Clinical Chemistry 53:4
000–000 (2007)

Molecular Diagnostics and Genetics

Preanalytical mRNA Stabilization of Whole Bone Marrow Samples

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Background: **Gene expression profiling is a useful tool for diagnosis and basic research of cancer. A major limitation is that, even during short-term storage of native specimens of peripheral blood or bone marrow (BM) and/or RNA isolation, significant changes of gene expression pattern can occur because of gene induction, repression, and RNA degradation.**

Methods: **We investigated the effectiveness of a newly developed RNA stabilization and preparation system for BM specimens (PAXgene™ Bone Marrow RNA System) over time. We analyzed 256 RNA samples, processed from 64 BM specimens.**

Results: **Although the overall RNA yield (normalized to 1×10^7 leukocytes) was not different, the RNA preparation using unstabilized reference samples had an ~3 times higher failure rate. With the PAXgene system, we observed significantly higher RNA integrity compared with the reference RNA preparation system ($P < 0.01$). In the stabilized samples, we found very high pairwise correlation in gene expression ($\Delta\Delta C_T$ 0.16–0.53) for the analyzed genes (*GATA1*, *RUNX1*, *NCAM1*, and *SPI1*) after 48-h storage compared with immediate preparation of RNA (2 h after BM collection). However, we found major differences in half of the analyzed genes using the reference RNA isolation procedure ($\Delta\Delta C_T$ 1.07 and 1.32).**

Conclusions: **The PAXgene system is able to stabilize RNA from clinical BM samples and is suitable to isolate high-quality and -quantity RNA.**

Gene expression analysis by quantitative reverse-transcription PCR (Q-RT-PCR)[3] is widely used in basic and clinical research of cancer. In patients with leukemia, bone marrow (BM) specimens—rather than peripheral blood (PB) specimens—are the source of material for initial diagnosis and follow-up evaluation of treatment response. Nearly all children with leukemia in Germany (96.9%; http://www.kinderkrebsregister.de) are treated in nationwide multicenter clinical trials with centralized laboratories for diagnosis and research questions. Anticoagulated BM specimens are commonly shipped to these laboratories by overnight mail at room temperature. Owing to gene induction, repression, and RNA degradation, storage of native specimens of PB or BM and method of RNA isolation have significant influence on gene expression *(1–3)*. However, the evaluation of gene expression levels using Q-RT-PCR or microarray technology is essential for the investigation of the molecular origin of leukemia *(4–8)* as well as for monitoring minimal residual disease *(9)*.

Acute leukemias are clonal disorders of the hematopoietic system that arise in the BM. The current hypothesis for leukemogenesis published by Bonnet and Dick *(10)* assumes a multistep process starting from a leukemic stem cell that undergoes hierarchical differentiation into leukemic blasts. The aberrant expression of different transcription factors that are crucial at specific stages of hematopoiesis is believed to have a dominant role in either generation or maintenance of the malignant clone. The *RUNX1* gene (runt-related transcription factor 1)[4] is the most frequent target gene of aberrant activity in human leukemias. It is involved in several chromosomal translocations, e.g., t(8;21) in childhood acute myeloid leukemia, t(12;21) in childhood acute lymphoblastic leukemia, and t(3;21) in chronic myeloid leukemia or myelo-

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Received August 16, 2006; accepted January 16, 2007.
Previously published online at DOI: 10.1373/clinchem.2006.078592

[3] Nonstandard abbreviations: Q-RT, quantitative reverse-transcription; RT, reverse-transcription; BM, bone marrow; PB, peripheral blood; RIN, RNA integrity number.

[4] Human genes: *RUNX1*, runt-related transcription factor 1; *GATA1*, GATA binding protein 1; *SPI1*, spleen focus forming virus proviral integration oncogene 1; *NCAM1*, neural cell adhesion molecule 1.

dysplastic syndrome. Furthermore, a high incidence of *RUNX1* point mutations has been revealed. Recently, a third mode of *RUNX1* involvement in leukemogenesis—an increased dosage of *RUNX1*—has been reported *(11)*. The adhesion molecule *NCAM1* (neural cell adhesion molecule 1) is commonly expressed at the surface of subsets of acute leukemias and furthermore contributes to hematopoiesis-supporting capacity of stromal cell lines *(12)*. *GATA1* (GATA binding protein 1) is essential for normal erythropoiesis with a crucial role in cell survival and maturation *(13)*. Mutations in exon 2 of *GATA1*, which are translated into a shorter GATA1s protein, are detectable in virtually all cases of myeloid leukemia of Down syndrome and transient myeloproliferative disease of newborns with Down syndrome *(14)*. PU.1, which is encoded by the gene *SPI1* (spleen focus forming virus proviral integration oncogene 1), serves as a suppressor of acute myeloid leukemia, and its down-regulation results in an aggressive form of acute myeloid leukemia *(15–17)*.

Recently, a commercially available in vitro diagnostic system for the stabilization of RNA in PB specimens (PAXgene™ Blood RNA System, PreAnalytiX) arrived on the market. This system inhibits RNA degradation and prevents samples from ex vivo changes in gene expression starting immediately at the time of sample collection *(18)*. RNA stabilization is a major prerequisite for reliable transcript analysis in whole blood samples. An important difference between PB and BM that is relevant for RNA stabilization is BM's significantly higher range in cellularity, especially in diagnostic samples of acute leukemias (>500 000 leukocytes/μL), thus defining a strong need to adapt the existing PAXgene Blood RNA system to BM. For research use only, PreAnalytiX has optimized the system for BM. The system enables the collection, stabilization, storage, and transportation of human BM specimens, together with a rapid and efficient protocol for isolation and purification of intracellular RNA.

Our aim was to investigate the influence of preanalytical steps of sample handling after aspiration and before RNA analysis, focusing on RNA yield and integrity and stability of gene expression profiles. We compared the PAXgene Bone Marrow RNA System with a method without RNA stabilization using standard tubes and considered a 2-day storage of BM, reflecting the average shipping time in common clinical settings. We used transcript concentrations of marker genes *GATA1*, *RUNX1*, *SPI1*, and *NCAM1*, commonly involved in leukemogenesis, to study the need for stabilization of BM samples in a clinical setting of disease monitoring.

Patients, Materials, and Methods

PATIENTS AND SAMPLES

We collected BM specimens (n = 64), using 5000 IU heparin per 5 mL BM as anticoagulant, from randomly selected children with acute leukemia at initial diagnosis or during treatment or from children without pathologic hematopoietic findings. BM specimens were obtained after informed consent from each patient or patient's guardian. All investigations were approved by the local ethics committee. Immediately after collection, we transferred 2.5 mL BM to 2 PAXgene Bone Marrow RNA tubes containing optimized RNA stabilization solution. After 2 h (P2) or 48 h (P48) of storage at room temperature after collection, the tubes were frozen (−20 °C) for a median of 5 weeks (range, 1–15 weeks). After thawing, we prepared RNA by use of the PAXgene Bone Marrow RNA Kit. Two specimens served as reference samples: 0.5 mL BM was transferred into sterile plastic tubes and processed using the QIAamp® RNA Blood Mini Kit (Qiagen) at the same time points [2 h (R2) and 48 h (R48); Fig. 1]. The time points were chosen to represent the operational procedures in many hospitals and laboratories. For the reference protocol, we took into account a lag of 2 h (for in-house transportation and sample receipt in the laboratory) between collection of BM and preparation of RNA in the laboratory. For the PAXgene system, a minimum precipitation time of 2 h has to be spent before the RNA precipitate can be centrifuged. The 48-h storage at room temperature of either the plastic tube with anticoagulated BM (reference protocol) or the PAXgene Bone Marrow RNA tube represents the time interval between collection of BM and shipping to an external laboratory.

BM specimens were eligible for evaluation if all 4 RNA preparation procedures (2 PAXgene samples and 2 reference samples) fulfilled the following selection criteria for downstream analysis: (*a*) RNA concentration >5 mg/L and (*b*) RNA integrity number (RIN) >7 or A_{260}/A_{280} between 1.9 and 2.1. We measured leukocytes in BM specimens by use of FlowCount™ Fluorospheres (Beckman Coulter) and a Cytomics FC 500 flow cytometer (Beckman Coulter).

RNA PREPARATION PROCEDURE

We isolated and purified RNA from PAXgene Bone Marrow RNA tubes using the PAXgene Bone Marrow RNA Kit according to manufacturer's instructions. In brief, we harvested nucleic acids by centrifugation of collection tubes. We washed, resuspended, and incubated pellets in optimized buffers together with proteinase K. Cell lysates were homogenized and cell debris removed by use of PAXgene Shredder columns. RNA binding conditions were adjusted with ethanol, and lysates were applied to RNA spin columns. After centrifugation through a silica-gel membrane, we removed contaminants with efficient wash steps, followed by DNase I treatment to remove traces of bound DNA. After additional wash steps, RNA was released in 80 μL elution buffer and heat-denatured. We prepared reference RNA samples from unstabilized BM aliquots from sterile standard plastic tubes using the QIAamp RNA Blood Mini Kit as described in the handbook, with an elution volume of 50 μL.

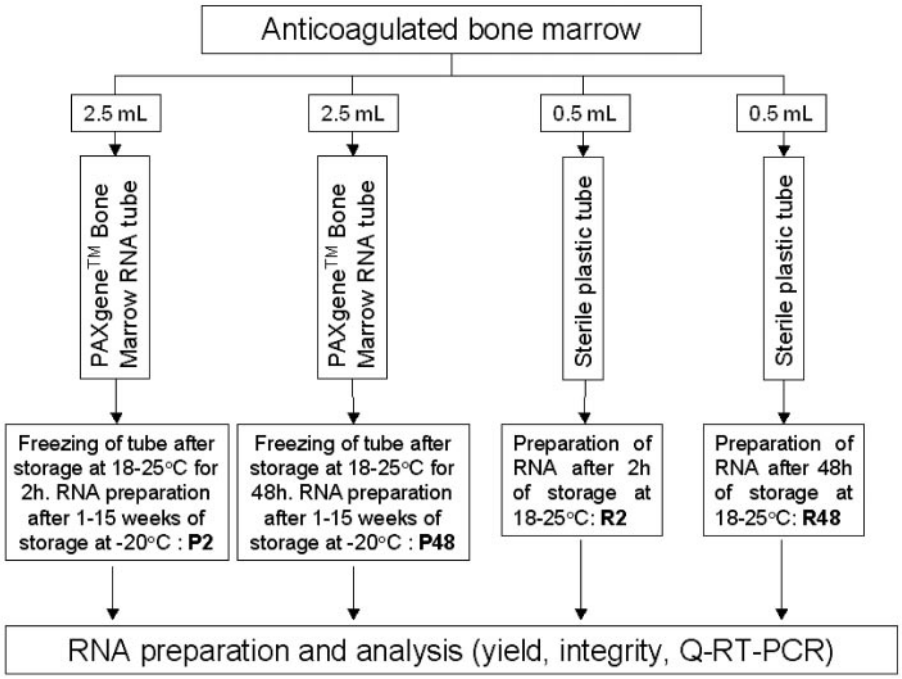

Fig. 1. Flowchart of the study design.

Immediately after collection, 2.5 mL anticoagulated BM was transferred to 2 PAXgene Bone Marrow RNA tubes containing optimized RNA stabilization solution. After 2 (*P2*) and 48 (*P48*) h of storage at room temperature after collection, the tubes were frozen (−20 °C) for a median of 5 weeks (range, 1–15 weeks). After thawing, RNA was prepared using the PAXgene Bone Marrow RNA Kit. Two other specimens of 0.5 mL BM each were transferred into sterile plastic tubes as reference samples and were processed using the QIAamp RNA Blood Mini Kit (Qiagen) at the same time points (*R2* and *R48*).

RNA yield and integrity

We diluted aliquots of RNA samples in 10 mmol/L Tris · Cl, pH 7.5, and quantified them using UV spectroscopy. We also used the buffers in which the RNA were isolated to zero the spectrophotometer in the final dilution of RNA aliquots to be quantified. We analyzed RNA integrity from aliquots of 1.5 μL RNA by use of RNA 6000 Nano reagents and chips on a Bioanalyzer 2100 device equipped with BioSizing software version A02.12 (Agilent Technologies). We applied the software RIN calculation algorithm to RNA fluorescence profiles after separation of RNA by capillary gel electrophoresis to establish RNA integrity with a score of 0 to 10 points (low to high RNA integrity).

Q-RT-PCR

Q-RT-PCR was performed in a 1-step method with 1 ng total RNA by use of the Quantitect® SYBR® Green reverse-transcription (RT)-PCR Kit and Quantitect Primer Assays (Qiagen) for the detection of *GATA1*, *RUNX1*, *NCAM1*, and *SPI1* and for control gene 18S rRNA. We chose 18S rRNA because general RNA degradation correlates very well with decreased amounts of 18S rRNA. We performed all assays in duplicate by use of the ABI™ 7300 Real-Time PCR System (Applied Biosystems). Calculation was done by relative quantification using the $2^{-\Delta\Delta CT}$ T method *(19)*. Only those data with a C_T value <35 (cutoff value) were included in the evaluation.

Feasibility study

Before the above study, we performed a feasibility study with a subset of 31 BM specimens. Along with the quantification of RNA yield and RNA integrity, we analyzed the expression of *IL8* gene (interleukin 8) using the Quantitect SYBR Green RT-PCR Kit (Qiagen) on the LightCycler® 2.0 System (Roche Applied Science). We chose *IL8* because of its known up-regulation after short-term storage of clinical samples *(20)*.

Results

Independent of the leukocyte count of the BM specimens (median, 17 151 leukocytes/μL; range, 2800–546 740), the handling of the PAXgene Bone Marrow RNA System was easy and convenient. There was no association between leukocyte count and the yield of total RNA in either preparation method (data not shown). Regarding the influence of 2-day storage on *IL8* gene expression, we found a huge difference in the unstabilized samples compared with the stabilized samples [median change in gene expression, 14.8-fold (R) vs 0.7-fold (P)]. As shown in Fig. 2, the C_T values of the R48 samples were consistently lower than the C_T values of the R2 samples, representing an increase in gene expression. Although the correlation between C_T values of P2 and P48 is not optimal (slope, 0.90; $R^2 = 0.64$), possibly because of missing normalization with transcripts of a housekeeping gene, the correlation for the reference samples was even worse (slope, 0.32; $R^2 = 0.08$).

Yield and integrity of RNA

Altogether, 180 RNA samples, processed from 45 BM specimens, fulfilled the inclusion criteria and were analyzed for yield, integrity, and gene expression profiles. We excluded 19 specimens because of low-yield or low-quality RNA samples: P2 (n = 3), P48 (n = 5), R2 (n = 12), and R48 (n = 15). In 26 samples, criteria for both yield and

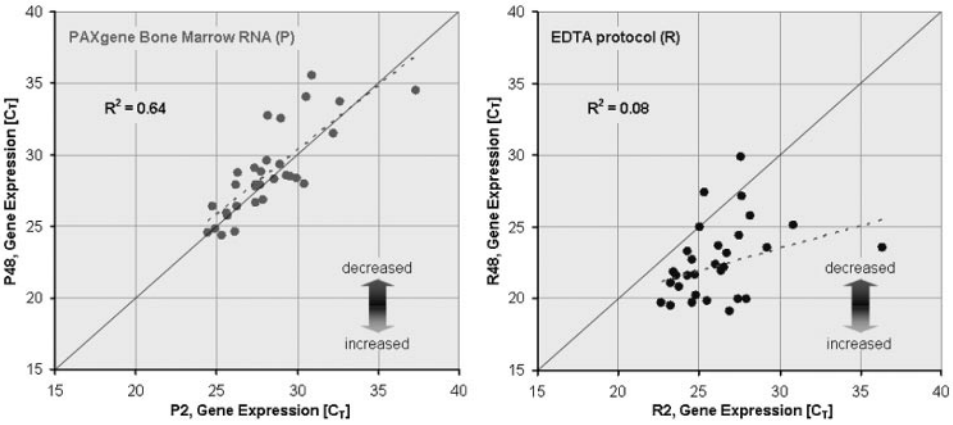

Fig. 2. *IL8* gene expression in RNA samples prepared from BM with 48 and 2 h of storage using either the PAXgene system (*P*) or the reference protocol without RNA stabilization (*R*).

The *diagrams* show the cycle threshold (C_T) of the Q-RT-PCR of *IL8* for the samples at 2 (*x axis*) and 48 (*y axis*) h of storage for either preparation method. The *black diagonal line* represents an ideal correlation, i.e., no change in gene expression between the 2 time points; the *dashed line* indicates the calculated regression line (*P*, $y = 0.90 \cdot x$, $R^2 = 0.64$; *R*, $y = 0.31 \cdot x$, $R^2 = 0.08$).There is a high increase in *IL8* expression after 48-h storage for the RNA prepared by the reference method compared with the PAXgene method.

quality were not fulfilled, 6 samples were of bad quality, and 3 samples (all reference samples) were of insufficient yield (1.2, 1.2, and 2.6 mg/L, respectively).

The number of invalid samples obtained with the reference method was ~3 times higher than with the PAXgene system (27 vs 8 samples), demonstrating higher overall RNA quality and quantity with PAXgene.

The RNA yield for each method was measured and normalized to 1×10^7 leukocytes in the input BM sample. As shown in Fig. 3A, the overall yield was similar in the 4 RNA isolation procedures. We found no differences in RNA yield at 2 and 48 h for each method separately (ANOVA).

The integrity of the isolated RNA using the PAXgene system was significantly higher at both time points compared with the reference system: P2, 8.6 (0.2) vs R2, 6.8 (0.4), $P = 0.0003$, and P48, 8.1 (0.2) vs R48 6.7 (0.5), $P = 0.008$. We found no statistically significant difference between R2 and R48, but a slight decrease between P2 and P48 ($P = 0.048$). As shown in Fig. 3B, the spreading of RIN is more distinct in the RNA prepared by the reference method with regard to the 25% to 75% confidence interval: R2, 5.2–9.1 and R48, 4.1–9.0 vs P2 8.4–9.3 and P48, 7.8–8.9. As an example, Fig. 3C illustrates the RNA integrity of a complete data set with 4 samples (P2, P48, R2, and R48).

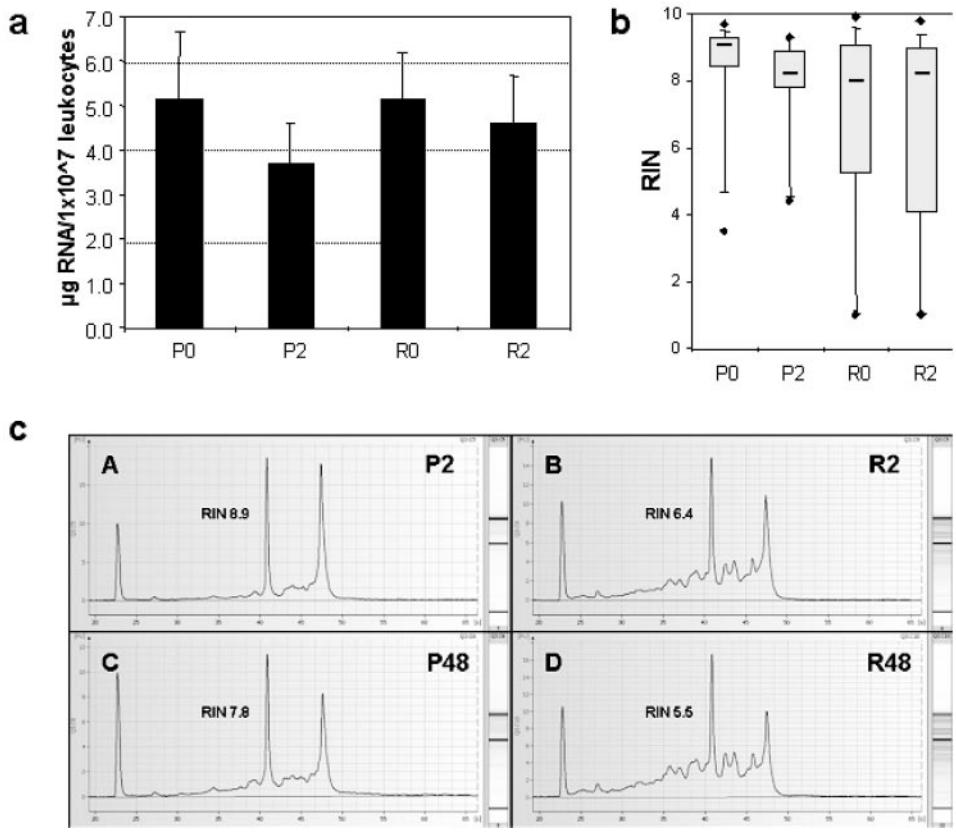

Fig. 3. RNA yield and integrity.

(*A*), RNA yield of BM aliquots with 2 and 48 h of storage at room temperature obtained using the PAXgene system (*P*) and the reference protocol without RNA stabilization (*R*) (mean +SE). (*B*), *box plot* of RIN values from RNA samples of BM aliquots with 2 and 48 h of storage at room temperature obtained using the PAXgene system (*P*) and the reference protocol without RNA stabilization (*R*) (*black bars*, median; *gray rectangles*, 25th to 75th percentile; *black lines*, 5th to 95th percentile; *rhombs*, minimum/maximum value). (*C*), representative RNA integrity analysis of 4 RNA samples of a single BM aliquot with 2 and 48 h of storage at room temperature obtained using the PAXgene system (*P*) and the reference protocol without RNA stabilization (*R*).

PAXgene stabilizes gene expression profiles

We analyzed mRNA levels of different genes after RNA preparation using the PAXgene procedure at P2 and P48. Differences of $\Delta\Delta C_T$, calculated as $\Delta C_T(P2) - \Delta C_T(P48)$ for the control gene (18S rRNA), were homogeneous within a tight range: *GATA1* 0.17 (0.10), *RUNX1* 0.27 (0.17), *NCAM1* 0.53 (0.23), and *SPI1* 0.16 (0.09). This results in a high pairwise correlation in gene expression for any gene at P48 compared with P2: *GATA1* 89 (6)%, *RUNX1* 83 (10)%, *NCAM1* 69 (12)%, and *SPI1* 89 (6)% (Fig. 4).

Gene-dependent change of expression level using standard RNA isolation techniques

Regarding expression levels of the same genes after RNA preparation from unstabilized BM samples using QIAamp RNA Blood Kit, the results are more heterogeneous. For 2 of the 4 analyzed genes, the differences in $\Delta\Delta C_T$, $\Delta C_T(R2) - \Delta C_T(R48)$, were also within the range of ± 0.5, whereas for the 2 other genes, the differences were >1: *GATA1* 1.32 (0.19), *RUNX1* 0.25 (0.18), *NCAM1* 1.07 (0.23), and *SPI1* −0.01 (0.14). The resulting expression levels at R48 for the latter genes were accordingly reduced to 40 (6)%, $P < 0.0001$ (*GATA1*) and 47 (8)%, $P = 0.005$ (*NCAM1*; Fig. 4).

Expression levels are not comparable using different RNA isolation protocols

Comparing the 2 RNA isolation procedures with each other, there were major differences in the expression levels of the analyzed genes. We observed lower mean (SD) gene expression at P2 vs R2 for all genes: *GATA1* 70 (12)%, *RUNX1* 50 (11)%, *NCAM1* 41 (8)%, and *SPI1* 51 (6)%. At the 48-h time point, the changes in gene expression for *RUNX1* and *SPI1* were similar at 45 (7)% and 43 (6)%, respectively, whereas for the other 2 genes (*GATA1* and *NCAM1*), the decrease in gene expression using the reference method resulted in significant discrepancy: 138 (26)% and 76 (16)% (Fig. 5).

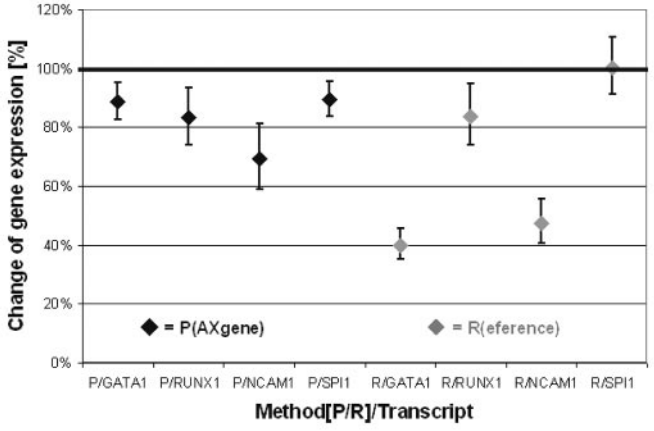

Fig. 4. Change of gene expression in RNA samples prepared from BM with 48 and 2 h of storage using either the PAXgene system (*P*) or the reference protocol without RNA stabilization (*R*).
Data are shown as mean (SE).

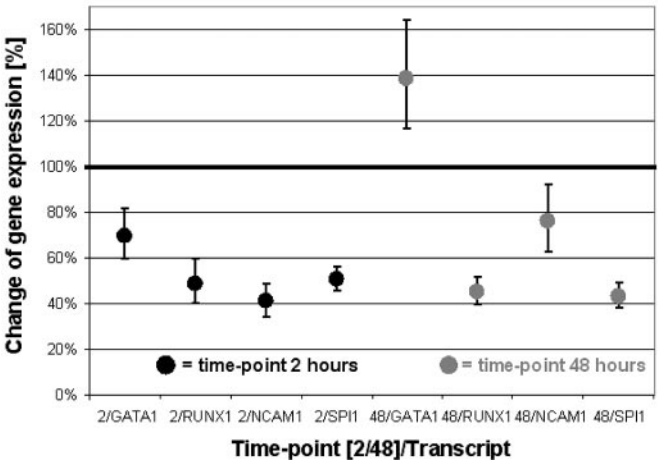

Fig. 5. Differences of transcript levels between RNA samples prepared from BM with the PAXgene system and the reference protocol without RNA stabilization at same test time point with 2 and 48 h of storage.
Data are shown as mean (SE).

Discussion

In multicenter clinical trials, a centralized analysis of patient samples is generally performed to minimize interlaboratory variations by applying a standardized method to all samples. Little is known, however, about the impact of preanalytical sample handling, particularly for molecular genetic analyses based on mRNA. High interindividual and temporal variations of gene expression patterns have been demonstrated because of genetic background, age, sex, and the time of day at which the sample was taken *(21)*. Other similarly important variables affecting gene expression profiles include mode of sample aspiration, use of different anticoagulants, variable temperature during storage, and time frame between aspiration and processing of the sample.

We carried out a comparative study of a newly developed system for RNA stabilization in whole BM specimens (PAXgene Bone Marrow RNA System) and unstabilized controls after different times of storage. The time delays before RNA preparation were chosen to represent the average shipping time between clinics and laboratories. Although alterations in gene expression may occur during a very short period of time, our study was designed to represent realistic operational procedures in a hospital, where BM punctures (especially in children) are often performed in an operating room, and a routine in-house transport time to the laboratory has to be taken into account.

The promising data from the feasibility study confirmed the known up-regulation of *IL8* gene expression in unstabilized specimens. The PAXgene-stabilized BM specimens with 48 and 2 h of storage showed high accordance of in terms of *IL8* gene expression, prompting us to perform a more comprehensive study. The analyses of purity (calculated as A_{260}/A_{280} ratio) and RNA integrity (calculated by RIN) served as tools to determine the

overall quality of the RNA preparation and purification procedure in terms of purity and possible degree of overall RNA degradation. Although the use of cDNA microarrays allows genomewide gene expression screening, their value for exact quantification is limited *(22)*. For more detailed information about the stabilization of RNA, Q-RT-PCR was performed to quantify mRNA concentrations. The genes to be analyzed were selected because of their attributes as key regulators during different stages of hematopoiesis and leukemogenesis, as well as their more or less distinct organ specificity: *NCAM1* and *RUNX1* as representatives of genes with functions (hematopoiesis, osteogenesis, cell adhesion) on many cell types (hematopoietic cells, neurons, muscles) of various differentiated tissues (embryonic cells, differentiated cells), *GATA1* and *SPI1* having more circumscribed compartments and cell types (erythropoiesis/megakaryopoiesis, myelopoiesis, and lymphopoiesis).

There were no major differences in the yield of isolated RNA between the 4 different procedures. Regarding the integrity of the RNA, however, the PAXgene system was superior to the reference method of unstabilized BM samples at both time points. Handling of samples with very high leukocyte counts was not impaired compared with samples with normal leukocyte counts. The unexpectedly high integrity of the RNA samples obtained after 48 h of storage under unstabilized conditions (R48) might be explained by the RNA preparation procedure: in contrast to a direct preparation from whole BM, the QIAamp procedure used here includes erythrocyte lysis as well as the generation of a cell pellet that contains only intact white BM cells. Therefore, possible RNA degradation products are removed with the supernatant.

Regarding gene expression profiling, the use of RNA stabilization reagents is still controversial: there are some reports of differences in gene expression resulting from poor stability of genes analyzed using either microarray technology *(23)* or Q-RT-PCR *(24, 25)*. In terms of RNA quality and gene expression, however, our results from BM samples show excellent pairwise correlation between different incubation times using quantitative RT-PCR and are in line with those derived from PB *(3, 26, 27)*. The PAXgene system is appropriate for quantification of gene expression levels in BM samples. For some genes, however, the reference method resulted in similar gene expression levels, so the use of a stabilization reagent does not seem to be essential for every gene. This has also been shown for the quantification of tissue factor (F3) and vascular endothelial growth factor, with comparable results derived from stabilized and unstabilized samples *(28)*. Therefore, each researcher must carefully evaluate the preanalytical handling of the samples and choose a system for RNA ex vivo stabilization in case of gene expression analysis in later project phases with unknown targets at start of study. In studies where RNA isolates are stored for a long time before analysis of currently unrevealed disease marker transcripts, BM stabilization using the PAXgene system is recommended.

Different principles of RNA isolation may lead to different gene expression levels: whereas the QIAamp RNA isolation procedure includes erythrocyte lysis, during the PAXgene protocol, the RNA of all BM cells is isolated. Therefore, it is not suitable to directly compare mRNA expression levels derived from different isolation procedures.

In conclusion, the PAXgene Bone Marrow RNA System is suitable to stabilize, isolate, and purify RNA in high quality and quantity from clinical BM samples. The ability to preserve gene expression is comparable to that of PB specimens reported in detail by different researchers with the PAXgene Blood RNA System.

We thank Carolin Augsburg and Heike Balven for excellent technical assistance and Douglas McGarvey for review of the manuscript. Conflicts of interest: K.G. and J.L. are full-time employees and participate in the stock options program of at Qiagen GmbH.

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