PAXgene®

Bone Marrow RNA Kit Handbook

For isolation and purification of intracellular RNA from whole bone marrow samples stabilized in PAXgene Bone Marrow RNA Tubes

Important: To be used only in conjunction with PAXgene Bone Marrow RNA Tubes.

For Research Use Only. Not for use in diagnostics procedures.

March 2016

PreAnalytiX
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The present product comes with a license under certain claims of US-7,270,953, and US-7,682,790, as well as EP-1820793 B1 and foreign equivalents of these patent claims to use the product to process the nucleic acid complex formed in the course of sample collection in a PAXgene Bone Marrow RNA Tube.

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Please see page 31 for contact information for your local PreAnalytiX distributor.

Contents

Kit Contents

PAXgene Bone Marrow RNA Kit	**(30)**
Catalog no.	**764133**
Number of preps	**30**
Buffer BMR1 (Resuspension Buffer)	20 ml
Buffer BMR2 (Binding Buffer)*	18 ml
Buffer BMR3 (Wash Buffer 1)*	25 ml
Buffer BMR4 (Wash Buffer 2 concentrate)†	7 ml
Buffer BMR5 (Elution Buffer)	5 ml
Buffer BMR Wash (bottle)	2 x 125 ml
Proteinase K (green lid)	1.4 ml
PAXgene RNA Spin Columns (red)	3 x 10
Processing Tubes (2 ml)	3 x 50 1 x 30
Secondary BD Hemogard™ Closures	30
Microcentrifuge Tubes (1.5 ml)	2 x 50 1 x 10
RNase-Free DNase Set: ■ DNase I, RNase-Free (lyophilized) ■ Buffer RDD (white lid) ■ RNase-Free Water (DNase resuspension buffer) (tube, red lid)	 1500 Kunitz units‡ 2 x 2 ml 2 ml
PAXgene Shredder Spin Columns (lilac)	3 x 10
Research Use Only Certification Card	1
Handbook	1

* Contains a guanidine salt. See page 6 for safety information.

† Buffer BMR4 is supplied as a concentrate. Before using for the first time, add ethanol (96–100%, purity grade p.a.) as indicated on the bottle to obtain a working solution.

‡ Kunitz units are commonly used units for measuring DNase I; see page 12 for definition.

Shipping and Storage

The PAXgene Bone Marrow RNA Kit is shipped at ambient temperature.

The RNase-Free DNase Set, in the PAXgene Bone Marrow RNA Kit, should be stored upon receipt at 2–8°C. All other components of the PAXgene Bone Marrow RNA Kit can be stored dry at room temperature (15–25°C).

Product Use Limitations

For Research Use Only. Not for use in diagnostics procedures. The performance characteristics of this product have not been fully established.

The PAXgene Bone Marrow RNA Kit is not for the isolation and purification of genomic DNA or viral nucleic acids from human whole bone marrow. Due to the limited number of transcripts validated for stabilization specifications, the performance characteristics have not been established for all transcripts. Laboratory personnel should review the manufacturer's data and their own data to determine whether validation is necessary for other transcripts.

Product Warranty and Satisfaction Guarantee

PreAnalytiX guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, PreAnalytiX will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a PreAnalytiX product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish.

A copy of PreAnalytiX terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call Technical Services or your local distributor (see page 31 or visit **www.preanalytix.com**).

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of PAXgene Bone Marrow RNA Kits is tested against predetermined specifications to ensure consistent product quality.

Technical Assistance

At PreAnalytiX and QIAGEN, we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of PreAnalytiX and QIAGEN products. If you have any questions or experience any difficulties regarding the PAXgene Bone Marrow RNA Kit or PreAnalytiX products in general, please do not hesitate to contact us.

PreAnalytiX customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at PreAnalytiX. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information, please contact Technical Services at **www.preanalytix.com** or call your local distributor (see page 31 or visit **www.preanalytix.com**).

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.preanalytix.com/resources** where you can find, view, and print the SDS for each PreAnalytiX kit and kit component.

CAUTION: DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

Buffer BMR2 and Buffer BMR3 contain guanidine thiocyanate, which can form highly reactive compounds when combined with bleach. If liquid containing these buffers is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

The RNA stabilizing solution and bone marrow mixture from the PAXgene Bone Marrow RNA Tube can be disinfected using 1 volume of commercial bleach solution (5% sodium hypochlorite) per 9 volumes of the RNA stabilizing solution and bone marrow mixture.

Waste from sample preparation, such as supernatants from centrifugation steps in the RNA purification procedure, is to be considered potentially infectious. Before disposal, the waste must be autoclaved or incinerated to destroy any infectious material. Disposal must be made according to official regulations.

Introduction

Aspiration and collection of whole bone marrow is the first step in many molecular assays used to study cellular RNA. However, a major problem in such experiments is the instability of the cellular RNA profile in vitro. Copy numbers of individual mRNA species in whole bone marrow can change significantly during storage or transport at room temperature. This is caused both by rapid RNA degradation and by induced expression of certain genes after the sample is aspirated from the donor. Such changes in the RNA expression profile make reliable studies of gene expression impossible. A method that preserves the RNA expression profile during and after sample aspiration is therefore essential for accurate analysis of gene expression in human whole bone marrow.

Principle and procedure

PreAnalytiX has developed a system that enables the collection, stabilization, storage, and transportation of whole human bone marrow specimens, together with a rapid and efficient protocol for isolation and purification of intracellular RNA. The system requires the use of PAXgene Bone Marrow RNA Tubes for bone marrow storage and RNA stabilization, followed by RNA isolation and purification using the PAXgene Bone Marrow RNA Kit.

Sample transfer and RNA stabilization

PAXgene Bone Marrow RNA Tubes contain a proprietary reagent composition based on a patented RNA stabilization technology. This protects RNA molecules from degradation by RNases and minimizes ex vivo changes in gene expression. PAXgene Bone Marrow RNA Tubes are intended for the storage of human whole bone marrow and stabilization of cellular RNA for up to 3 days at 15–25°C or up to 5 days at 2–8°C. Currently available data shows stabilization of cellular RNA for several months at –20°C or –70°C. For more information from ongoing studies evaluating stability for longer time periods, please contact QIAGEN Technical Services.

The actual duration of RNA stabilization may vary depending upon the specific transcript and the downstream application used. The performance characteristics have not been established for all transcripts.

RNA purification

The PAXgene Bone Marrow RNA Kit is for the isolation and purification of total RNA from 2 ml human whole bone marrow collected in a PAXgene Bone Marrow RNA Tube. The procedure is shown in the flowchart on page 9. RNA preparation begins with a centrifugation step to pellet nucleic acids in the PAXgene Bone Marrow RNA Tube.

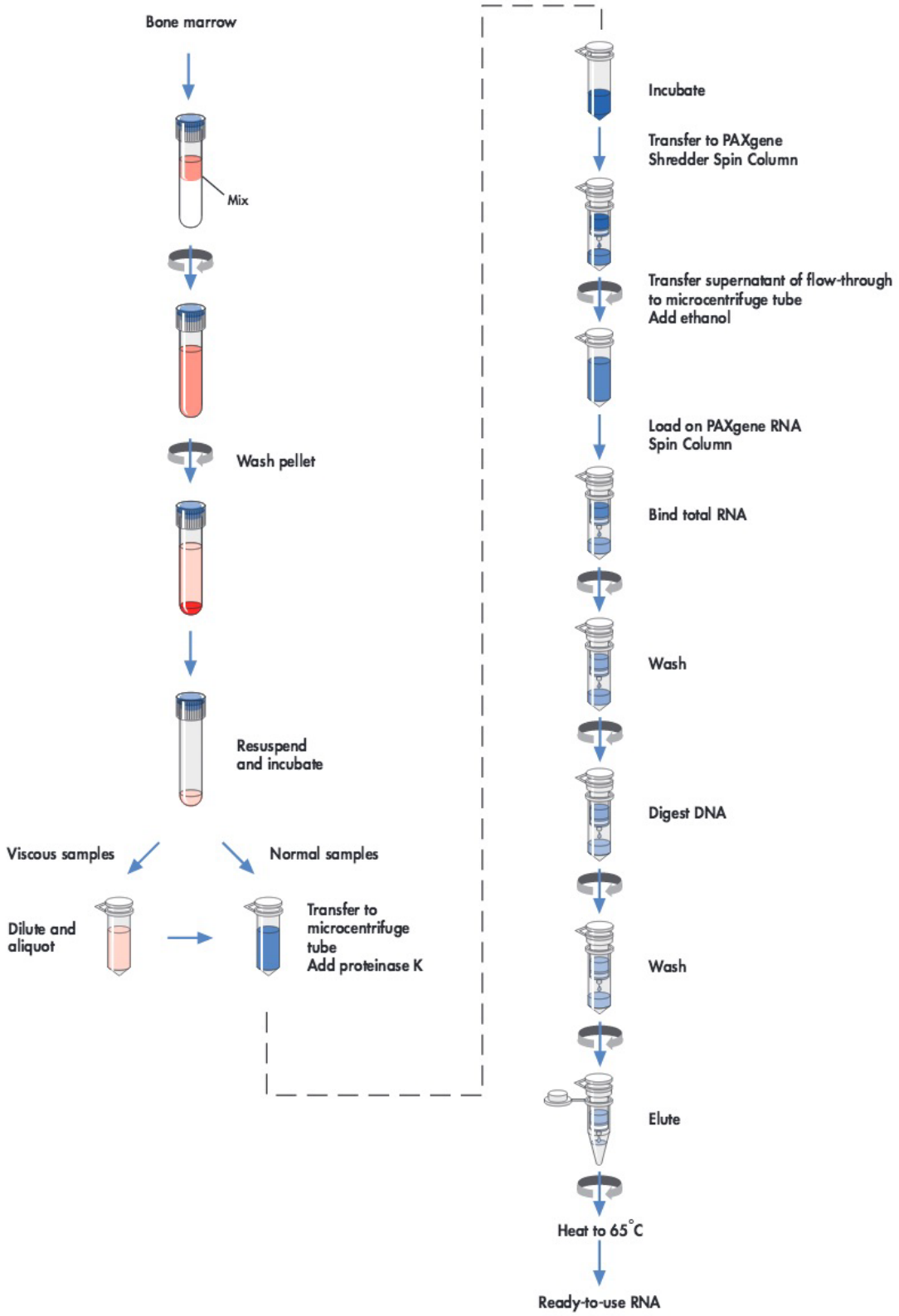
The pellet is washed and resuspended. The viscosity of the sample is checked, and viscous samples are diluted and re-aliquoted. The sample is then incubated in optimized buffers together with proteinase K to digest proteins. An additional centrifugation through the PAXgene Shredder spin column is carried out to homogenize the cell lysate and remove residual cell debris, and the supernatant of the flow-through fraction is transferred to a fresh microcentrifuge tube. Ethanol is added to adjust binding conditions, and the lysate is applied to a PAXgene RNA spin column. During a brief centrifugation, RNA is selectively bound to the PAXgene silica-gel membrane as contaminants pass through. Remaining contaminants are removed in several efficient wash steps. Between the first and second wash steps, the membrane is treated with DNase I to remove trace amounts of bound DNA. After the wash steps, RNA is eluted in a low-salt elution buffer and heat-denatured.

Total RNA purified using the PAXgene Bone Marrow RNA Kit is highly pure. Genomic DNA contamination is minimized, and purified RNA is ready to use in downstream applications, with no detectable PCR inhibition.

Bone marrow samples are extremely heterogeneous, consisting of varying amounts of cells, tissue, and solid matter.* RNA yields are highly donor-dependent and can vary greatly from sample to sample as well as between replicates from the same donor.

* Batinic´, D. et al. (1990) Relationship between differing volumes of bone marrow aspirates and their cellular composition. Bone Marrow Transplant. **6**, 103.

The Paxgene Bone Marrow RNA procedure



Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (SDSs), available from the product supplier.

- PAXgene Bone Marrow RNA Tubes (cat. no. 764114)
- Ethanol (96–100%, purity grade p.a.).
- Pipets (10 µl–4ml)*
- Sterile, aerosol-barrier, RNase-free pipet tips†
- Graduated cylinder‡
- Centrifuge* capable of attaining 3000–5000 x g, and equipped with a swing- out rotor and buckets to hold PAXgene Bone Marrow RNA Tubes (e.g., QIAGEN Centrifuge 4-16KS or Centrifuge 4-16S, see page 28 for Ordering Information).

  Note: Use only a swing-out rotor. Centrifugation using a fixed-angle rotor does not form a compact pellet and results in reduced yields. If the rotor used cannot accommodate PAXgene Bone Marrow RNA Tubes, the contents of the tube can be transferred to a round-bottomed centrifuge tube and centrifuged using a swing-out rotor. Centrifugation in conical tubes is not recommended since it is difficult to resuspend the pellets in conical tubes.
- Variable-speed microcentrifuge* capable of attaining 1000–8000 x *g* and equipped with a rotor for 2 ml microcentrifuge tubes
- Shaker–incubator* capable of incubating at 55°C and 65°C and shaking at >400 rpm, not exceeding 1400 rpm (e.g., Eppendorf® Thermomixer Compact, or equivalent)
- Vortex mixer*
- Crushed ice
- Permanent pen for labeling

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

† Ensure that you are familiar with the guidelines on handling RNA (Appendix A, page 21).

‡ For the addition of ethanol to Buffer BMR4 concentrate.

Protocol: Purification of RNA from Whole Bone Marrow in PAXgene Bone Marrow RNA Tubes

Important points before starting

- If working with RNA for the first time, read "Appendix A: General Remarks on Handling RNA", page 21.
- Ensure that the kit boxes are intact and undamaged, and that buffers have not leaked. Do not use a kit that has been damaged.
- When using a pipet, ensure that it is set to the correct volume, and that liquid is carefully and completely aspirated and dispensed.
- To avoid transferring samples to the wrong tube or spin column, ensure that all tubes and spin columns are properly labeled. Label the lid and the body of each tube. For spin columns, label the body of its processing tube. Close each tube or spin column after liquid is transferred to it.
- Spillages of samples and buffers during the procedure may reduce the yield and purity of RNA.
- Unless otherwise indicated, all steps of this protocol, including centrifugation steps, should be carried out at room temperature (15–25°C).

Things to do before starting

- Bone marrow must be transferred to PAXgene Bone Marrow RNA Tubes according to the instructions in the PAXgene Bone Marrow RNA Tube Product Circular.
- Ensure that the PAXgene Bone Marrow RNA Tubes are incubated for at least 2 h at room temperature after bone marrow collection to ensure complete lysis of cells. Incubation of the PAXgene Bone Marrow RNA Tube overnight may increase yields. If the PAXgene Bone Marrow RNA Tube was stored at 2–8°C or –20°C directly after bone marrow collection, first equilibrate it to room temperature, carefully invert the tube 10 times, and then store it at room temperature for 2 h before starting the procedure. Do not thaw frozen PAXgene Bone Marrow RNA Tubes at temperatures above 25°C.
- A shaker–incubator is required in steps 9 and 24. Set the temperature of the shaker–incubator to 55°C.
- Buffer BMR2 may form a precipitate upon storage. If necessary, warm to 37°C to dissolve.
- Buffer BMR4 is supplied as a concentrate. Before using for the first time, add ethanol (96–100%, purity grade p.a.) as indicated on the bottle to obtain a working solution.

- If using the RNase-Free DNase Set for the first time, prepare DNase I stock solution. Dissolve the solid DNase I (1500 Kunitz units)* in 350 µl of the DNase resuspension buffer (RNase-free water) provided with the set. Take care that no DNase I is lost when opening the vial. Do not vortex the reconstituted DNase I. DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube.
- Current data shows that reconstituted DNase I can be stored at 2–8°C for up to 6 weeks. For long-term storage of DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots (use the 1.5 ml microcentrifuge tubes supplied with the kit), and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.
- When reconstituting and aliquoting DNase I, ensure that you follow the guidelines for handling RNA (Appendix A, page 21).

* Kunitz units are commonly used units for measuring DNase I, defined as the amount of DNase I that causes an increase in A_{260} of 0.001 per minute per milliliter at 25°C, pH 5.0, with highly polymerized DNA as the substrate (Kunitz, M. (1950) J. Gen. Physiol. **33**, 349 and 363).

Procedure

1. **Centrifuge the PAXgene Bone Marrow RNA Tube for 10 min at 3000–5000 x *g* using a swing-out rotor.**

   Note: Ensure that the bone marrow sample has been incubated in the PAXgene Bone Marrow RNA Tube for a minimum of 2 h at room temperature (15–25°C), in order to achieve complete lysis of cells.

   Note: The rotor must allow the PAXgene Bone Marrow RNA Tubes to fully swing out during centrifugation and contain tube adapters for round-bottom tubes. If other types of tube adapter are used or if the tubes do not swing out fully, the tubes may break during centrifugation.

2. **Remove the supernatant by decanting or pipetting. Add 7 ml Buffer BMR Wash to the pellet, and close the tube using a fresh secondary Hemogard closure.**

   If the supernatant is decanted, take care not to disturb the pellet, and dry the rim of the tube with a clean paper towel.

   Note: Occasionally, an upper lipid layer is present, especially when PAXgene Bone Marrow RNA Tubes were stored frozen. Make sure to remove and discard this layer with the supernatant.

3. **Vortex until the pellet is visibly dissolved, and centrifuge for 10 min at 3000–5000 x *g* using a swing-out rotor. Remove and discard the entire supernatant.**

   Small amounts of debris remaining in the supernatant after vortexing but before centrifugation will not affect the procedure.

   Note: Incomplete removal of the supernatant will inhibit lysis and dilute the lysate, and therefore affect the conditions for binding RNA to the PAXgene membrane.

4. **Add 350 µl Buffer BMR1 and vortex until the pellet is visibly dissolved.**

   Note: If the pellet does not fully dissolve after vortexing, dissolve by pipetting up and down using a cutoff pipet with a large diameter.

5. **Add 300 µl Buffer BMR2, mix by vortexing for 10 s and incubate for 3 min at room temperature (15–25°C).**

6. **Check the viscosity of the solution by gently flicking the tube. If the solution remains capable of pipetting and did not become highly viscous, pipet the entire sample into a 1.5 ml microcentrifuge tube and proceed directly with step 9. Otherwise, for highly viscous samples proceed with step 7.**

   Note: Do not omit the incubation in step 5 or the evaluation of viscosity in step 6. This could make processing of some viscous samples impossible later in the protocol.

7. **Add another 350 µl Buffer BMR1 and vortex until the viscous solution is clearly dissolved.**

   Up to 1 min of vortexing at maximum speed may be required for some samples.

8. **Pipet an aliquot of 240 µl diluted sample (approximately 20% of the total) into a 1.5 ml microcentrifuge tube. Add 250 µl Buffer BMR1 and 270 µl Buffer BMR2 to the sample aliquot. Mix by vortexing for 5 s.**

   Normally, this aliquot (approximately 20%) of a diluted viscous sample will give high yields of RNA, sufficient for most downstream applications. If desired, additional aliquots of the remaining sample (approximately 830–960 µl) can be archived for later processing. Dilute these aliquots with Buffer BMR1 and Buffer BMR2, and mix by vortexing as described above. Then store at –20°C or –70°C. To process these samples later, thaw and continue with step 9.

9. **Add 40 µl proteinase K. Mix by vortexing for 5 s, and incubate for 10 min at 55°C using a shaker–incubator at 400–1400 rpm. After incubation, set the temperature of the shaker–incubator to 65°C (for step 24).**

10. **Pipet the lysate directly into a PAXgene Shredder spin column (lilac) placed in a 2 ml processing tube, and centrifuge for 3 min at maximum speed (but not to exceed 20,000 x g).**

    Note: Carefully pipet the lysate into the PAXgene Shredder spin column and visually check that the lysate is completely transferred to the spin column, but do not use more than 800 µl lysate.

    To prevent damage to columns and tubes, do not exceed 20,000 x g.

11. **Carefully pipet the entire supernatant of the flow-through fraction to a fresh 1.5 ml microcentrifuge tube without disturbing the pellet in the processing tube.**

    Note: Sometimes no solid pellet forms during centrifugation. In this case, transfer the complete solution, which may be gelatinous.

    Note: Occasionally, a brown, gelatinous, upper lipid layer is present. Do not transfer this upper layer.

12. **Add 350 µl ethanol (96–100%, purity grade p.a.). Mix by vortexing and centrifuge briefly (1–2 s at 500–1000 x *g*) to remove drops from the inside of the tube lid.**

    Note: The length of the centrifugation must not exceed 1–2 s, as this may result in pelleting of nucleic acids and reduced yields of total RNA.

13. **Pipet 700 µl sample into the PAXgene RNA spin column (red) placed in a 2 ml processing tube, and centrifuge for 1 min at 8000–20,000 x *g*. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.***

14. **Pipet the remaining sample into the PAXgene RNA spin column, and centrifuge for 1 min at 8000–20,000 x *g*. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.***

    Note: Carefully pipet the sample into the spin column and visually check that the sample is completely transferred to the spin column.

15. **Pipet 350 µl Buffer BMR3 into the PAXgene RNA spin column. Centrifuge for 1 min at 8000–20,000 x *g*. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.***

16. **Add 10 µl DNase I stock solution to 70 µl Buffer RDD in a 1.5 ml microcentrifuge tube. Mix by gently flicking the tube, and centrifuge briefly to collect residual liquid from the sides of the tube.**

    If processing, for example, 10 samples, add 100 µl DNase I stock solution to 700 µl Buffer RDD. Use the 1.5 ml microcentrifuge tubes supplied with the kit.

    Note: DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently flicking the tube. Do not vortex.

17. **Pipet the DNase I incubation mix (80 µl) from step 16 directly onto the PAXgene RNA spin column membrane, and incubate at room temperature (15–25°C) for 15 min.**

    Note: Ensure that the DNase I incubation mix is placed directly onto the membrane. DNase digestion will be incomplete if part of the mix is applied to and remains on the walls or the O-ring of the spin column.

* Flow-through contains Buffer BMR2 or Buffer BMR3 and is therefore not compatible with bleach. See page 6 for safety information.

18. **Pipet 350 µl Buffer BMR3 into the PAXgene RNA spin column, and centrifuge for 1 min at 8000–20,000 x *g*. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.***

19. **Pipet 500 µl Buffer BMR4 to the PAXgene RNA spin column, and centrifuge for 1 min at 8000–20,000 x *g*. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.**

    Note: Buffer BMR4 is supplied as a concentrate. Add ethanol to Buffer BMR4 before use (see "Things to do before starting", page 11).

20. **Add another 500 µl Buffer BMR4 to the PAXgene RNA spin column. Centrifuge for 3 min at 8000–20,000 x *g*.**

21. **Discard the tube containing the flow-through and place the PAXgene RNA spin column in a new 2 ml processing tube. Centrifuge for 1 min at 8000–20,000 x *g*.**

22. **Discard the tube containing the flow-through. Place the PAXgene RNA spin column in a 1.5 ml microcentrifuge tube, and pipet 40 µl Buffer BMR5 directly onto the PAXgene RNA spin column membrane. Centrifuge for 1 min at 8000–20,000 x *g* to elute the RNA.**

    It is important to wet the entire membrane with Buffer BMR5 in order to achieve maximum elution efficiency.

23. **Repeat the elution step (step 22) as described, using 40 µl Buffer BMR5 and the same microcentrifuge tube.**

24. **Incubate the eluate for 5 min at 65°C in the shaker–incubator (from step 9) without shaking. After incubation, chill immediately on ice.**

    Note: This incubation at 65°C denatures the RNA for downstream applications. Do not exceed the incubation time or temperature.

* Flow-through contains Buffer BMR2 or Buffer BMR3 and is therefore not compatible with bleach. See page 6 for safety information.

25. If the RNA samples will not be used immediately, store at –20°C or –70°C.

Since the RNA remains denatured after freezing and thawing, it is not necessary to repeat the incubation at 65°C. If using the RNA samples in a commercial assay, follow the instructions supplied by the manufacturer.

For accurate quantification of RNA by absorbance at 260 nm, we recommend diluting samples with 10 mM Tris-HCl, pH 7.5.* Diluting the sample in RNase-free water may lead to inaccurately low values.

Zero the spectrophotometer using a blank consisting of the same proportion Buffer BMR5 and Tris-HCl buffer as in the samples to be measured. Buffer BMR5 has high absorbance at 220 nm, which can lead to high background absorbance levels if the spectrophotometer is not properly zeroed.

Note: For quantification in Tris-HCl buffer, use the relationship $A_{260} = 1 \Rightarrow 44\ \mu g/ml$. See Appendix B, page 24.

For RT-PCR and real-time RT-PCR with the purified RNA, QIAGEN offers a range of optimized, ready-to-use kits that provide highly specific and sensitive results. For details, visit **www.qiagen.com/PCR.**

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions on the respective product page at www.preanalytix.com or our Technical Support Center at www.qiagen.com/FAQ/FAQList.aspx. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information see page 31 or visit **www.preanalytix.com**).

	Comments and suggestions
RNA degraded	
a) RNase contamination	Be careful not to introduce any RNases into the reagents during the procedure or later handling (see Appendix A, page 21).
b) Cells in sample are not intact	Cells in the bone marrow may be apoptotic prior to collection. RNA from these cells may be degraded.
c) More than 2 ml bone marrow collected in the PAXgene Bone Marrow RNA tube	Do not use more than 2 ml bone marrow. Use of more than 2 ml will interfere with the stabilization properties of the PAXgene Bone Marrow RNA tube, leading to reduced RNA yields and degraded RNA.
Low RNA yield	
a) Less than 2 ml bone marrow transferred into the PAXgene Bone Marrow RNA tube	Ensure that 2 ml bone marrow is transferred to the PAXgene Bone Marrow RNA Tube (see the *PAXgene Bone Marrow RNA Tube Product Circular*). Bone marrow samples can vary considerably in the number of cells that they contain. RNA yields are highly donor- dependent and can vary greatly from sample to sample as well as between replicates from the same donor.
b) More than 2 ml bone marrow collected in the PAXgene Bone Marrow RNA Tube	Do not use more than 2 ml bone marrow. Use of more than 2 ml will interfere with the stabilization properties of the PAXgene Bone Marrow RNA tube, leading to reduced RNA yields and degraded RNA.

	Comments and suggestions
c) RNA concentration measured in water	RNA must be diluted in10 mM Tris-Cl, pH 7.5* for accurate quantification (see Appendix B, page 24).
d) Fixed-angle centrifuge rotor used in steps 1 and 3	Use only a swing-out rotor. Centrifugation using a fixed-angle rotor does not form a compact pellet and results in reduced yields.
e) Contents of PAXgene Bone Marrow RNA Tube centrifuged in conical centrifuge tube	If the rotor used cannot accommodate PAXgene Bone Marrow RNA Tubes, alternatively, the contents of the tube can be transferred to a round-bottomed centrifuge tube and centrifuged using a swing-out rotor. Centrifugation in conical tubes is not recommended since it is difficult to resuspend the pellets in conical tubes.
f) Cell debris transferred to the PAXgene RNA spin column in steps 13 and 14	Avoid transferring large particles when pipetting the supernatant in step 11 (transfer of small debris will not affect the procedure).
g) Supernatant not completely removed in step 3	Ensure the entire supernatant is removed. If the supernatant is decanted, remove drops from the rim of the tube by dabbing onto a paper towel. Take appropriate precautions to prevent cross-contamination.
h) Centrifugation for more than 1–2 s in step 12	Samples in step 12 should only be centrifuged briefly, no more than 1–2 s. Longer centrifugation may result in pelleting of RNA and reduced yields.
i) After collection in the PAXgene Bone Marrow RNA Tube, bone marrow is incubated for less than 2 h at room temperature	Incubate bone marrow in the PAXgene Bone Marrow RNA Tube for at least 2 h at room temperature after collection.

* Wilfinger, W.W., Mackey, M., and Chomczynski, P. (1997) Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. BioTechniques **22**, 474.

	Comments and suggestions
j) Very low number of intact cells in sample	Samples from some donors may have very low numbers of intact cells, depending on their disease state or drug therapy. These samples therefore only contain small amounts of intact RNA, and higher yields cannot be obtained.
Low A_{260}/A_{280} ratio	
a) RNA diluted in water before purity is measured	Use 10 mM Tris-HCl, pH 7.5 to dilute RNA before measuring purity* (see Appendix B, page 24).
b) Spectrophotometer not properly zeroed	Zero the spectrophotometer using a blank consisting of the same proportion Buffer BMR5 and 10 nM Tris-HCl, pH 7.5, as in the samples to be measured. Buffer BMR5 shows high absorbance at 220 nm, which can lead to high background absorbance levels if the spectrophotometer is not properly zeroed.

Appendix A: General Remarks on Handling RNA

Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to destroy RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Great care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the purification procedure. In order to create and maintain an RNase-free environment, precautions must be taken during pretreatment and use of disposable and non-disposable vessels and solutions while working with RNA.

General handling

Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible. Keep purified RNA on ice when aliquots are pipetted for downstream applications.

Disposable plasticware

The use of sterile, disposable polypropylene tubes is recommended throughout the procedure. These tubes are generally RNase-free and do not require pretreatment to inactivate RNases.

Nondisposable plasticware

Nondisposable plasticware should be treated before use to ensure that it is RNase-free. Plasticware should be thoroughly rinsed with 0.1 M NaOH, 1 mM EDTA* followed by RNase-free water (see "Solutions", page 22). Alternatively, chloroform- resistant plasticware can be rinsed with chloroform* to inactivate RNases.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

Glassware

Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent,* thoroughly rinsed, and oven baked at 240°C for at least 4 hours (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware can be treated with DEPC* (diethyl pyrocarbonate). Fill glassware with 0.1% DEPC (0.1% in water), allow to stand overnight (12 hours) at 37°C, and then autoclave or heat to 100°C for 15 minutes to eliminate residual DEPC.

Electrophoresis tanks

Electrophoresis tanks should be cleaned with detergent solution (e.g., 0.5% SDS),* thoroughly rinsed with RNase-free water, and then rinsed with ethanol† and allowed to dry.

Solutions

Solutions (water and other solutions) should be treated with 0.1% DEPC. DEPC is a strong, but not absolute, inhibitor of RNases. It is commonly used at a concentration of 0.1% to inactivate RNases on glass or plasticware or to create RNase-free solutions and water. DEPC inactivates RNases by covalent modification.

Add 0.1 ml DEPC to 100 ml of the solution to be treated and shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 hours at 37°C. Autoclave for 15 minutes to remove any trace of DEPC. DEPC will react with primary amines and cannot be used directly to treat Tris buffers*.

DEPC is highly unstable in the presence of Tris buffers and decomposes rapidly into ethanol and CO_2. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris to make the appropriate buffer. Trace amounts of DEPC will modify purine residues in RNA by carbethoxylation. Carbethoxylated RNA is translated with very low efficiency in cell-free systems. However, its ability to form DNA:RNA or RNA:RNA hybrids is not seriously affected unless a large fraction of the purine residues have been modified.

Residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 minutes.

Note: PAXgene RNA buffers are guaranteed RNase-free without using DEPC treatment and are therefore free of any DEPC contamination.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

† Plastics used for some electrophoresis tanks are not resistant to ethanol. Take proper care and check the supplier's instructions.

Avoiding cross-contamination

Because of the sensitivity of nucleic acid amplification technologies, the following precautions are necessary when handling samples to avoid cross-contamination:

- Carefully pipet the sample into the spin column without moistening the rim of the column.
- Always change pipet tips between liquid transfers. Use aerosol-barrier pipet tips.
- Avoid touching the spin column membrane with the pipet tip.
- After vortexing or heating a microcentrifuge tube, briefly centrifuge it to remove drops from the inside of the lid.
- Wear gloves throughout the entire procedure. In case of contact between gloves and sample, change gloves immediately.
- Close the spin column before placing it in the microcentrifuge. Centrifuge as described in the procedure.
- Open only one spin column at a time, and take care to avoid generating aerosols.
- For efficient parallel processing of multiple samples, fill a rack with processing tubes to which the spin columns can be transferred after centrifugation. Discard the used processing tubes containing flow-through, and place the new processing tubes containing spin columns directly in the microcentrifuge.

Appendix B: Storage, Quantification and Determination of Quality of Total RNA

Storage of RNA

Purified RNA may be stored at –20°C or –70°C in Buffer BMR5.

Quantification of RNA

The concentration of RNA can be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer (see "Spectrophotometric quantification of RNA" below). For small amounts of RNA, however, it may not be possible to accurately determine amounts photometrically. Small amounts of RNA can be quantified using the Agilent® 2100 Bioanalyzer, fluorometric quantification, or quantitative, real-time RT-PCR.

Spectrophotometric quantification of RNA

The concentration of RNA should be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. To ensure significance, readings should be in the linear range of the spectrophotometer. An absorbance of 1 unit at 260 nm corresponds to 44 µg of RNA per ml (A_{260} = 1 ⇒ 44 µg/ml). This relation is valid only for measurements in 10 mM Tris-HCl,* pH 7.5. Therefore, if it is necessary to dilute the RNA sample and this should be done in 10 mM Tris-HCl. As discussed below (see "Purity of RNA," page 26), the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity.

When measuring RNA samples, be certain that cuvettes are RNase-free. Zero the spectrophotometer using a blank consisting of the same proportion Buffer BMR5 and Tris-HCl buffer as in the samples to be measured. Buffer BMR5 has high absorbance at 220 nm, which can lead to high background absorbance levels if the spectrophotometer is not properly zeroed.

An example of the calculation involved in RNA quantification is shown below:

Volume of RNA sample = 80 µl

Dilution = 10 µl of RNA sample + 140 µl 10 mM Tris-HCl, pH 7.5 (1/15 dilution)

Measure absorbance of diluted sample in a cuvette (RNase-free).

A_{260} = 0.3

Concentration of RNA sample = 44 x A_{260} x dilution factor
= 44 x 0.3 x 15
= 198 µg/ml

Total yield = concentration x volume of sample in milliliters
= 198 µg/ml x 0.08 ml
= 15.8 µg RNA

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

Purity of RNA

The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV, such as protein. However, the A_{260}/A_{280} value is influenced considerably by pH. Lower pH results in a lower A_{260}/A_{280} value and reduced sensitivity to protein contamination.* For accurate values, we recommend measuring absorbance in 10 mM Tris·Cl, pH 7.5. Pure RNA has an A_{260}/A_{280} value of 1.8–2.2† in 10 mM Tris·Cl, pH 7.5. Use the buffer in which the RNA is diluted to zero the spectrophotometer, and make sure to add the same volume of Buffer BMR5 as the volume of eluted RNA to be diluted. Buffer BMR5 has high absorbance at 220 nm, which can lead to high background absorbance levels if the spectrophotometer is not properly zeroed.

DNA contamination

No currently available purification method can guarantee that RNA is completely free of DNA, even when it is not visible on an agarose gel. While the vast majority of cellular DNA will be removed by the DNase digestion step, trace amounts may still remain in the purified RNA.

For analysis of very low abundance targets, any interference by residual DNA contamination can be detected by performing real-time RT-PCR control experiments in which no reverse transcriptase is added prior to the PCR step.

To prevent any interference by DNA in real-time RT-PCR applications, such as with Rotor-Gene® Q and Applied Biosystems® instruments, we recommend designing primers that anneal at intron splice junctions so that genomic DNA will not be amplified. QuantiTect® Primer Assays from QIAGEN (**www.qiagen.com/GeneGlobe**) are designed for SYBR Green–based real-time RT-PCR analysis of RNA sequences (without detection of genomic DNA) where possible. For real-time RT-PCR assays where amplification of genomic DNA cannot be avoided, the QuantiTect Reverse Transcription Kit provides fast cDNA synthesis with integrated removal of genomic DNA contamination (see Ordering Information, page 28).

* Wilfinger, W.W., Mackey, M., and Chomczynski, P. (1997) Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. BioTechniques **22**, 474.

† Values up to 2.3 are routinely obtained for pure RNA (in 10 mM Tris-HCl, pH 7.5) with some spectrophotometers.

Integrity of RNA

The integrity and size distribution of total RNA purified with PAXgene Bone Marrow RNA Kit can be checked, for example, by denaturing agarose gel electrophoresis and ethidium bromide staining* or using the Agilent 2100 Bioanalyzer. The respective ribosomal RNAs should appear as sharp bands or peaks. The apparent ratio of 28S rRNA to 18S rRNA should be approximately 2:1.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

Ordering Information

Product	Contents	Cat. no.
Products that can be ordered from QIAGEN		
PAXgene Bone Marrow RNA Kit (30)	For 30 RNA preps: 30 PAXgene Spin Columns, 30 PAXgene Shredder Spin Columns, Processing Tubes, RNase-Free DNase I, RNase-Free Reagents and Buffers. To be used in conjunction with PAXgene Bone Marrow RNA Tubes	764133
PAXgene Bone Marrow RNA Tubes (50)	50 Bone Marrow Collection Tubes. To be used in conjunction with the PAXgene Bone Marrow RNA Kit	764114
QIAGEN Centrifuges		
Centrifuge 4-16KS (220–240 V)	Refrigerated universal laboratory centrifuge with brushless motor (220–240 V; 50/60 Hz)	81610
Centrifuge 4-16KS (200 V; 50/60 Hz)	Refrigerated universal laboratory centrifuge with brushless motor (200 V; 50/60 Hz)	81600
Centrifuge 4-16KS (220 V, 50 Hz)	Refrigerated universal laboratory centrifuge with brushless motor (220 V; 50 Hz)	81620
Centrifuge 4-16S (120 V; 60 Hz)	Universal laboratory centrifuge with brushless motor (120 V; 60 Hz)	81510
Centrifuge 4-16S (100 V; 50/60 Hz)	Universal laboratory centrifuge with brushless motor (100 V; 50/60 Hz)	81500
Centrifuge 4-16S (220 V, 50 Hz)	Universal laboratory centrifuge with brushless motor (220 V, 50 Hz)	81520

Product	Contents	Cat. no.
Related products		
QuantiTect Kits		
QuantiTect Reverse Transcription Kit (50)	For 50 x 20 µl reverse-transcription reactions: gDNA Wipeout Buffer, Quantiscript® Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, RNase-Free Water	205311
QuantiTect Multiplex RT-PCR Kit (200)	For 200 x 50 µl reactions: 3 x 1.7 ml 2x QuantiTect Multiplex RT-PCR Master Mix (with ROX dye), 100 µl QuantiTect Multiplex RT Mix, 2 x 2 ml RNase-free Water	204643
QuantiTect Primer Assay (200)*	For 200 x 50 µl reactions or 400 x 25 µl reactions: 10x QuantiTect Primer Assay (lyophilized)	Varies
Rotor-Gene Q — for outstanding performance in real-time PCR		
Rotor-Gene Q 5plex HRM System	Real-time PCR cycler and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories, 1-year warranty on parts and labor, installation and training	9001650

For up-to-date licensing information and product-specific disclaimers, see the respective PreAnalytiX or QIAGEN kit handbook or user manual. PreAnalytiX kit handbooks and product circulars are available at **www.preanalytix.com**. QIAGEN kit handbooks and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

* Larger kit sizes available; please inquire.

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