RNAgard® Blood RNA Purification Kit

User Manual

REF **CE-12000-001**

CE IVD

For in vitro diagnostic use.

Biomātrica®

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Contents

RNAgard Blood System Products and Ordering Information

Description	Quantity	Catalog Number
RNAgard® Blood Tubes	50 tubes	CE-12006-050
RNAgard® Blood RNA Purification Kit	50 preps	CE-12000-001
RNAgard® Blood Reagent	100 mL	CE-12100-001
RNAgard® Blood Precipitation Buffer	50 preps	CE-12180-001

Orders may be placed online at www.biomatrica.com, via email at orders@biomatrica.com or via phone to Biomatrica Customer Support at 00 1 858 550 0308.

RNAgard Blood RNA Purification Kit Contents

RNAgard Blood RNA Purification Kit (50 preps) Catalog No. CE-12000-001	
Component	**Amount**
RNAgard Blood Precipitation Buffer	180 mL
Resuspension Buffer‡	21 mL
Wash Buffer 1*	25 mL
Wash Buffer 2*	15 mL
RNase-Free Water Bottle	12 mL
DNase I, RNase-Free; Lyophilized Powder	1 X vial
DNase I Buffer	2 X 2.5 mL
Sterile Water (DNase I Resuspension Water)	1 mL
Purification Columns	50
2ml Collection Tubes	2 X 100

‡ Not compatible with disinfecting reagents containing bleach. Contains guanidine isothiocyanate. See page 5 for safety information.
* Wash Buffer 1 and Wash Buffer 2 are supplied as concentrates. Before using for the first time, dilute with ethanol (96-100%, Analytical Reagent grade or purity grade p.a.) according to the volumes indicated on the bottles.

Storage Conditions

- Upon receipt of the Kit (shipped at ambient temperature), remove the vial of lyophilized DNase I, RNase-Free Lyophilized Power and store at 2-8°C.
- After reconstituting the lyophilized DNAse I, aliquot and store the resulting solution at -20°C.
- Store all other RNAgard Blood RNA Purification Kit components at ambient temperature (18-25°C). Do not freeze.

Safety Information

Practice Universal Precautions when handling this product.
Contents of this kit may cause irritation to eyes, respiratory system and skin.

1. If accidental inhalation occurs, supply fresh air and seek medical advice.
2. In case of skin contact, immediately wash with water and soap and rinse thoroughly.
3. In case of eye contact, rinse immediately with plenty of water for at least 15 minutes and seek medical advice.
4. If accidental swallowing occurs, immediately seek medical advice.
5. Refer to SDS in case of accidental ingestion or skin contact. All SDS information is available at http://biomatrica.com/support_sds.php.

Intended Use

The RNAgard Blood RNA Purification Kit is intended for the purification of intracellular RNA from human blood samples collected and preserved in RNAgard Blood Tubes. When used in combination with RNAgard Blood Tubes as part of the RNAgard Blood System, the RNAgard Blood RNA Purification Kit provides high yields of pure RNA, with very low levels of genomic DNA. The purified RNA is suitable for RT-PCR applications used in molecular diagnostic testing. **The performance of the RNAgard Blood System has only been characterized for C-FOS and IL1-β gene transcripts. The user is responsible for characterizing performance of the RNAgard System for other applications.**

Introduction

Clinical Research studies often require blood sample collection at multiple geographic sites under a wide range of conditions. RNAgard Blood Tubes are designed for the immediate preservation of intracellular RNA in human blood samples, providing an efficient method for standardized collection, transport and storage of whole blood specimens. RNA integrity and gene transcript levels are maintained for up to 14 days at room temperature when whole blood is stored in RNAgard tubes, and can easily be purified with the RNAgard Blood RNA purification kit.

Sample Collection and Preservation

RNAgard Blood Tubes contain a proprietary chemical preservation reagent that lyses blood cells, inactivates nucleases and stops RNA synthesis, therefore preserving the levels of RNA transcripts. Once RNA is purified using the RNAgard Blood RNA Purification Kit, it is suitable gene expression analysis using technologies such as RT-PCR and expression arrays.

RNA Purification Procedure

Materials and Equipment Required but Not Provided

- RNAgard Blood Tubes (Cat. No. CE-12006-050)
- Ethanol (96-100%)
- Pipets * (5 μL - 3 mL)
- Sterile, aerosol-barrier, RNase-free pipet tips
- 50 mL Conical Tubes
- 1.5 mL Microfuge Tubes
- Vortex Mixer*
- Centrifuge* - variable-speed capable of attaining 1,000-14,000 x g
- Shaker* - incubator ≥ 400 rpm
- Water bath or heat block at 70°C

* Ensure that instruments have been checked, maintained, and calibrated regularly according to the manufacturer's recommendations.

Important Notes Before Starting:

- Ensure that blood was collected in RNAgard Blood Tubes according to the instructions in the "Procedure for Specimen Preparation for Analysis" section of the RNAgard Blood Tube *Instructions For Use*.
- Allow at least 2 hours after blood collection to start the purification process. To maximize RNA yield, allow at least 8-12 hours of storage at room temperature prior to RNA purification.
- Wash Buffer 1 and Wash Buffer 2 are provided as concentrates; reconstitute with the amounts of 96-100% ethanol indicated on the bottle labels prior to use.
- DNase I is sensitive to physical denaturation. When reconstituting the lyophilized powder, mix gently by inversion; do not vortex the DNase I solution.
- Ensure that the RNAgard Blood Tubes are equilibrated to room temperature before starting the RNA purification process.

Protocol for RNA purification

1. Invert the RNAgard Blood Tube at least 5 times to ensure proper mixing, then remove the cap and pour the contents of the tube into a clean 50 mL conical tube.
2. Pipet 3 mL of RNAgard Blood Precipitation Buffer into the 50 mL conical tube to bring the total volume to ~12 mL and close the cap on the tube.
3. Incubate 15 minutes at room temperature with shaking (500-750 rpm).

4. Vortex the 50 mL conical tube vigorously (maximum speed) for at least 30 seconds, ensuring that the solution travels to the top of the tube to achieve proper mixing of the contents.
5. Centrifuge the tube at 3,600 – 4,000 x g for 30 minutes at room temperature in a swinging bucket rotor.

   Note**:** *Centrifugation can also be* performed *at 10,000 - 12,000 x g for 10 minutes using a fixed-angle rotor* without *impact on RNA yield or quality.*
6. Carefully pour off the supernatant fraction and leave the tube inverted on absorbent paper for 1-2 minutes.

   Note: *A translucent reddish pellet should be visible at the bottom of the tube.*
7. Blot the remaining drops of liquid off the tube rim with clean absorbent paper.
8. Add 350 µL of Resuspension Buffer into the tube and pulse-vortex 5-10 times to re-suspend the pellet.
9. Add 250 µL of 100% ethanol into the tube and pulse-vortex 3-5 times to mix the contents of the tube.
10. Transfer solution to a clean purification column, close the lid and centrifuge for 1 minute at 6,700 – 7,000 x g.

    Note*: For any centrifugation (steps 10 to 23) use bench-top microfuge.*
11. Discard the collection tube and place column in a clean 2 mL collection tube.
12. Add 400 µL of Wash Buffer 1 into the column, close the lid and centrifuge at 6,700 – 7,000 x g for 1 minute.
13. Discard the flow-through, place column into the same 2 mL collection tube and centrifuge at 18,000 - 20,000 x g (or maximum speed) for 1 minute to dry the column.
14. Discard the collection tube and place column in a clean 2 mL collection tube.
15. Perform DNase treatment:
    a. If using DNase I for the first time, prepare DNase I stock solution by adding 300 µL of sterile, RNase-free Water (provided) to the DNase I (RNase-Free) lyophilized powder and mix gently by inversion; **do not vortex**. Aliquot the DNase I enzyme and store at -20°C for long term storage. **Note: The DNase I stock can be freeze/thawed up to three times without loss of activity.**
    b. Prepare diluted DNase I solution by combining 5 µL of DNase I and 95 µL of DNase I Buffer for each sample, then mix gently by inversion. (Add 10% when preparing solutions for multiple samples).
    c. Add 100 µL of the DNase I Mix into each column and incubate at room temperature for 15-20 minutes.
    d. During the DNase I treatment, preheat the RNase-free water to 70°C for elution of RNA.
16. Add 400 µL of Wash Buffer 1 to the column, close the lid, incubate at room temperature for 30 seconds and centrifuge at 6,700 – 7,000 x g for 1 minute.

17. Discard the collection tube and place column in a clean collection tube.
18. Add 350 µL of Wash Buffer 2 to the column, close the lid and centrifuge at 6,700 – 7,000 x g for 1 minute.
19. Repeat step 18 with a second 350 µL of Wash Buffer 2**,** without changing collection tube.
20. Discard the collection tube, place column in a clean 2 mL collection tube and centrifuge at 18,000 - 20,000 x g (or maximum speed) for 2 minutes to dry the column.
21. Remove the purification column from the tube and place in an RNase-free 1.5 mL microfuge tube (not provided in the kit) for eluting the RNA.
22. Add 100 µL of RNase-free water pre-heated to 70°C onto the column, close the lid, and incubate for 1 minute at room temperature and centrifuge for 1 minute at 6,700 – 7,000 x g to elute RNA.
23. For maximum RNA concentration, re-apply the eluted solution back to the column and centrifuge 1 minute at 6,800 x g. For maximum RNA yield, repeat the step 22 with new 100 µL of RNase-free water.

Product Use Limitations

1. The RNAgard Blood RNA Purification Kit has not been characterized for purification of RNA from blood samples not collected and stored in RNAgard Blood Tubes.
2. The RNAgard Blood RNA Purification Kit has been optimized for purification from 2.5 mL of blood collected into RNAgard Blood Tubes. If the full 2.5 mL of blood has not been drawn into RNAgard Blood Tubes, RNA yield and quality might be affected.
3. The RNAgard Blood RNA Purification Kit has not been optimized for purification of small RNA species such as miRNA.

RNA Purification Procedure Summary

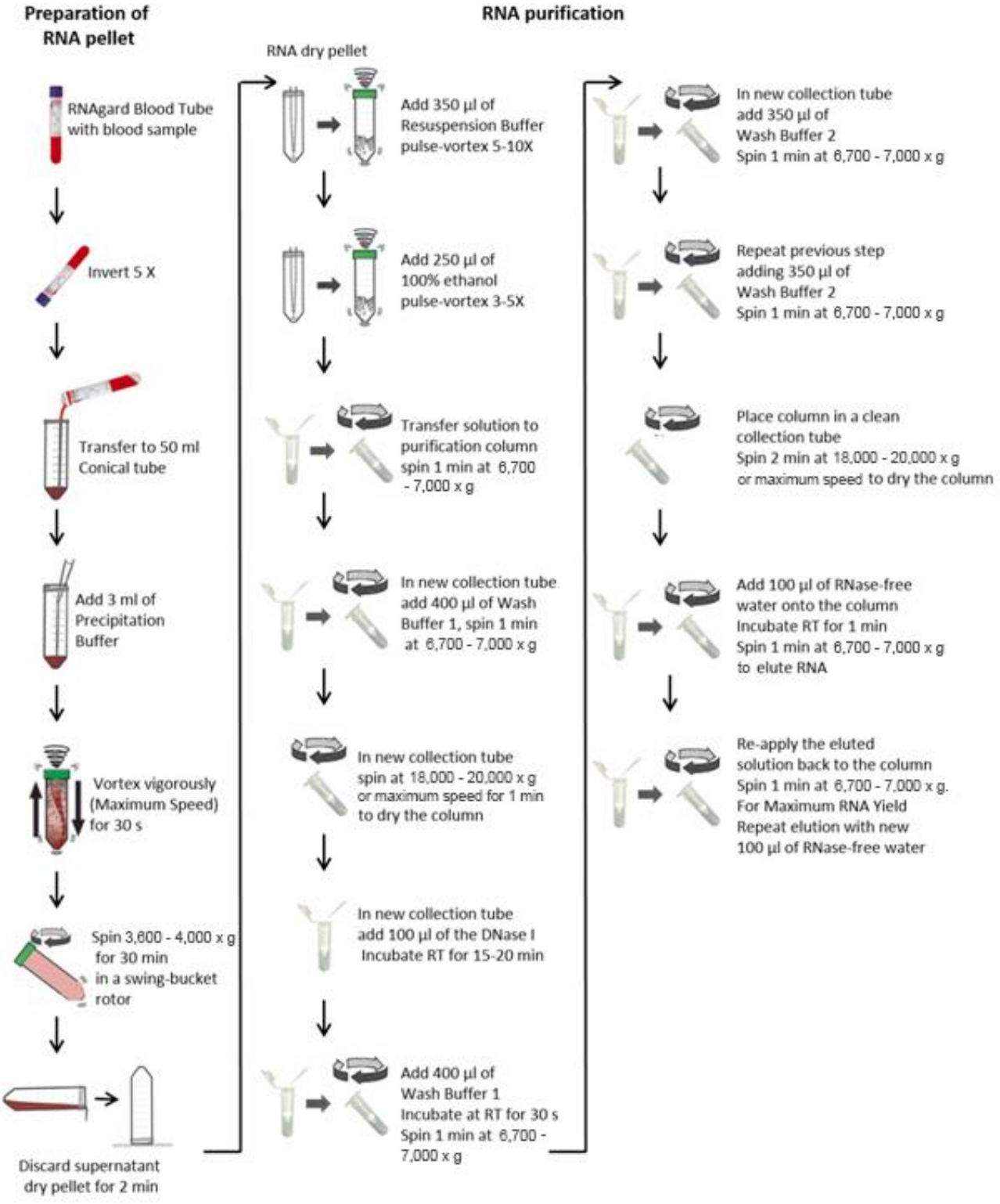

Figure 3: RNA purification procedure. Schematic view of RNA purification from human blood samples collected in RNAgard Blood Tubes using the RNAgard Blood RNA Purification Kit.

Appendix A: Stability of Gene Expression

Preservation of gene expression patterns in whole blood for up to 14 days at room temperature was evaluated by whole genome array expression analysis for multiple blood donors. Figure 2 shows a representative comparison of expression profiles for more than 47,000 transcripts between fresh whole blood (X-axis) and after 3 days (A) or 14 days (B) of ambient temperature storage (Y-axis). The green lines define the transcripts from stored whole blood with expression levels within a 2-fold change relative to freshly collected blood samples.

Preservation of RNA levels by the RNAgard Blood System was further characterized by RT-PCR analysis of the transcripts for the inflammatory cytokines IL-1β and C-Fos. The RNA levels for these two genes, was measured by the Ct values relative to the 18S RNA reference gene (ΔCt). Changes in the ΔCt values relative to Day 0 values were expressed as ΔΔCt. ΔCt values were maintained over 14 days of blood sample storage at room temperature; ΔΔCt values were between -1.0 and +1.0 (Figure 3). Similar results were obtained for blood samples stored for up to a month at 2-8°C, or frozen at -20°C or -80°C (data not shown).

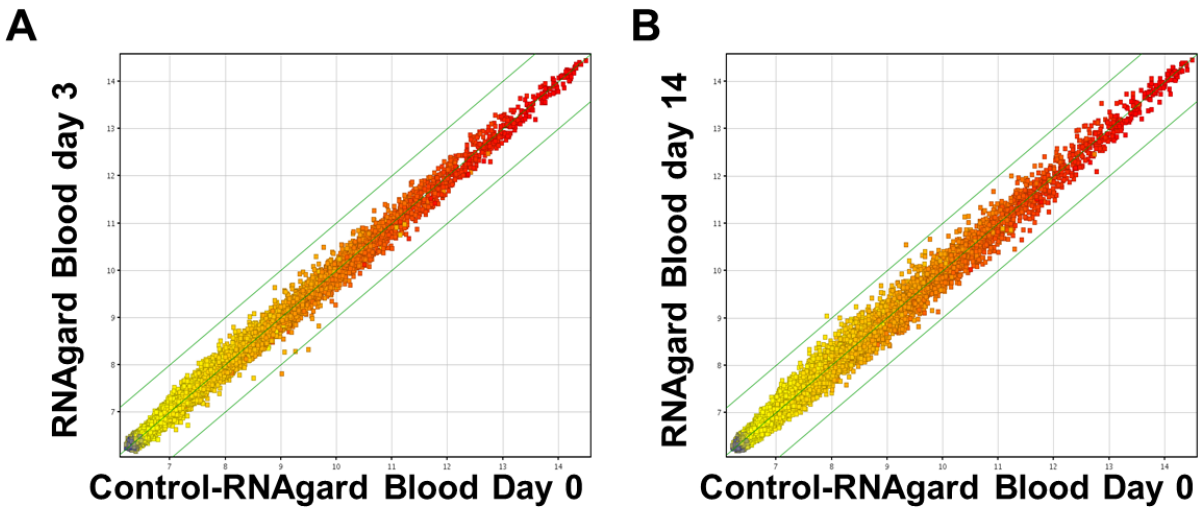

Figure 1: Microarray analysis of whole transcriptome expression in RNA from human whole blood samples. Human blood was drawn in RNAgard Blood tubes from a healthy donor. RNA was purified after 0, 3 or 14 days of room temperature storage. Whole transcriptome expression was analyzed using the Human HT12 Bead Array (Illumina Corp., San Diego, CA), and normalized transcript levels from samples stored for 3 (A) or 14 (B) days (Y-axis) were compared to transcript levels from the fresh sample whole blood from the same donor (X-axis).

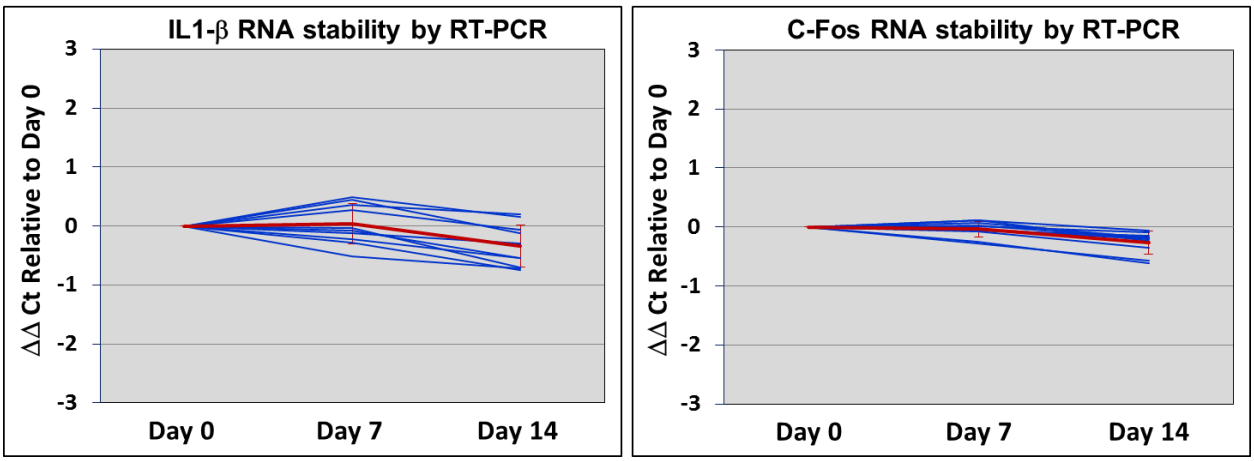

Figure 2: Analysis by RT-PCR of IL1-β and C-Fos RNA obtained from human blood samples collected and stored in RNAgard Blood Tubes. Blood was drawn from 5 healthy donors in RNAgard Blood Tubes and stored at room temperature (18-25 °C). At days 0, 7 and 14, RNA was purified with RNAgard Blood RNA Purification Kit. C-Fos and IL1-β transcript levels, normalized to 18S transcripts, were determined by RT-PCR and expressed as **Δ**Ct values. The change in **Δ**Ct relative to levels of the same transcript in day 0 samples from the same donor was expressed as **ΔΔ**Ct.

Appendix B: High RNA yield and purity

The RNAgard Blood RNA Purification Kit allows for isolation of at least 3 µg of RNA for at least 97% of blood samples collected and stored in RNAgard Blood Tubes. As shown in Figure 4, repeatable high RNA yields (Figure 4, A) with high purity (Figure 4, B) were obtained from blood samples from the same 8 donors over 2 weeks of room temperature blood sample storage.

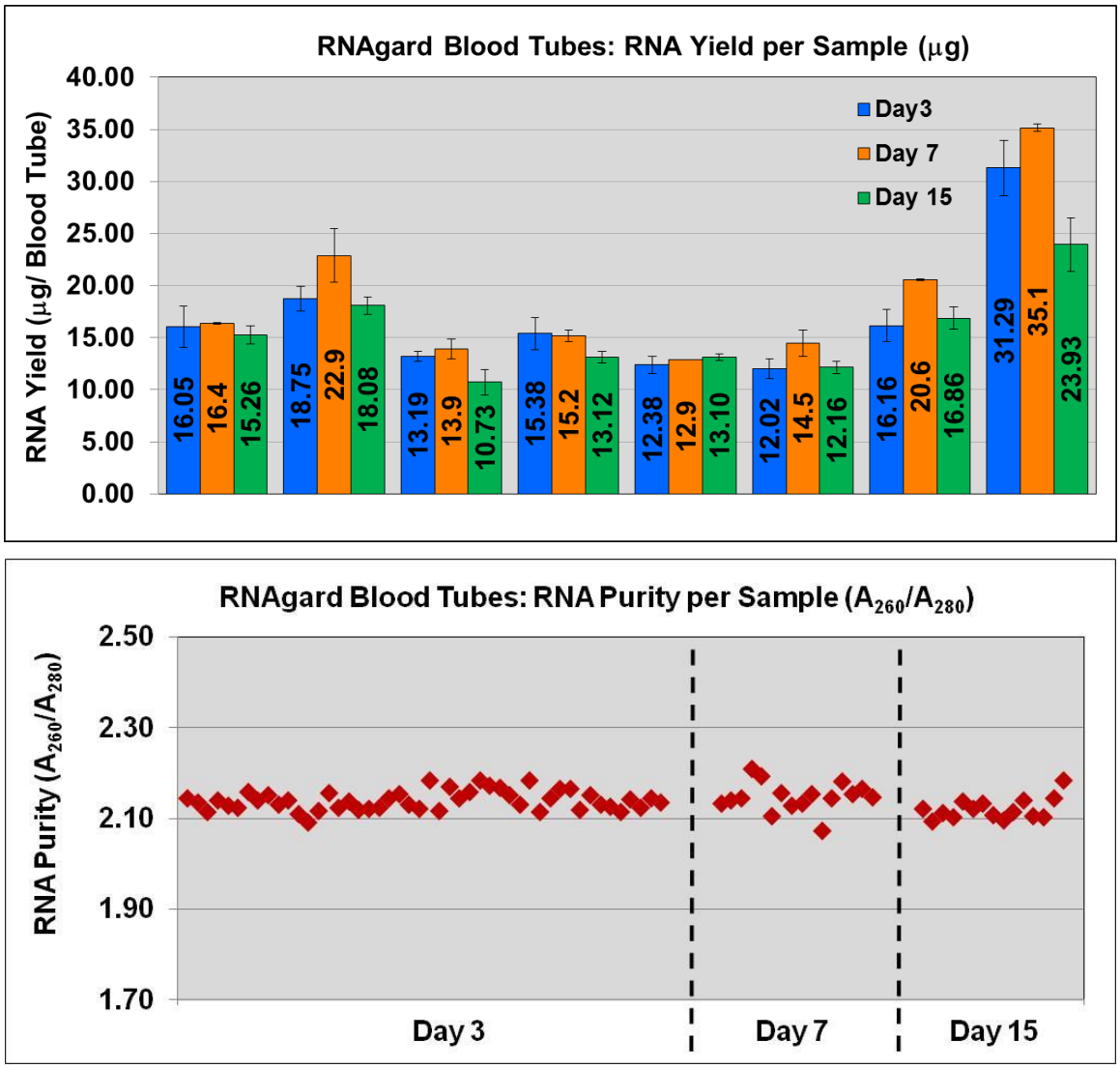

Figure 4: High RNA yield and purity. Blood was drawn from 8 healthy donors in RNAgard Blood Tubes and stored at room temperature (18-25°C). After 3, 7 and 15 days, RNA was purified from duplicate blood samples per donor, using the RNAgard Blood RNA Purification Kit. A: RNA yield per sample was determined by UV spectrophotometry and shown as average values from the duplicate samples per donor. B: RNA purity for all samples was determined by UV spectrophotometry (A260/A280).

Appendix C: Repeatable and reproducible purification of RNA

RNA purification from blood samples collected in RNAgard Blood Tubes using the RNAgard Blood RNA Purification Kit is highly reproducible, as shown by the similar RNA yields obtained from the same blood donors by 3 different operators (Figure 5). Similarly to the RNA yield, the high purity and quality of the isolated RNA is very reproducible, as shown by the A260/A280 values consistently between 1.8 and 2.2 (Figure 6), as well as the genomic DNA content below 0.10 % (w/w) (Figure 7), independently of which one of the 3 different operators purified the RNA from the blood samples.

The reproducibility of the RNAgard Blood RNA Purification Kit was also verified by RT-PCR analysis of the RNA transcript levels for C-Fos and IL1-β. As shown in Figure 8, the RNA levels of both C-Fos and IL1-β transcripts relative the control 18S RNA transcript are similar, independently of the operator that processed the samples.

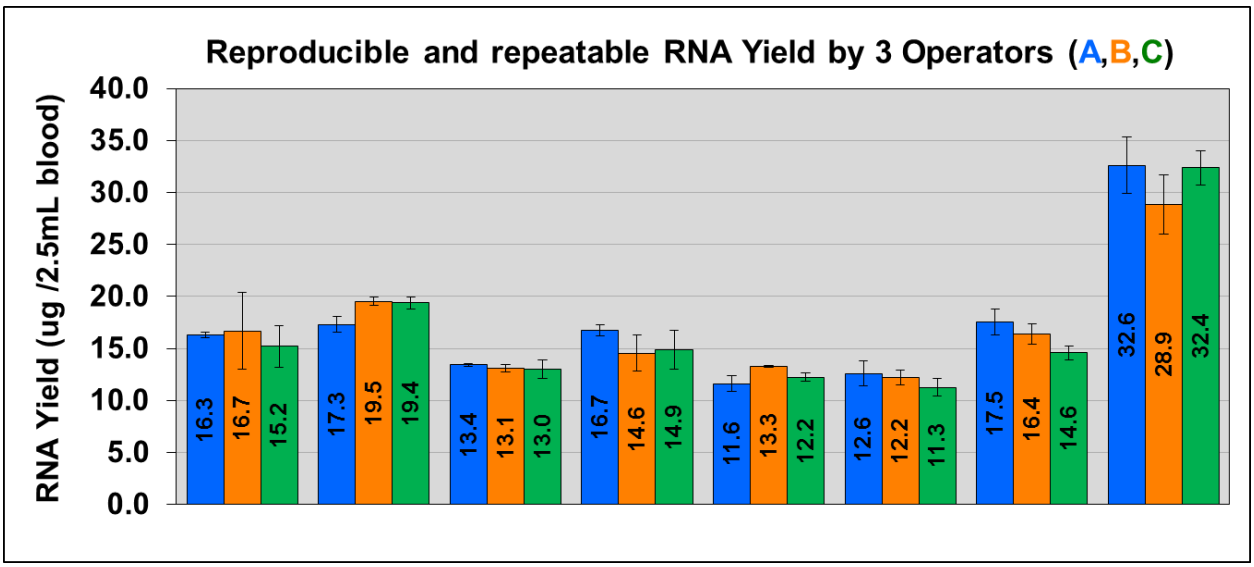

Figure 5: Reproducible RNA yield. Blood was drawn from 8 healthy donors in RNAgard Blood Tubes and stored at ambient temperature (18-25°C) for 3 days. RNA was purified from duplicate blood samples per donor, by 3 different operators, using the RNAgard Blood RNA Purification Kit. RNA yield per sample was determined by UV spectrophotometry and shown as average values from the duplicate samples per donor.

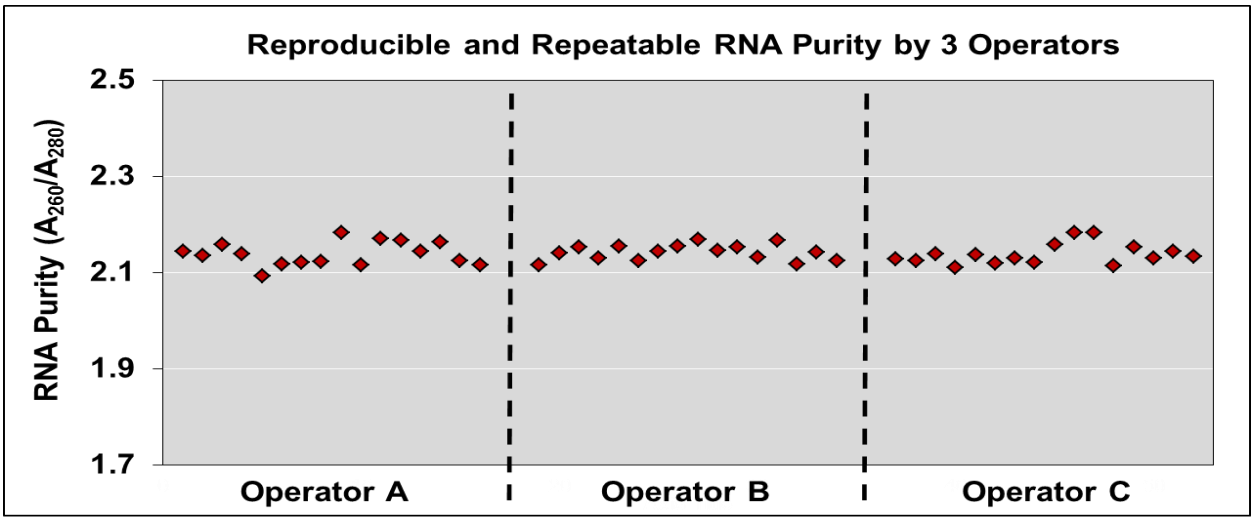

Figure 6: Reproducible purity of RNA. Blood was drawn from 8 healthy donors in RNAgard Blood Tubes and stored at ambient temperature (18-25°C). After 3 days of storage, samples were processed by 3 operators using the RNAgard Blood RNA Purification Kit. RNA purity for all samples was determined by UV spectrophotometry (A260/A280).

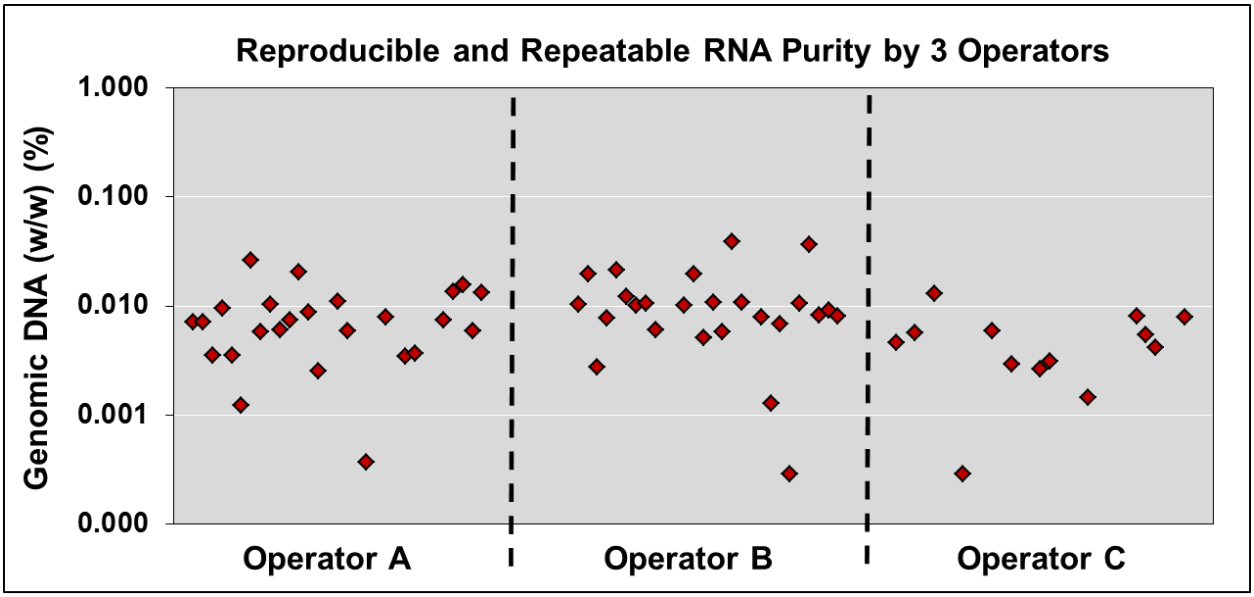

Figure 7: Reproducible purity of RNA. Blood was drawn from 8 healthy donors in RNAgard Blood Tubes and stored at ambient temperature (18-25°C). After 3 days of storage, samples were processed by 3 operators using the RNAgard Blood RNA Purification Kit. Percentage of genomic DNA contamination was determined for all RNA samples by qPCR amplification of an RNAse P genomic DNA amplicon, relative to amplification of the same target from known inputs of genomic DNA samples.

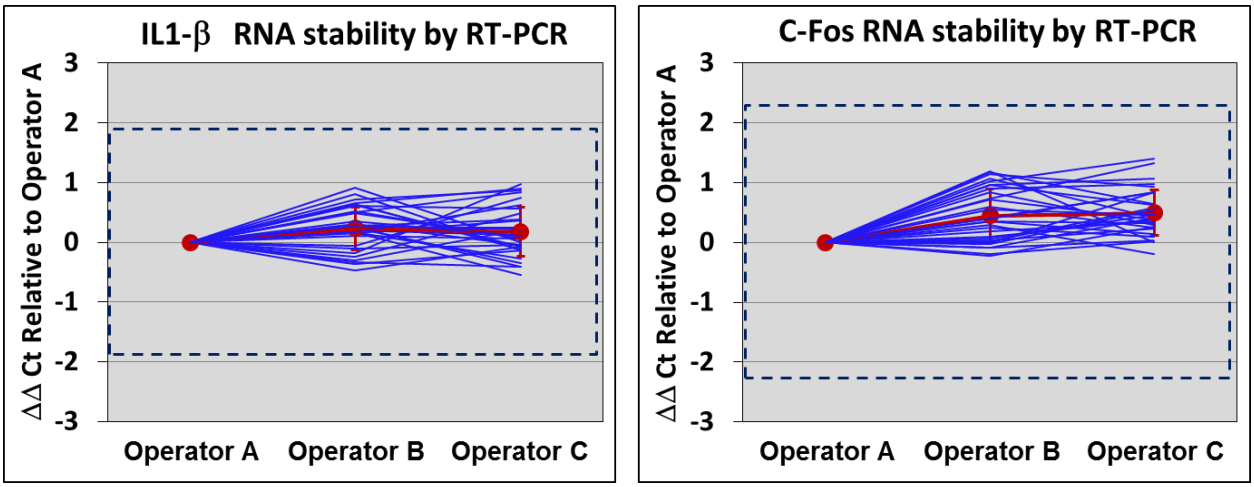

Figure 8: Characterization of C-Fos and IL1-β transcript levels by RT-PCR assay. Blood was drawn from 8 healthy donors in RNAgard Blood Tubes and stored at ambient temperature (18-25^{o}C). After 3 days of storage, samples were processed by 3 operators using the RNAgard Blood RNA Purification Kit. C-Fos and IL1-β transcript levels, normalized to 18S transcripts were determined by RT-PCR in samples processed by operator B and operator C and shown relative to levels of the same transcripts, in samples processed from the same donor by operator A, expressed as **ΔΔ**Ct.

Troubleshooting Guide

This troubleshooting guide includes suggestions for solving the following potential situations. Biomatrica's Technical Support scientists are available to answer questions about the information and protocols in this user manual (see pg. 16 for contact information or visit www.biomatrica.com).

Situation	Comment	Reason/Suggestion
The blood looks coagulated or precipitated after storage or shipment.	RNAgard Blood formulation denatures proteins, which will precipitate over time. Precipitation does not affect the RNA stabilization properties of the formulation.	Ensure that sample is mixed before RNA purification procedure, by inverting tube 3-5 times.
Low RNA yield	Possible reasons: - Low concentration of leukocytes in the blood sample. - RNAgard Blood Tube sample not mixed thoroughly prior to RNA purification. - Choice of blood collection tube.	 - Leukocyte concentrations can vary 10-fold between donors resulting in wide range of RNA yield. - Invert the RNAgard Blood Tube 3-5 times immediately prior to RNA isolation. - RNA isolation with RNAgard Blood RNA Purification Kit has been optimized with the RNAgard Blood Tube. We do not guarantee the quality of RNA stabilized in other blood collection tubes.
Isolated RNA is impure	Possible reasons: - Choice of blood collection tube. - Inactivation of DNAse I leading to genomic DNA contamination. - Loss of activity of DNAse I leading to genomic DNA contamination.	 - RNA isolation from RNAgard Blood Tubes has been optimized with the RNAgard Blood RNA Purification Kit. Purification of RNA collected in RNAgard Blood tubes with other products is not recommended. - After reconstitution of DNAse I mix gently do not vortex. - After reconstitution of DNAse I limit to three freeze/thaw cycles.

Technical Assistance

Biomatrica, Inc. is committed to providing outstanding technical support. Biomatrica's Technical Service Department is staffed by experienced scientists with hands-on experience in molecular biology and the use of Biomatrica's products. Please contact Biomatrica directly with any questions regarding the RNAgard Blood RNA Purification Kit.

Technical Service Department
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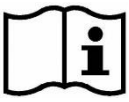
Glossary of Harmonized Symbols

	Item number		Do Not Reuse
	LOT number: Batch number		Hazard
	Expiry Date. Use by the end of the month indicated		Manufacturer
	Temperature limitation		Sterilization using irradiation
	Keep away from direct sunlight		Sterilization using aseptic technique
	Consult instructions for use		Authorized representative in the European Community

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