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

Kalle Guenther, Claudia Langebrake, Juergen Lauber, Dirk Reinhardt

1 QIAGEN GmbH, Hilden, Germany
2 Hannover Medical School, Department of Pediatric Hematology and Oncology, Germany

Introduction
Gene expression analysis of human bone marrow is a useful tool for the diagnosis and research of cancer and monitoring disease. 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 e.g. 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 QIAGEN Bone Marrow RNA Tube** to a commonly used procedure.

This study was designed to reflect the workflow in the reference laboratory of the A6-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.

Material and methods
In accordance with institutional procedure, a total of 45 bone marrow specimens of unrelated 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 unchanged standard procedure using appropriate aseptic 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 performed using the PAXgene Bone Marrow RNA Kit according to manufacturer’s handbook and from standard plastic tubes using the reference method, a silicon 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 spectrometry and capillary gel electrophoresis with calculation of RNA integrity numbers (RIN, Agilent® 2100 Bioanalyzer with NanoChip).

Results
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. As an example of RNA integrity obtained using both procedures, Figure 3 illustrates a complete dataset of fluorescence profiles of capillary gel electrophoresis of RNA from one donor with all aliquots samples (PAX, 2 h, PAX, 48 h, Reflex, 2 h, and Reflex, 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.

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 (RAX, 2 h; RAX, 48 h; p = 0.003 and 0.002; PAX, 2 h; PAX, 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 total RNA RIN values of 25th to 75th percentile 1.3–9.5/6.5–9.9 (Ref., 2 h) and 1.6–9.5/5.3–9.0 (Ref., 48 h) vs. 0.0–9.4/8.3–9.0 (PAX, 2 h) and 4.8–9.7/8.0–9.9 (PAX, 48 h). As an example of RNA integrity obtained using both procedures, Figure 3 illustrates a complete dataset of fluorescence profiles of capillary gel electrophoresis of RNA from one donor with all aliquots samples (PAX, 2 h, PAX, 48 h, Reflex, 2 h, and Reflex, 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 RNA, 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 h compared to 2 hours demonstrated by low ΔΔ C_{T} (Figure 4A) and nearly constant relative transcript changes of transcript levels detected using a common reference method.

The PAXgene Bone Marrow RNA System in comparison to the reference procedure.

Conclusions
The study presented here demonstrates that the new PAXgene Bone Marrow RNA System (FRG) enables the collection, stabilization, storage, and transportation of human bone marrow samples, 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, one-step, real-time, quantitative, duplex, RT-PCR uses for RNA preparation (QIAGEN Group); PAXgene RNA was quantified using UV spectroscopy. Means (boxes) and standard errors of the mean (SEM, vertical bars) of transcript levels were calculated for each sample (C_{T}) + standard deviation (SD). Changes in gene expression were calculated in comparison to the reference method. The reference time-point (2 h) was chosen as reference (ΔΔC_{T} = 0).

RNA integrity

Material and methods (continued)
Applicability of prepared RNA to downstream analysis was investigated by real-time PCR. Transcripts of GATA1, RUNX1, NCAM, and SPI1 were selected on the assumption that they play a dominant role in either the generation or maintenance of malignant cell clones in AL. Transcription levels at both 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.

Using 18S rRNA as an internal reference, one-step, real-time, quantitative, duplex, RT-PCR assays (QIAGEN Quantitect Probes RT-PCR Kit) were used on an ABI PRISM® 7700 SDS instrument (Applied Biosystems) to calculate ΔΔ C_{T}. (18S rRNA – C_{T} target transcript) and ΔΔ C_{T} (ΔΔ C_{T} target transcript) 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-U/Wilcoxon test (U test) in WinSTAT software package.

Results
Independent of the white blood cell (WBC) count (median: 17.2 ± 10^6, range: 2.8 to 546.7 ± 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).

Conclusions
- The study presented here demonstrates that the new PAXgene Bone Marrow RNA System (FRG) enables the collection, stabilization, storage, and transportation of human bone marrow samples, 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, one-step, real-time, quantitative, duplex, RT-PCR uses for RNA preparation (QIAGEN Group); PAXgene RNA was quantified using UV spectroscopy. Means (boxes) and standard errors of the mean (SEM, vertical bars) of transcript levels were calculated for each sample (C_{T}) + standard deviation (SD). Changes in gene expression were calculated in comparison to the 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.