratories in stabilized ($Q_{RNA\ 1}$–$Q_{RNA\ 2}$) or unstabilized ($Q_{RNA\ 3}$–$Q_{RNA\ 4}$) blood samples. In Panels A and B, each box shows the 25th and 75th percentiles, the horizontal line inside the box indicates the median, and the limits of the two whiskers correspond to minimum and maximum of the difference distribution. The horizontal dotted line represents the expected value in absence of difference between the compared samples (RNA 1 - RNA 2 and RNA 3 - RNA 4).

RIN value was similar for both sample types (8.0 and 8.4 RNA A and RNA B, respectively). However the variability was higher for RNA B (IQR, 1.5) compared to RNA A (IQR, 0.9).

### 3.2.4. RNA expression data

The minimum, median, and maximum values of the measured transcript levels expressed as $\log_{10}$ (copies/µg RNA) of the studied genes in all the samples extracted by the participating laboratories are reported in Supplemental Table 4 and in Fig. 5.

For all measured variables RNA B (unstabilized blood) showed the largest variation in comparison to RNA A, whereas the REF RNA showed the lowest variation. Analysis of the selected genes revealed that, for the housekeeping gene GAPDH, (Panel A), the median value of the REF sample was comparable with that of RNA A and RNA B, but the variability of measured GAPDH levels was highest in RNA B. For IL1B (Panel B), a lower median value for RNA B is observed when compared to RNA A and REF RNA. In contrast higher transcript levels, for FOS and IL8 (Panels C and D) were found in RNA B compared to RNA A and REF RNA. Generally,

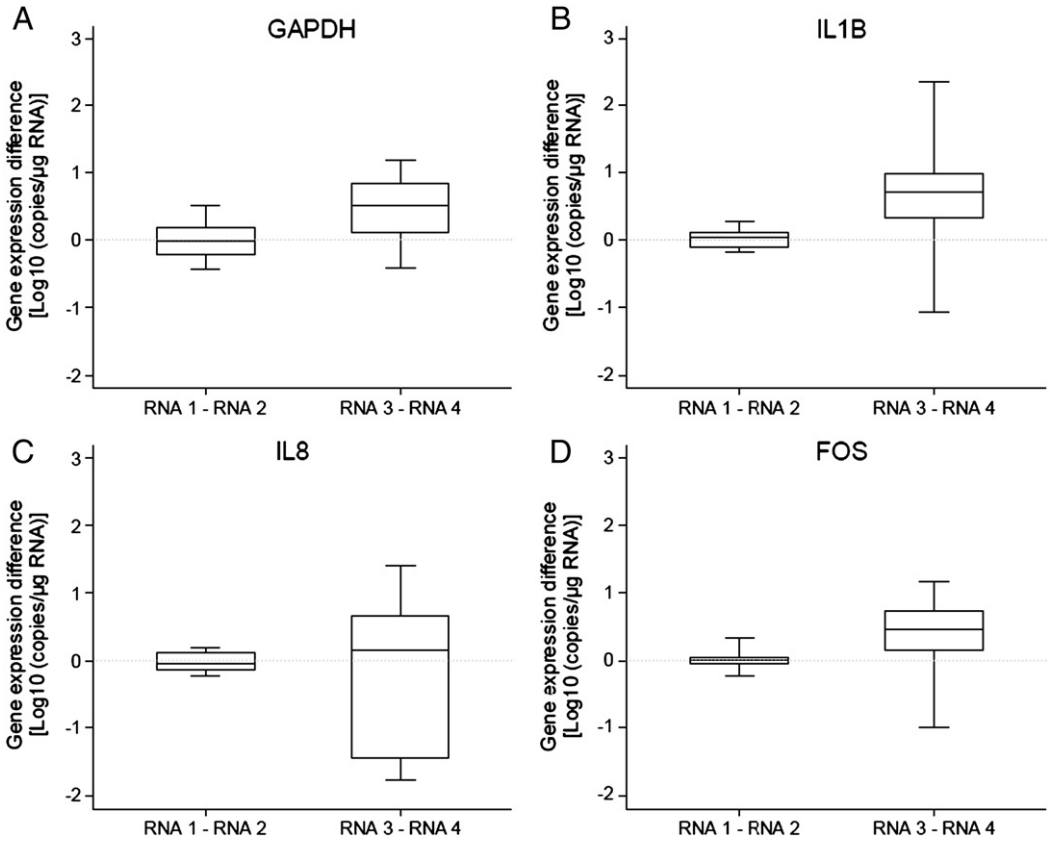


**Fig. 4.** Exp A. Difference, in terms of quantity, of the RNA extracted by the participating laboratories in stabilized (RNA 1–RNA 2) or unstabilized (RNA 3–RNA 4) blood samples regarding the mRNA expression of the genes: GAPDH (Panel A), IL1B (Panel B), IL8 (Panel C), and FOS (Panel D). Each box shows the 25th and 75th percentiles, the horizontal line inside the box indicates the median, and the limits of the two whiskers correspond to minimum and maximum of the difference distribution. The horizontal dotted line represents the expected value in absence of difference between the compared samples.

**Table 1**
Purity, yield, RIN, gene expression and kinetic values of REFpool RNA and REF RNA.

| Parameter | REFpool RNA n = 1 | REF RNA n = 8 | | | | p-value[b] |
|---|---|---|---|---|---|---|
| | | Min. | Median | Max. | IQR[a] | |
| RNA purity (R) | 2.237 | 2.130 | 2.184 | 2.347 | 0.121 | 0.727 |
| RNA total yield (Q) (ng/μl) | 2.477 | 2.429 | 2.694 | 3.251 | 0.348 | 0.070 |
| RNA integrity number (RIN) | 8.400 | 7.900 | 8.380 | 8.500 | 0.100 | 0.375 |
| GAPDH [$\log_{10}$ (copies/μg RNA)] | 7.039 | 6.845 | 7.212 | 7.569 | 0.278 | 0.289 |
| FOS [$\log_{10}$ (copies/μg RNA)] | 6.822 | 6.749 | 6.854 | 6.958 | 0.103 | 0.289 |
| IL1B [$\log_{10}$ (copies/μg RNA)] | 6.434 | 6.429 | 6.617 | 6.736 | 0.105 | 0.070 |
| IL8 [$\log_{10}$ (copies/μg RNA)] | 5.756 | 5.673 | 5.787 | 5.994 | 0.156 | 0.727 |
| GAPDH kinetics distance | 4.123 | 0.440 | 1.635 | 9.357 | 1.827 | 0.070 |
| FOS kinetics distance | 0.825 | 0.347 | 1.912 | 9.563 | 0.978 | 0.289 |
| IL1B kinetics distance | 2.713 | 0.687 | 1.715 | 3.487 | 1.127 | 0.070 |
| IL8 kinetics distance | 1.877 | 1.430 | 2.030 | 2.333 | 0.417 | 0.289 |

Note: Two laboratories did not return REF RNA to the SPIDIA UNIFI laboratory.
[a] IQR: interquartile range (75th percentile–25th percentile).
[b] p-value of Sign Test for the comparison of REFpool RNA with REF RNA values.

the measured transcript levels in RNA A are more similar to those in the REF RNA sample than those in RNA B.

*3.2.5. Interferences in RT-qPCR as reflected by kinetics analysis*

The distribution of the KD values, reflecting the interference in the qPCRs among the samples, was skewed (see Fig. 6). For all the genes, KD was lower for RNA A compared to RNA B. The box-plot rule was used to identify outliers which are indicated in the figure by a dot. Also the scatter reflected by the interquartile range (spread of the box) of the KD is larger for RNA B, indicating heterogeneity in the qPCR performance of RNA B, most likely indicating larger variations in PCR efficiency due to interference.

## 4. Discussion

The EN ISO 15189 document, developed to support medical laboratories in checking their competence and quality management, states that the laboratory must take part in an interlaboratory comparison program. For molecular diagnostics, most of the PT programs in 33 published studies [18] are aimed to test the ability of the laboratory to identify or quantify a particular target gene. Two of these studies [18,19] were focused on qPCR by evaluating the performance in testing gene expression analysis. In the study of Raggi et al. [19] the challenge samples were cDNA retrieved from three pools of total RNA. Ramsden et al. [20] used, as unknown sample, a cell line (K562) stabilized in RNAlater® reagent, and the evaluation included RNA extraction, reverse transcription, and qPCR quantification. When planning PT programs for molecular methods the test samples can either be derived from biological materials (*e.g.* blood), or can be based on synthetic materials such as purified DNA, RNA transcripts and recombinant plasmids. There are a number of advantages and disadvantages associated with each approach and, depending upon the objectives of the PT program, these factors need to be considered in advance [21]. As the SPIDIA-RNA PT program is focused on the evaluation and standardization of the pre-analytical phase, only the use of a biological material is recommended, and thus, the present study represents the first attempt to use a blood sample in a PT program with the aim to monitor the pre-analytical workflow for RNA based tests. Due to these characteristics, this PT should be considered more as a proof of principle model than a formal PT program offered by a certified PT organization.

In an attempt to cover all the possible sources of pre-analytical variation for RNA analysis in blood, in this study we propose a broad panel of RNA quality parameters for the evaluation of the RNA samples; these include UV spectrophotometric analysis for RNA

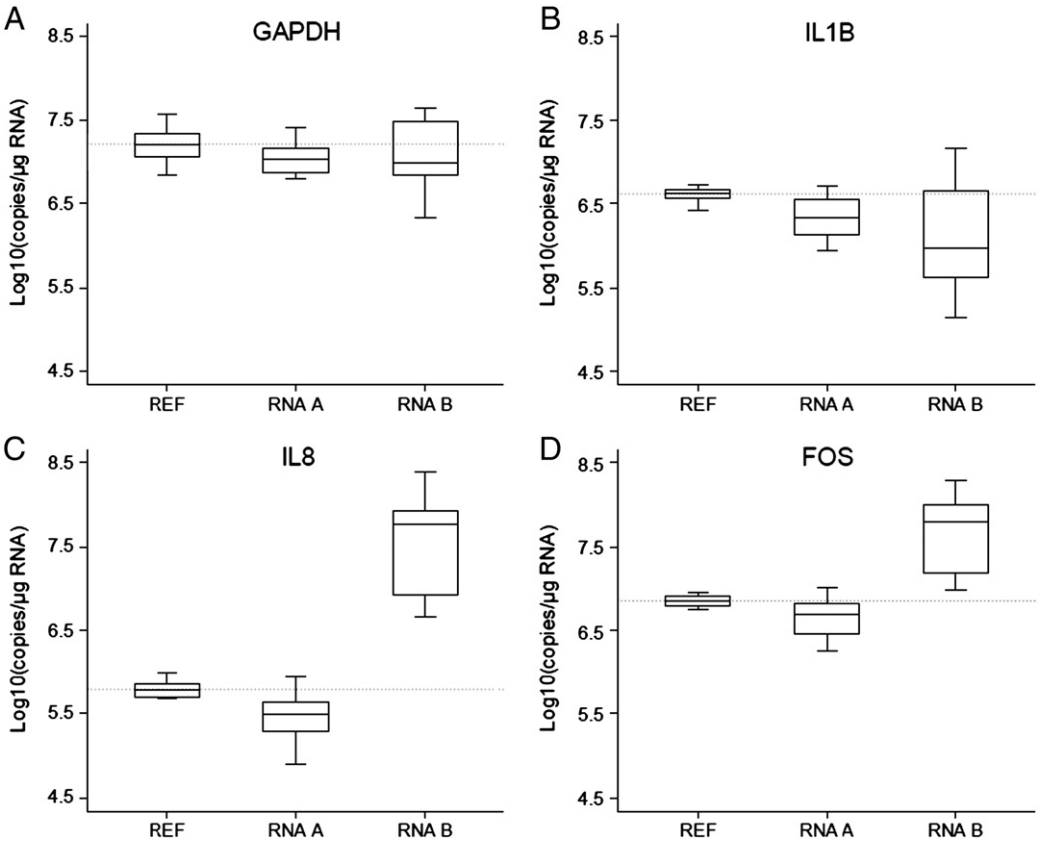


**Fig. 5.** Exp B. Box plots reflecting the mRNA expression of GAPDH (Panel A), IL1B (Panel B), IL8 (Panel C), and FOS (Panel D) measured in the three sample types REF, RNA A and RNA B. Each box indicates the 25th and 75th percentiles. The horizontal line inside the box indicates the median, and the whiskers indicate the extreme measured values. The dotted horizontal line indicates the median value of the REF samples and serves for comparison.

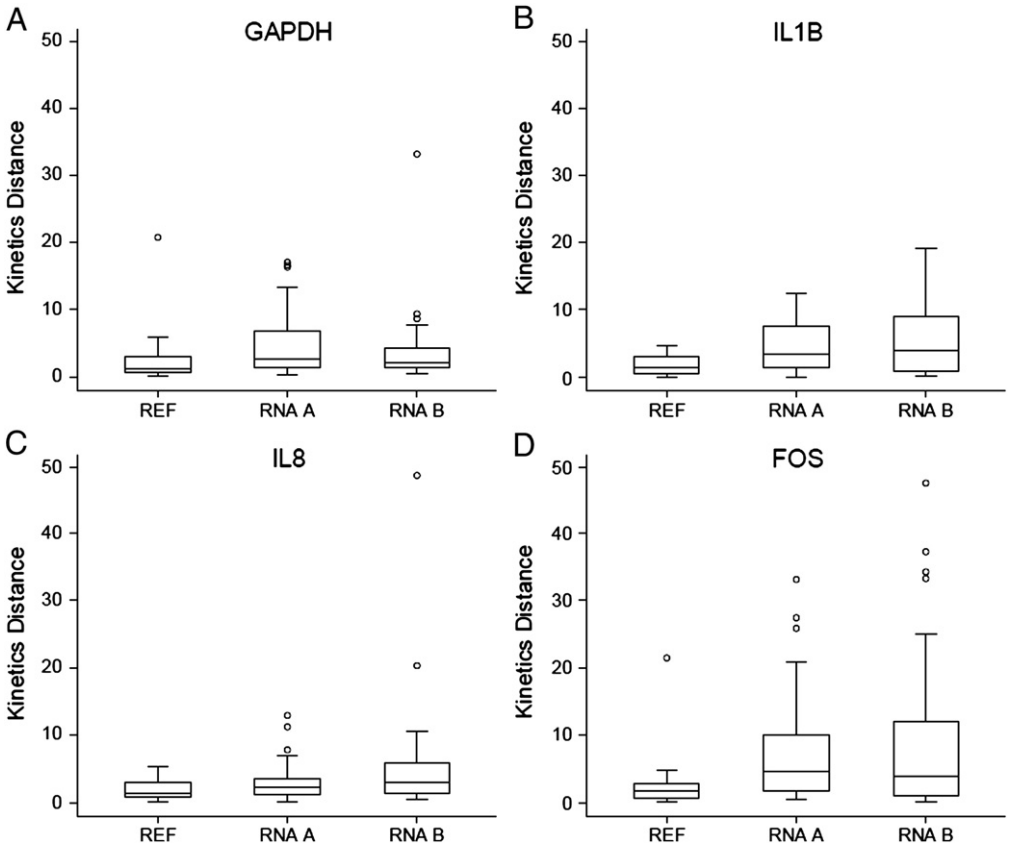


**Fig. 6.** Exp B. Box plot referred to the KDs of the qPCR technical replicates of the four genes analyzed in the three sample types (REF, RNA A and RNA B). Each box shows the 25th and 75th percentiles. The horizontal line inside the box indicates the median, and the limits of the two whiskers correspond to the most extreme observations, which exceeded 1.5 times the IQR from the box. Observations falling outside the whiskers are represented by circles.

purity and yield, the RIN value for an overall evaluation of integrity, the measurement of the expression of FOS, IL1B, IL8 and GAPDH for evaluating the gene expression stability, and kinetics analysis of the RT-qPCR data for the detection of the presence of heterogeneous interfering substances.

With the aim to cover all the main pre-analytical steps for RNA analysis in blood, two different experimental models have been planned. The first model (Exp A) has been designed to evaluate the role of the pre-analytical variables from blood collection to storage and RNA extraction and in particular: I) the role of the blood collection tube and II) the role of the time interval from blood collection to RNA extraction. For this model each laboratory collected four blood samples (RNAs 1–4) from an own donor.

The second experimental model (Exp B) has been focused on: I) the influence of RNA shipment and II) the role of the blood collection tube. In this model the participant laboratories received two blood samples (Tube A and Tube B) and an extracted RNA sample (REF RNA), all of them derived from the same donor.

The first important result of this study was the comparison between REF RNA and REFpool RNA data. The results for yield, purity, RIN, gene expression profile and KD were not statistically significantly different between REF RNA and REFpool RNA (Table 1) supporting a fundamental requirement for planning a PT programs for RNA analysis, *i.e.* there is no significant shipment effect on RNA extracted by the participating laboratories when it is sent on dry ice. Consequently we can assume that the RNA evaluation (performed by SPIDIA) reflects the performance of the RNA extraction of the participating laboratories, and this procedure will be adopted for the pan-European trial.

From the other data obtained from both Exp A and Exp B, we can also get important information to be used for planning the SPIDIA-RNA ring trial.

They can be summarized as follows. For all quality parameters investigated in the extracted RNA samples of Exp A and Exp B, we have always observed a higher variability when the blood sample is collected in unstabilized blood collection tubes. This finding can be due to the different procedures used by the participating laboratories for RNA extraction (Supplemental Table 1A), by the time interval from blood collection to RNA extraction (Supplemental Table 1B) and/or by the storage conditions (Supplemental Table 1C). For the blood collected in PAXgene Blood RNA Tube the variability is reduced because, not only the cells are immediately lysed, RNases are inactivated and the nucleic acids are complexed to protect them from nucleolytic degradation, but also because the RNA must be extracted following a single standardized procedure and reagents.

The RIN value, in EDTA blood collection tubes, becomes significantly worse when more than 24 h are spent between blood collection and RNA extraction (Supplemental Table 3). The RNA expression profiles are also significantly affected by the time interval from blood collection to RNA extraction in EDTA blood collection tubes showing in some cases overexpression (Fig. 5 Panels C and D) or downregulation (Fig. 5 Panel B).

Due to the limited number of participating laboratories the value of these results is limited and we expect to clarify the role of these pre-analytical variables when the data of the SPIDIA-RNA ring trial will be available, however we can anticipate that these selected genes are appropriate markers reflecting changes in expression levels and transcripts occurring post sampling.

## 5. Conclusion

Taken together, all these data are going to have a profound impact when planning the main SPIDIA-RNA ring trial. We assume that

laboratories performing RNA based analysis are currently using a collection tubes either with or without stabilizers, therefore both kinds of blood collection tubes will be used for the main ring trial. Those laboratories that collect blood using stabilizing tubes, that preserve cells and RNA (*e.g.* PAXgene Blood RNA Tubes), will extract RNA by the same procedure, as these tubes must be used in conjunction with a dedicated RNA extraction kit. On the other hand, we expected a broader variety of extraction procedures to be used in conjunction with the EDTA tubes as this is currently a less standardized workflow in routine laboratories. In order to be able to monitor all possible effects of sample storage, shipment and RNA extraction methods on the RNA quality, an internal time course experiment will be run in parallel with the sample shipment to the participating laboratories within SPIDIA facilities following defined storage conditions and using a panel of RNA extraction methods. Moreover, in order to limit the number of the variables that can influence the RNA quality, the protocol for the participants to the ring trial will include strict conditions for storage and time intervals for RNA extraction. Finally another important aspect, related to the implementation of this ring trial, will regard the choice of reference values that ideally should correspond to the true value (*i.e.* gold standard). If a reference value is available, a ring trial can answer the question 'Can the results of a laboratory be deemed accurate?' On the other hand, in the absence of this value, a ring trial can only answer the more humble question 'may the results of a laboratory be deemed consistent with the majority of the results provided by all the participating laboratories?'. As the latter will represent the context of the planned ring trial, the most suitable reference value will be estimated by applying appropriate statistical procedures we previously developed [19, 20, 22]. According to these procedures, reference values will be derived from the results distribution of participants whose performance was not questioned in order to obtain "a correct result as a consensus of testing participants' result" in accordance with international guidelines [21].

Supplementary materials related to this article can be found online at doi:10.1016/j.cca.2012.01.015.

## Conflicts of interest

K. G. and R. W. are full-time employees of QIAGEN and participate in the stock options program of QIAGEN.

## Acknowledgments

Grant/funding support. The SPIDIA RNA pilot study has been supported by European Union (FP7. Title "SPIDIA: Standardization and improvement of generic pre-analytical tools and procedures for *in vitro* diagnostic", Grant agreement no. 222916).

We gratefully acknowledge the contribution of all partners and participant laboratories.

## References

[1] Härtel C, Bein G, Müller-Steinhardt M, Klüter H. Ex vivo induction of cytokine mRNA expression in human blood samples. J Immunol Methods 2001;249:63–71.
[2] Pahl A, Brune K. Gene expression changes in blood after phlebotomy: implications for gene expression profiling. Blood 2002;100:1094–5.
[3] Kim SJ, Dix DJ, Thompson KE, et al. Effects of storage, RNA extraction, genechip type, and donor sex on gene expression profiling of human whole blood. Clin Chem 2007;53:1038–45.
[4] Bustin SA. Why the need for qPCR publication guidelines? — The case for MIQE. Methods 2010;50:217–26.
[5] Rainen L, Oelmüller U, Jurgensen U, et al. Stabilization of mRNA expression in whole blood samples. Clin Chem 2002;48:1883–90.
[6] Baechler EC, Batliwalla FM, Karypis G, et al. Expression levels for many genes in human peripheral blood cells are highly sensitive to ex vivo incubation. Genes Immun 2004;5:347–53.
[7] Batliwalla FM, Li W, Ritchlin CT, et al. Microarray analyses of peripheral blood cells identifies unique gene expression signature in psoriatic arthritis. Mol Med 2005;11:21–9.
[8] Sharma P, Sahni NS, Tibshirani R, et al. Early detection of breast cancer based on gene-expression patterns in peripheral blood cells. Breast Cancer Res 2005;7:634–44.
[9] Vlasova TI, Stratton SL, Wells AM, Mock NI, Mock DM. Biotin deficiency reduces expression of SLC19A3, a potential biotin transporter, in leukocytes from human blood. J Nutr 2005;135:42–7.
[10] Tan FK, Zhou X, Mayes MD, et al. Signatures of differentially regulated interferon gene expression and vasculotrophism in the peripheral blood cells of systemic sclerosis patients. Rheumatology 2006;45:694–702.
[11] Carrol ED, Salway F, Pepper SD, et al. Successful downstream application of the Paxgene Blood RNA system from small blood samples in paediatric patients for quantitative PCR analysis. BMC Immunol 2007;8:20.
[12] Tanner MA, Berk LS, Felten DL, Blidy AD, Bit SL, Ruff DW. Substantial changes in gene expression level due to the storage temperature and storage duration of human whole blood. Clin Lab Haematol 2002;24:337–41.
[13] Langebrake C, Günther K, Lauber J, Reinhardt D. Preanalytical mRNA stabilization of whole bone marrow samples. Clin Chem 2007;53:587–93.
[14] Hao S, Baltimore D. The stability of mRNA influences the temporal order of the induction of genes encoding inflammatory molecules. Nat Immunol 2009;10:281–8.
[15] Tichopad A, Bar T, Pecen L, Kitchen RR, Kubista M, Pfaffl MW. Quality control for quantitative PCR based on amplification compatibility test. Methods 2010;50:308–12.
[16] Verderio P, Pizzamiglio S, Gallo F, Ramsden SC. FCI: an R-based algorithm for evaluating uncertainty of absolute real-time PCR quantification. BMC Bioinformatics 2008;9:13.
[17] Hollander M, Wolfe DA. Nonparametric statistical methods. Second ed. New York: John Wiley & Sons; 1999.
[18] Sun F, Bruening W, Uhl S, Ballard R, Tipton K, Schoelles K. Quality, regulation and clinical utility of laboratory-developed molecular tests. AHRQ-Technology Assessment Report, (010) 1–205.
[19] Raggi CC, Verderio P, Pazzagli M, et al. An Italian program of external quality control for quantitative assays based on real-time PCR with Taq-Man probes. Clin Chem Lab Med 2005;43:542–8.
[20] Ramsden SC, Daly S, Geilenkeuser WJ, et al. EQUAL-quant: an international external quality assessment scheme for real-time PCR. Clin Chem 2006;52:1584–91.
[21] Madej RM, Cao Z, Hall L, Neuwald PD, Williams LO. Proficiency testing (external quality assessment) for molecular methods, approved guideline. CLSI document MM14-A, Vol. 25, No. 24. Clinical and Laboratory Standards Institute; 2005. p. 1–51.
[22] Verderio P, Ramsden SC, Orlando C, et al. External quality assessment schemes for real-time PCR: a statistical procedure to corrective actions. Clin Chem Lab Med 2008;46:717–21.