Performance evaluation study of the PAXgene™ Blood RNA System with regulatory compliance

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1. Introduction

Gene expression analysis in peripheral blood is an important tool in molecular research, pharmacogenomics, and diagnostics. Reliable analysis is challenged by *ex vivo* changes of expression patterns starting immediately at the time of blood collection. In 2001, PreAnalytiX successfully introduced the PAXgene Blood RNA System intended for the collection, storage, and transport of blood, stabilization of RNA and subsequent isolation and purification of intracellular total RNA from whole blood for research use only. The system consists of a blood collection tube (PAXgene Blood RNA Tube) and nucleic acid purification kit (PAXgene Blood RNA Kit).

This system is widely used for clinical research applications and drug trials. In addition, as RNA-based detection of minimal residual disease in leukemias and monitoring diseases on the molecular level become more important in managing patients, the need increases for an FDA-cleared (US) and CE-marked (EU) preanalytical RNA system for in vitro diagnostic (IVD) applications.

The aim of this study was to evaluate and verify the performance characteristics of a new, optimized PAXgene Blood RNA System (IVD) to support product claims specified in the submission to regulatory authorities. The product was submitted to the FDA using the de novo 510(k) process.

Here we describe a small subset of design verification experiments of the optimized new PAXgene Blood RNA System (IVD) to verify the system's performance, reliability, repeatability, and reproducibility claims.

2. Materials and methods

According to its intended use, system performance was verified with human whole blood with white blood cell counts in the range of 4.8 x 10^6 – 1.1 x 10^7 cells/ml, collected from different donors and stored in PAXgene Blood RNA Tubes (BD, cat. no. 762162). RNA was subsequently extracted using the PAXgene Blood RNA Kit (762174 in Europe; 762164 in the US and Canada) according to the kit handbook.

For stability experiments, a reference method was used, combining standard blood collection tubes with EDTA as an anticoagulant and total RNA purification using a standard acid-phenol organic-extraction method (QIAzol Lysis Reagent) with silica-membrane–based RNA cleanup (QIAamp® RNA Blood Mini Kit). One-step, real-time, duplex RT-PCR assays were developed and used to analyze transcript levels by normalizing C_T (threshold cycle) values of analytes (IL1B) to those of 18S rRNA using the comparative $\Delta\Delta C_T$ method.

RNA was quantified and purity determined using UV spectrophotometric analysis. Genomic DNA contamination was assayed by real-time PCR assays of β-actin sequences and comparison with a standard curve. Inhibition of RT-PCR was analyzed by one-step real-time RT-PCR assays of β-actin sequences using a constant amount of template RNA.

All assays were validated according to method validation guidelines given in USP 27 <1225> "Validation of compendial methods" and ICHQ2A and ICHQ2B "Validation of analytical procedures". This study was conducted in compliance with regulatory guidelines to support product performance claims, and results were submitted to authorities in a 510(k) clearance process, followed by approval of the complete system by The Center for Devices and Radiological Health (CDRH) of the Food and Drug Administration (FDA) to be used as an IVD cleared product in April, 2005 (Regulation Number 21 CFR 866.4070).

3. Results — stabilization at 18–25°C

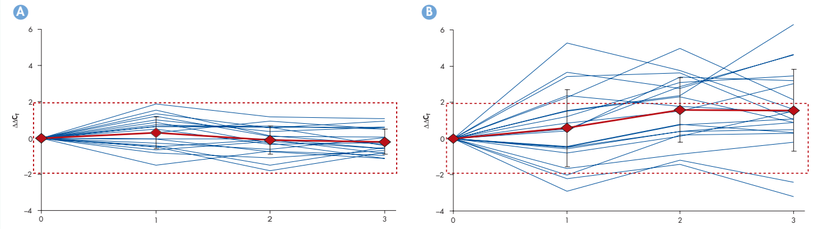

Figure 1 Duplicate blood samples were drawn from 10 donors and stored at 18–25°C for the indicated number of days, followed by total RNA purification. (A) Blood was collected and stored in PAXgene Blood RNA Tubes, and total RNA was purified using the PAXgene Blood RNA Kit. (B) Blood was collected and stored in standard blood collection tubes with EDTA as an anticoagulant, and total RNA was purified using a standard organic-extraction method with silica-membrane-based RNA cleanup. Relative transcript levels of IL1B were determined by real-time, duplex RT-PCR, using 18S rRNA as an internal standard. The values for all samples are plotted with means and standard deviations of all samples shown. The dashed lines indicate the ±3x total precision of the assay (1.93 C_T).

- Stability: Transcripts remained stable for 3 days at 18–25°C using the PAXgene Blood RNA System, in contrast to significant changes using the reference method.
- Yield: 100% of samples purified using the PAXgene Blood RNA System had RNA yields ≥3 µg (n = 80).
- Purity: 100% of samples purified using the PAXgene Blood RNA System had A_{260}/A_{280} values in the range 1.8–2.2 (n = 80).

4. Results — stabilization at 2–8°C

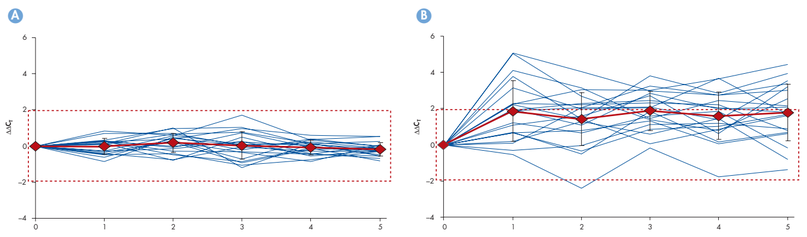

Figure 2 Duplicate blood samples were drawn from 10 donors and stored at 2–8°C for the indicated number of days, followed by total RNA purification. (A) Blood was collected and stored in PAXgene Blood RNA Tubes, and total RNA was purified using the PAXgene Blood RNA Kit. (B) Blood was collected and stored in standard blood collection tubes with EDTA as an anticoagulant, and total RNA was purified using a standard organic-extraction method with silica-membrane-based RNA cleanup. Relative transcript levels of IL1B were determined by real-time, duplex RT-PCR, using 18S rRNA as an internal standard. The values for all samples are plotted, with means and standard deviations of all samples shown. The dashed lines indicate the ±3x total precision of the assay (1.93 C_T).

- Stability: Transcripts remained stable for 5 days at 2–8°C using the PAXgene Blood RNA System, in contrast to significant changes using the reference method.
- Yield: 100% of samples purified using the PAXgene Blood RNA System had RNA yields ≥3 µg (n = 120).
- Purity: 100% of samples purified using the PAXgene Blood RNA System had A_{260}/A_{280} values in the range 1.8–2.2 (n = 120).

5. Results — stabilization at –20°C and –70°C

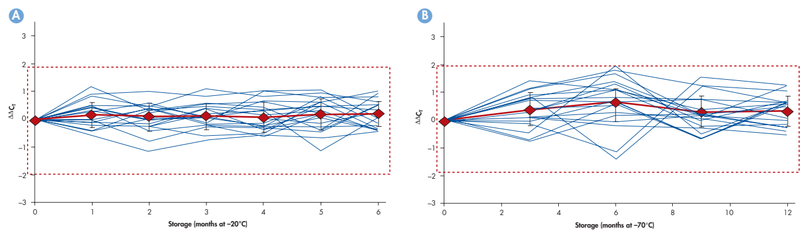

Figure 3 Duplicate blood samples were drawn from 10 donors into PAXgene Blood RNA Tubes and stored at –20°C or –70°C for the indicated number of months, followed by total RNA purification using the PAXgene Blood RNA Kit. (A) PAXgene Blood RNA Tubes were stored at –20°C. Relative transcript levels of IL1B were determined by real-time, duplex RT-PCR, using 18S rRNA as an internal standard. (B) PAXgene Blood RNA Tubes were stored at –70°C. Relative transcript levels of IL1B were determined by real-time, duplex RT-PCR, using 18S rRNA as an internal standard. The values for all samples are plotted, with means and standard deviations of all samples shown. The dashed lines indicate the ±3x total precision of the assay (1.93 C_T).

- Stability: Transcripts remained stable for 6 months at –20°C and 12 months at –70°C using the PAXgene Blood RNA System. (The reference method was not tested since freeze–thawing of unpreserved blood is known to result in heavy degradation or total loss of RNA.)
- Yield: 100% of samples stored at –20°C (n = 140) and 99% of samples stored at –70°C (n = 100) had RNA yields ≥3 µg.
- Purity: 100% of samples stored at –20°C (n = 140) and 99% of samples stored at –70°C (n = 100) had A_{260}/A_{280} values in the range 1.8–2.2.

6. Results — minimal genomic DNA and no RT-PCR inhibition

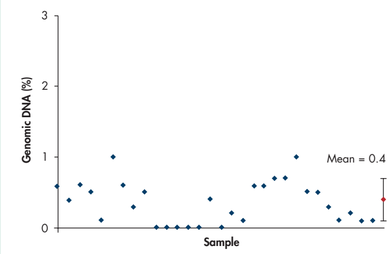

Figure 4 Duplicate blood samples were drawn from 15 donors into PAXgene Blood RNA Tubes and stored at 18–25°C for 1 day, followed by total RNA purification using the PAXgene Blood RNA Kit. Genomic DNA was detected by real-time PCR of the β-actin gene and quantified by comparison with a standard curve. Genomic DNA is reported as the percentage (w/w) of the total nucleic acids.

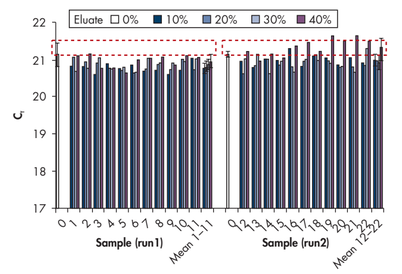

Figure 5 The PAXgene Blood RNA purification procedure was carried out 22 times using distilled water as the starting material instead of the pellet resulting from centrifugation of PAXgene Blood RNA Tubes. Each of the resulting "blank" eluates was used in real-time RT-PCR of the β-actin transcript with a constant amount of template RNA. The eluate comprised the indicated percentage of the reaction volume.

- Genomic DNA: All samples (100%, n = 30) fulfilled the design input specification with ≤1.0% genomic DNA (mean = 0.4%).
- RT-PCR inhibition: All samples (100%, n = 22) showed minimal effects on RT-PCR (<0.5 C_T), with eluates comprising up to 30% of the total reaction volume.

7. Results — lot-to-lot and user-to-user reproducibility

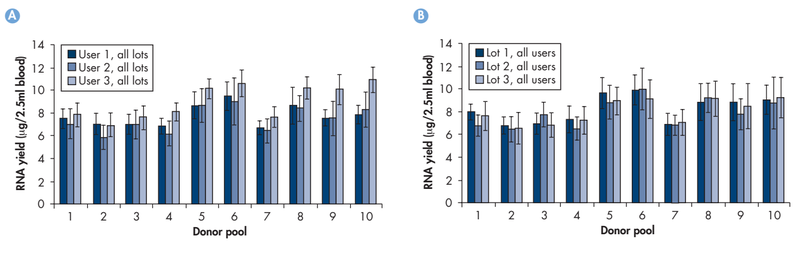

Figure 6 Blood samples from 30 different donors were collected in PAXgene Blood RNA Tubes (12 tubes per donor, 360 tubes in total). The contents of the tubes from 3 donors were pooled and subsequently realiquoted into 36 samples. These 36 samples per 3-donor-pool were processed by 3 different operators. Each operator used 3 different PAXgene Blood RNA Kit lots for the extraction and processed quadruplicate samples from each of the 10 donor pools. (A) RNA yield and standard deviation are given for each operator (**User 1**, **User 2**, **User 3**) using all 3 lots. (B) RNA yield and standard deviation are given for each lot (**Lot 1**, **Lot 2**, **Lot 3**) used by all 3 operators.

- Lot-to-lot reproducibility (within each user and between all lots calculated as CV of RNA yields per 12 replicates): CVs were ≤30% for 100% of all CVs.
- User-to-user reproducibility (within each lot and between all users per 12 replicates): CVs were ≤30% for 100% of all CVs.

8. Results — repeatability and reproducibility

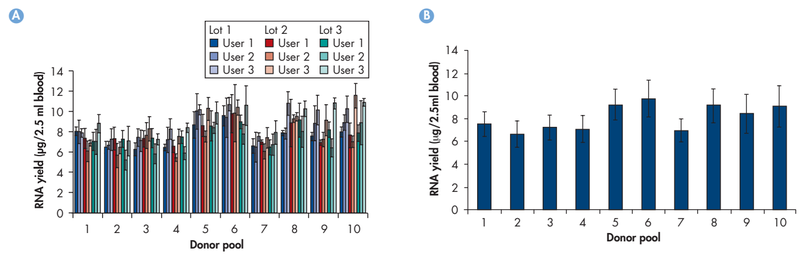

Figure 7 Individual and overall results from Figure 6. (A) Repeatability within each lot and within each user. (B) Reproducibility between all users and all lots.

- Repeatability (within each lot and within each user calculated as CV of RNA yield per quadruplicate sample preps): CVs were ≤25% for 98% (88/90) of all CVs.
- System reproducibility (between all users and all lots per 36 replicates calculated as CV of RNA yield per quadruplicate sample preps): CVs were ≤30% for 100% of all CVs.
- Yield and purity: All samples (n = 360) had RNA yields ≥3 µg (8.0 ± 1.7 µg per 2.5 ml blood sample [mean ± SD, range 4.5–13.7]) and A_{260}/A_{280} values of 2.0 ± 0.1 (mean ± SD, range 1.8–2.2), indicating the high reliability of the complete system.

9. Conclusions

This study demonstrates the high performance of the PAXgene Blood RNA System for the collection, storage, and transport of blood and stabilization of intracellular RNA in a closed tube and subsequent isolation and purification of intracellular RNA from whole blood for RT-PCR used in molecular diagnostic testing. Among the parameters tested were the following.

- In situ RNA stability: 3 days at 18–25°C, 5 days at 2–8°C, 6 months at –20°C, and 12 months at –70°C
- RNA purity: A_{260}/A_{280} values of 1.8–2.2 for ≥95% of samples
- RNA yield: ≥3 µg for ≥95% of samples (per 2.5 ml blood)
- Genomic DNA contamination: ≤1.0% for ≥95% of samples
- No significant RT-PCR inhibition when the eluate contributes up to 30% of reaction volume
- Repeatability of RNA yield: ≥95% of all CVs of donor-dependent yield are ≤25%
- Reproducibility of RNA yield: ≥95% of all CVs of donor-dependent yield are ≤30%

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