RNA stabilization of human whole bone marrow aspirates for high reliability of gene expression analysis

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Introduction

Gene expression analysis of human bone marrow is a useful tool for the diagnosis and research of cancer and disease monitoring. One of the major challenges for reliable analysis is the alteration of gene expression starting immediately at the time of aspiration, even after short-term storage of native specimens, due to ex vivo gene induction, repression, and RNA degradation.

PreAnalytiX developed and launched a product system* that enables the collection, stabilization, storage, and transportation of human bone marrow specimens, together with a rapid and efficient protocol for isolation and purification of cellular RNA. The system comprises bone marrow tubes and an RNA isolation and purification kit†.

The objective of this study was to investigate and compare the performance of the new PAXgene™ Bone Marrow RNA System*† to a commonly used procedure.

This study was designed to reflect the workflow in the reference laboratory of the AML-BFM study group (Department of Pediatric Hematology and Oncology of Hannover Medical School, Germany) with bone marrow samples retrieved from in-house and external clinics for RNA analysis. Two major test time-points of bone marrow storage were chosen prior to RNA preparation and analysis: up to 2 h of storage to simulate in-house transportation and 48 h to simulate shipment using overnight courier service.

* For Research Use Only. Not for use in diagnostic procedures. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

† PAXgene Bone Marrow RNA Tube (50), PreAnalytiX (Hombrechtikon, Switzerland), cat. no. 764114
PAXgene Bone Marrow RNA Kit (30), PreAnalytiX (Hombrechtikon, Switzerland), cat. no. 764133

Material and methods

In accordance with institutional procedure, a total of 45 bone marrow specimens of unselected children with acute leukemia (AL) at initial diagnosis, during treatment or without pathological findings in hematopoiesis, were aspirated at the Hannover Medical School (Germany) after informed consent from each patient or patient´s guardian. All investigations were approved by the local ethics committee*. Aspiration was done following an in-house standard procedure using common aspiration devices, syringes, and heparin as anti-coagulant.

Specimens were immediately divided into quadruplicate sample aliquots and transferred into two PAXgene Bone Marrow RNA Tubes or left untreated in two sterile standard plastic tubes (two reference aliquots).

From each pair of aliquots, the first one was processed within 2 h of storage at room temperature (18–25°C). RNA preparation from PAXgene Bone Marrow RNA Tubes was perfomed using the PAXgene Bone Marrow RNA Kit according to manufacturer's handbook and from standard plastic tubes using the reference method, a silica-membrane-based RNA preparation (QIAamp® RNA Blood Mini Kit with red blood cell lysis). Corresponding second aliquots were stored at room temperature for a total of 48 h prior to processing as described above.

The resulting total of 180 RNA samples were analyzed for RNA yield and integrity using UV spectroscopy and capillary gel electrophoresis with calculation of RNA integrity numbers (RIN, Agilent® 2100 Bioanalyzer with Nanochips).

* Study approval by ethics committee of the medical association Westfalen-Lippe (Germany). Registration number: 3XIIRein

Material and methods (continued)

Applicability of prepared RNA to downstream analysis was investigated by RT-PCR. Transcripts of GATA1, RUNX1, NCAM, and SPI1 were selected on the assumption that they play a dominant role in either the generation or the maintenance of malignant cell clones in AL. Transcript levels at both test time-points served to monitor transcript profiles over time of storage and to verify the stabilization properties of the PAXgene Bone Marrow RNA System in comparison to the reference procedure.

Utilizing 18S rRNA as an internal reference, one-step, real-time, quantitative, duplex, RT-PCR assays (QIAGEN QuantiTect® Probe RT-PCR Kit) were used on an ABI PRISM® 7700 SDS instrument (Applied Biosystems) to calculate ΔC_T (C_T18S rRNA – $C_T$target transcript) and $\Delta\Delta C_T$ (C_T2 h – C_T48 h) that were converted to the relative differences of transcript levels between paired RNA samples (2 h vs. 48 h of bone marrow storage prior to RNA preparation) within each procedure (PAXgene system vs. reference procedure).

Tests for statistical significance were performed using the Mann-Whitney-Wilcoxon test (U test) in WinSTAT software package.

Results

Independant of the white blood cell (WBC) count (median:17.2 x 10^6, range: 2.8 to 546.7 x 10^6 WBC/ml), the handling of the PAXgene Bone Marrow RNA System was easy and convenient with no failures in sample processing, thus demonstrating high reliability of the product.

The overall RNA yield (normalized to 10 x 10^6 WBC) was statistically not different in all four samples per specimen in an analysis of variance. However, decreases of yield were detected as the result of sample storage at room temperature with both procedures (Figure 1).

Results (continued)

Analysis of RNA quality resulted in significantly higher RNA integrity (represented by means of RIN) using the PAXgene Bone Marrow RNA System at both time-points compared with reference procedure: 8.5 ± 0.2 (PAX, 2 h) vs. 6.6 ± 0.5 (Ref., 2 h), p = 0.0003 and 8.0 ± 0.2 (PAX, 48 h) vs. 6.8 ± 0.5 (Ref., 48 h), p = 0.008. As shown in Figure 2, the variability of RIN was higher in the RNA prepared by the reference procedure with regard to 5th to 95th/ 25th to 75th percentile: 1.7–9.5/ 4.5–8.9 (Ref., 2 h) and 1.4–9.3/ 5.3–9.0 (Ref., 48 h) vs. 6.0–9.4/ 8.3–9.3 (PAX, 2 h) and 4.8–9.3/ 7.8–8.9 (PAX, 48 h). As an example of RNA integrity obtained using both procedures, Figure 3 illustrates a complete dataset of fluorescence profiles of capillar gel electrophoretic separation of RNA from one donor with all aliquot samples (PAX, 2 h, PAX, 48 h, Ref., 2 h, and Ref., 48 h). Degraded RNA from RNA samples of the reference procedure is indicated by a fraction of RNA fragments of intermediate size between 28S and 18S rRNA and below, also reflected by lower RIN values.

The PAXgene Bone Marrow RNA System showed high pairwise correlation in gene expression for each gene at 48 compared to 2 hours demonstrated by low $\Delta\Delta C_T$ (Figure 4A) and nearly constant relative transcript levels (Figure 4B): GATA1 89 ± 6%, RUNX1 83 ± 10%, NCAM 69 ± 12%, and SPI1 89 ± 6%. In contrast, significant differences were detected in two of the four analyzed genes using the reference procedure: 40 ± 6%, p <0.0001 (GATA1) and 47 ± 8%, p = 0.005 (NCAM), respectively (Figure 4B).

RNA yield

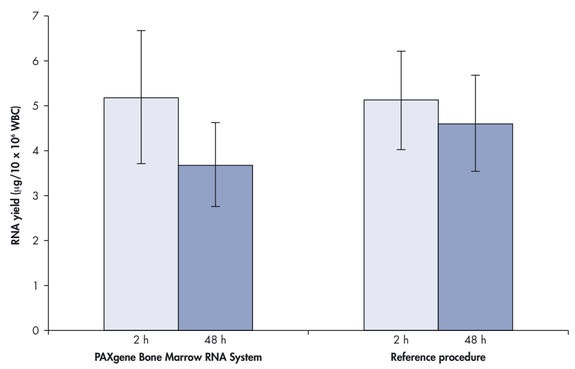

Figure 1. RNA yield of bone marrow aliquots with 2 h and 48 h of storage at room temperature obtained using the PAXgene Bone Marrow RNA System and the reference procedure without RNA stabilization. Isolated RNA was quantified using UV spectroscopy. Means (boxes) and standard errors of the mean (SEM, vertical lines) of RNA yield are given for all samples.

RNA integrity

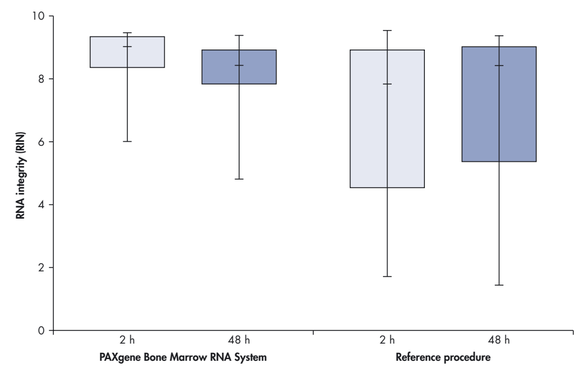

Figure 2. Boxplot of RNA integrity from RNA samples of bone marrow aliquots stabilized in PAXgene Bone Marrow RNA Tubes and standard plastic tubes without stabilization (reference). RNA was purified from pairs of samples using the PAXgene Bone Marrow RNA Kit and the reference protocol within 2 h and after 48 h, each. RNA integrity was analyzed using RNA integrity numbers (RIN). Horizontal lines in rectangles: median. Rectangles: 25th to 75th percentile. Vertical lines: 5th to 95th percentile.

RNA integrity (example)

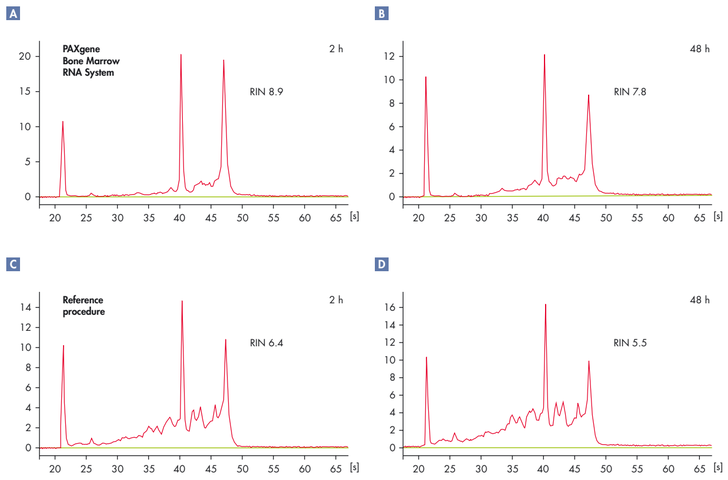

Figure 3. Representative results from integrity analysis of RNA prepared from all four aliquots of one bone marrow sample (patient ID#42), stored for the indicated time in A B PAXgene Bone Marrow RNA Tubes and C D standard plastic tubes without stabilization (reference). RNA was isolated using the PAXgene Bone Marrow RNA Kit and the reference protocol.

Gene expression levels

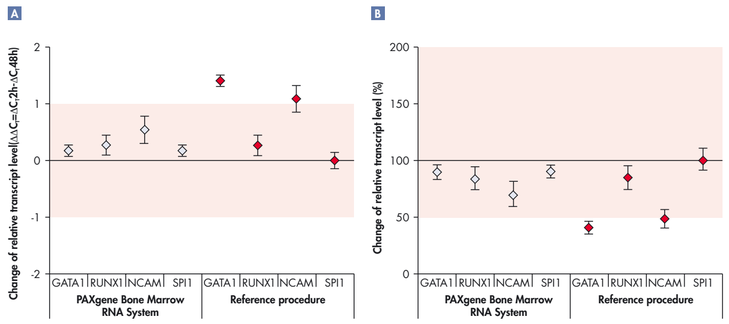

Figure 4. Gene expression analysis of RNA prepared from bone marrow samples after storage using the PAXgene Bone Marrow RNA System and the reference procedure (without stabilization).

A Real-time, quantitative, duplex RT-PCR was performed for transcripts of GATA1, RUNX1, NCAM, and SPI1 with internal normalization to 18S rRNA. ΔC_T values were calculated for each RNA sample (C_T18S rRNA – $C_T$target transcript). Changes of target transcript levels in paired samples as the effect of bone marrow storage were calculated with $\Delta\Delta C_T$ (C_T2 h – C_T48 h) shown as means (rhombi) and SEM (vertical lines). The red-shaded area shows a tolerance of +/- 1 C_T.

B Data from panel A were used to calculate the changes of relative transcript level. Note that the tolerance of +/- 1 C_T from panel A is equivalent to the bisection (50%)/doubling (200%) of transcript levels.

Conclusions

- The study presented here demonstrates that the new PAXgene Bone Marrow RNA System (FRUO) enables the collection, stabilization, storage, and transportation of human bone marrow specimens, together with a reliable, rapid and efficient protocol for isolation and purification of RNA from samples even of high white blood cell counts.
- The system provides stabilization of intracellular RNA for molecular downstream applications such as real-time, quantitative, RT-PCR and preserves gene expression patterns in comparison to significant changes of transcript levels detected using a common reference method.
- RNA was stabilized in PAXgene Bone Marrow RNA Tubes for two days at room temperature with essentially higher RNA integrity than the reference method.

PAXgene Blood RNA System, QuantiTect Probe RT-PCR Kit: For Research Use Only. Not for use in diagnostic procedures. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

The QIAamp RNA Blood Mini Kit is intended for general laboratory use. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

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