RNA Stabilization in Whole Blood: High Quality RNA with Room Temperature Handling of Human Blood Samples.

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Abstract

RNA transcripts purified from whole blood samples are increasingly being used as sensitive and accurate biomarkers for the diagnostic and prognostic measure of disease progression and patient nutrition. The sensitivity of gene expression analysis offers the possibility of early disease detection and dependable assessment of patient response to treatment. However, the utility of blood tissue for RNA-based molecular diagnostics depends on a reliable and standardized system for blood collection, preservation and processing. *Ex vivo* transcription profiles are known to change rapidly in blood cells, often with changes in relative transcript levels continuing during sample transport and storage. Therefore, consistent and dependable technology and methodology is critical if RNA biomarkers from blood are to reach their diagnostic potential. In this report we describe the development and testing of the RNAgard® Blood System, a fully-integrated system for the collection, ambient or room temperature management, and purification of RNA from human blood. This system consists of a stabilization reagent incorporated into a blood collection tube, providing immediate preservation of RNA levels upon blood draw. RNA is purified by a rapid, column-based method. Our data demonstrates the reproducibility of the RNAgard Blood System in providing high yields of pure total RNA from human blood stored for up to 14 days at ambient temperatures post blood draw. The quality and levels of RNA in blood extracted with the RNAgard Blood System are equivalent to freshly collected blood as demonstrated by a variety of analytical techniques including RT-qPCR, gene expression arrays and Bioanalyzer.

Materials and Methods

Sample preparation and nucleic acid analysis:

Human whole blood was collected in RNAgard Blood Tubes or competitor's RNA preservation tubes and stored for the times and temperatures stated in each figure. Samples were processed for RNA isolation using the Biomaxi™ RNA isolation kit or competitor's RNA isolation kit. Integrity of RNA was assessed by agarose gel electrophoresis, using the Agilent 2100 Bioanalyzer and the RNA 6000 Nano Kit or by RT-qPCR and Illumina Human HT-12 gene expression array.

Gene expression analysis:

Total RNA from samples collected and stored as indicated above were isolated and the RNA was quantified by absorbance spectroscopy. The RNA was then reverse transcribed and specific cDNAs were amplified using the iScript Reverse Transcription Supermix (Biorad), with the stated primer sets. Fold-change in IL1-β and c-Fos gene expression was calculated relative to expression at time "0" using the ΔΔCt method of relative quantification. For the Illumina Human HT-12 gene expression array, RNA integrity and quality was assessed by agarose gel electrophoresis and Agilent 2100 Bioanalyzer. Duplicate RNA samples per set were processed for Illumina gene expression array and analyzed according to the manufacturer's instructions.

RNA sequencing analysis:

Blood from a single human donor was collected in RNAgard Blood Tubes and stored at room temperature. Total blood RNA was purified on Day "0" and Day 14. PolyA cDNA preparation and.sequencing were conducted by Otogenetics (Norcross, GA). Sequencing was performed using the HiSeq 2000 platform (Illumina) and PE100 paired ends. Results were aligned to human genome assembly hg19.

Results

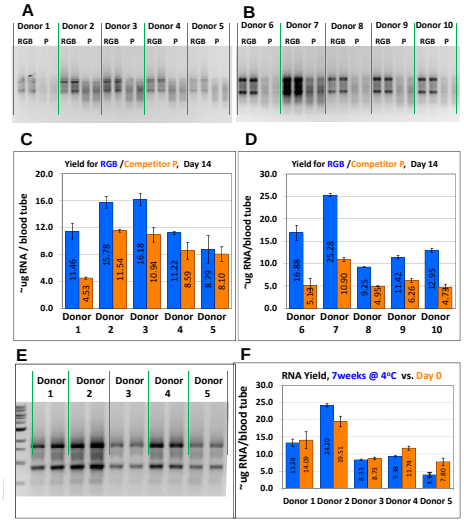


Figure 1. RNAgard Blood Tubes stabilize blood samples at room temperature for at least 14 days and at 4ºC for more than one month. (A-F): Blood from 10 healthy human donors (two separate 5-donor studies) was collected in RNAgard Blood (RGB) Tubes or in a competitor's blood tubes (Competitor P), and was stored at room temperature for 14 days. RNA was then purified from RGB samples using Biomatrica's BioMaxi RNA Kit and from the competitor's tubes using their recommended purification kit. 5% of the purified RNA from each sample was analyzed by agarose gel electrophoresis **(A,B)**. Total RNA yield per blood tube was determined by UV spectrophotometry, **(C,D)**. Blood from 5 healthy human donors was collected in RGB Tubes and stored at 4°C for 7 weeks. RNA was then purified, and RNA quality **(E)** and yield **(F)** were analyzed as described above for **(A-D)**.

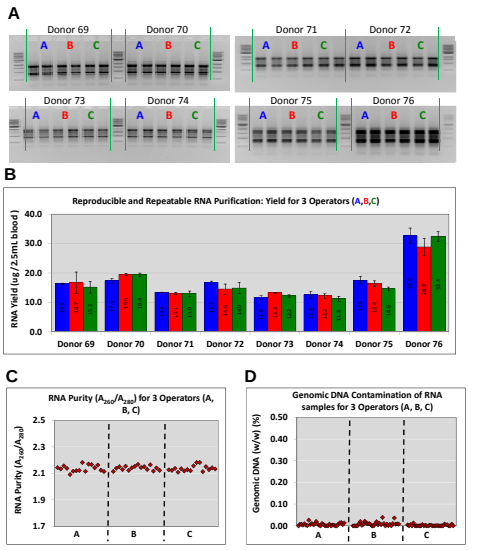


Figure 2. RNA with high quality and purity can be isolated from blood samples collected in RNAgard Blood (RGB) Tubes using the BioMaxi RNA extraction kit. Blood from 8 healthy human donors was collected in RGB Tubes and stored at room temperature for 3 days. RNA was isolated by 3 different operators (A,B,C), using the BioMaxi RNA kit. 5% of the purified RNA was analyzed by agarose gel electrophoresis **(A)**. Total RNA yield per blood tube and RNA purity (A_{260}/A_{280}) was determined by UV spectrophotometry **(B,C)**. Percentage of genomic DNA contamination in RNA samples was determined against a standard curve by qPCR amplification of an RNAse P amplicon **(D)**.

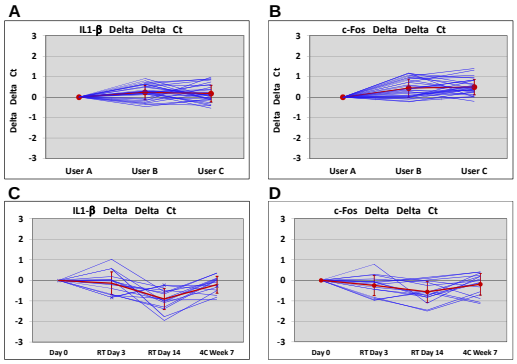
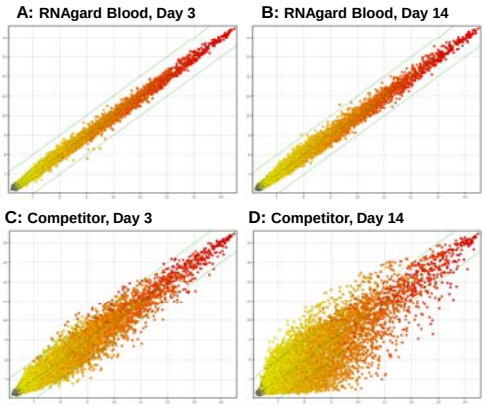


Figure 3. Real-time RT-PCR analysis in blood stored in RNAgard Blood Tubes. Human blood from multiple donors was collected in RNAgard Blood Tubes. RNA was isolated using the BioMaxi RNA Purification Kit, and relative transcript levels of c-Fos and IL1-β were determined by real-time RT-PCR using 18S rRNA as an internal control. **A,B:** Blood was collected from 8 healthy donors. After 3 days of room temperature sample storage, RNA was isolated from duplicate samples by 3 different users (User A, User B, User C) and used for real-time RT-PCR. The values for IL1- β **(A)** and c-Fos **(B)** transcripts for all samples are plotted, relative to the values for user A (8 donors, 2 samples, 2 real-time RT-PCR replicates = 32 data sets for each gene, per user), with mean (red lines) and standard deviation (red bars) for all samples shown. **(C,D):** Blood was collected from 5 healthy donors. After 0, 3 and 14 days of room temperature storage and 7 weeks of 4ºC sample storage, RNA was purified from duplicate samples and used for real-time RT-PCR. The relative transcript levels of IL1- β **(C)** and c-Fos **(D)** for all samples are plotted relative to the values for Day 0 (4 donors, 2 samples, 3 time points, 2 real-time RT-PCR replicates = 48 data sets for each gene) .



E: Number of genes > 2-fold expression variation

Blood Source	Blood Sample Storage time	RNAgard Blood Tube	Competitor
Donor 1	3 Days	10	62
	14 Days	11	1679
Donor 2	3 Days	2	131
	14 days	2	2004

Figure 4. Microarray analysis in blood stored in RNAgard Blood Tubes. Human blood from 2 healthy donors was collected in RNAgard Blood Tubes or a competitor's RNA preservation tubes, and RNA was isolated on collection day and after 3 or 14 days of room temperature storage, as stated in Materials and Methods. **A-D:** Representative analysis for Donor 2 of gene expression profiles of ~34,000 genes using the Illumina Human HT12 Bead Array for RNA extracted from blood collected in RNAgard Blood Tubes or a competitor's RNA preservation tubes after 3 days **(A,C)** or 14 days **(B,D)** of room temperature storage, relative to RNA extracted from freshly collected blood, using RNA isolation methods recommended for each product. **E:** Table summarizing the microarray results. Displayed are the number of genes with >2-fold change in expression levels relative to samples processed in the day of blood collection.

	Day 0	Day 14
Total Reads	31,866,716 (100.00%)	35,266,634 (100.00%)
Total # Bases	3,186,671,600 (100.00%)	3,526,663,400 (100.00%)
Mapped Reads	13,313,067 (41.79%)	13,905,158 (39.43%)
Reads Mapped Repetitively	4,655,032 (14.61%)	4,032,847 (11.44%)
% GC Content	53.60 ± 11.62%	52.73 ± 11.48%
% Reads Containing rRNA	1.09%	0.84%
% Poly-A Reads	0.01%	0.01%

Figure 5. RNA-seq analysis in blood stored in RNAgard Blood Tubes. Human blood from a single healthy donor was collected in RNAgard Blood Tubes. The RNA was isolated on collection day (Day 0) and after 14 days of room temperature storage using the BioMaxi Blood RNA Purification Kit. RNA sequencing was performed using the HiSeq 2000 sequencing platform with PE100 paired end reads. Sequencing results from both samples were essentially identical based on every parameter measured, indicating the stability of transcription profiles in blood stored in the RNAgard Blood Tubes.

Summary

- **RNAgard Blood Tubes protect RNA in blood samples stored at room temperature for up to 14 days and at 4ºC for at least 1 month, outperforming a leading competitor (Fig. 1).**
- **RNA with high yield, quality and purity can be reproducibly isolated from blood samples in RNAgard Tubes using the Biomaxi RNA isolation kit (Fig. 2).**
- **Gene expression remains unaltered in blood samples stored in RNAgard Blood Tubes for at least 14 days at room temperature and 7 weeks at 4ºC (Fig. 3,4).**
- **The total gene expression profile of blood stored for 3 or 14 days at room temperature in Biomatrica's RNAgard Blood Tubes remains unaltered, comparable to that of freshly collected blood samples, clearly outperforming a leading competitor (Fig. 4).**
- **RNA-seq analysis reveals that transcript sequences remain unaltered in whole blood specimens stored for 14 days at room temperature in RNAgard Blood (Fig. 5)**