PAXgene®

Tissue miRNA Kit Handbook

For isolation and purification of total RNA, including miRNA, from tissue samples stabilized in PAXgene Tissue Containers

Important: To be used only in conjunction with PAXgene Tissue Containers

For research use only. Not for use in diagnostics procedures.

December 2014

PreAnalytiX
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Kit Contents

PAXgene Tissue miRNA Kit	(50)
Catalog no.	**766134**
Number of preps	**50**
Buffer TM1 (Binding Buffer)*	18 ml
Buffer TM2 (Wash Buffer 1 concentrate)* †	12 ml
Buffer TM3 (Wash Buffer 2 concentrate)†	11 ml
Buffer TM4 (Elution Buffer)	5 ml
RNase-Free Water (bottle)	125 ml
Proteinase K (green lid)	1.4 ml
PAXgene RNA MinElute® Spin Columns (red) with Processing Tubes	5 x 10
PAXgene Shredder Spin Columns (lilac) with Processing Tubes	5 x 10
Processing Tubes (2 ml)	5 x 50
Microcentrifuge Tubes (1.5 ml)	3 x 50, 1 x 10
DNase I, RNase-Free (lyophilized, 1500 Kunitz units‡)	1 glass vial
Buffer RDD (white lid)	2 x 2 ml
RNase-Free Water (tube, lilac lid) (DNase Resuspension buffer)	2 ml
Carrier RNA (red lid)§	310 μg
Handbook	1

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

† Buffer TM2 and TM3 are supplied as concentrate. Before using for the first time, add 3 volumes of isopropanol as indicated on the bottle to TM2 (96–100%, purity grade p.a.) and 4 volumes of ethanol (96–100%, purity grade p.a.) to TM3 to obtain working solutions.

‡ Kunitz units are the commonly used units for measuring DNase I; see page 16 for definition.

§ Carrier RNA is not required for the protocols in this handbook.

Shipping and Storage

The PAXgene Tissue miRNA Kit is shipped at ambient temperature.

PAXgene RNA MinElute spin columns and the RNase-Free DNase Set in the PAXgene Tissue miRNA Kit should be stored upon receipt at 2–8°C. All other components of the PAXgene Tissue miRNA Kit can be stored dry at room temperature (15–25°C). Under these conditions, the kit is stable for at least 9 months.

Proteinase K is stable for at least 1 year after delivery when stored at room temperature. For longer storage or if ambient temperatures often exceed 25°C, we recommend storing proteinase K at 2–8°C.

Product Use Limitations

For Research Use Only. Not for use in diagnostics procedures. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

It is the user's responsibility to validate the performance of the PAXgene Tissue miRNA Kit for any particular use, since the performance characteristics of these kits have not been validated for any specific organism. The performance characteristics of this product have not been fully established.

Quality Control

In accordance with QIAGEN's ISO-certified Total Quality Management System, each lot of PAXgene Tissue miRNA Kit is tested against predetermined specifications to ensure consistent product quality.

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 QIAGEN Technical Services or your local distributor (see last page or visit www.qiagen.com).

Technical Assistance

At 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 products. If you have any questions or experience any difficulties regarding the PAXgene Tissue miRNA 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 see our Technical Support Center at www.qiagen.com/Support or call one of the QIAGEN Technical Service Departments or local distributors (see last page or visit www.qiagen.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.qiagen.com/safety where you can find, view, and print the SDS for each QIAGEN or PreAnalytiX kit and kit component.

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

Buffer TM1 and Buffer TM2 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.

Introduction

Current tissue fixation methods used in traditional histology are of limited use for molecular analysis. Fixatives that contain formaldehyde cross-link biomolecules and modify nucleic acids and proteins. During tissue fixation, storage, and processing, cross-links lead to degradation of nucleic acids. Since cross-links can not be removed completely, the resulting chemical modifications can cause inhibition in sensitive downstream applications such as quantitative PCR or RT-PCR. In order to enable both molecular and traditional pathology testing from the same specimen, a method is needed for stabilization of molecular content and preservation of morphology.

Principle and procedure

PreAnalytiX has developed the PAXgene Tissue System to meet such needs. The system consists of a tissue collection device (the PAXgene Tissue Container for collection, stabilization, storage, and transportation of human tissue specimens) and kits for purification of total RNA, DNA, or miRNA. PAXgene Tissue Containers provide tissue fixation for histopathology studies and enable purification of high-quality nucleic acids from the same sample for molecular analysis. The fixation and stabilization method preserves tissue morphology and the integrity of nucleic acids without destructive cross-linking and degradation found in formalin-fixed tissues.

For purification of total RNA, including miRNA, the system requires the use of PAXgene Tissue Containers for tissue collection and stabilization, followed by RNA isolation and purification using the PAXgene Tissue miRNA Kit. Together the container and kit provide a complete preanalytical solution for collection, fixation, and stabilization of tissue and for purification of high-quality RNA, including miRNA, for molecular analysis.

Sample collection and stabilization

PAXgene Tissue Containers are dual-chamber containers prefilled with 2 reagents. PAXgene Tissue Fix rapidly penetrates and fixes the tissue. After fixation, the tissue is removed from the PAXgene Tissue Fix and transferred to PAXgene Tissue Stabilizer in the second chamber of the same container. When the tissue is stored in PAXgene Tissue Stabilizer, nucleic acids and morphology of the tissue sample are stable for a minimum of 3 and a maximum of 7 days at room temperature (15–25°C) or for a minimum of 2 and a maximum of 4 weeks at 2–8°C, depending on the type of tissue. Storage at –30 to –15°C is

also possible for at least 3 months without any negative effects on the morphology of the tissue or the integrity of the nucleic acids.*

Stabilized samples can be embedded in paraffin for histological studies. Nucleic acids can be isolated from the stabilized samples either before or after embedding in paraffin. See the *PAXgene Tissue Container Product Circular* for information about tissue fixation, stabilization, processing, and paraffin embedding.

RNA purification

The PAXgene Tissue miRNA Kit provides 3 protocols for purification of total RNA from tissue fixed and stabilized in PAXgene Tissue Containers, including RNA molecules smaller than 200 nucleotides, such as 5.8S rRNA, 5S rRNA, tRNAs, and miRNAs. Optimized binding and washing conditions ensure the purification of RNA molecules as small as 18 nucleotides. As a prerequisite, the tissue must be fixed and stabilized in PAXgene Tissue Containers (see "Description of protocols" below and flowchart, page 10).

Disruption and homogenization of the tissue sample is performed in the binding buffer, Buffer TM1 (see "Disrupting and homogenizing starting materials", page 13). After a centrifugation step to remove residual cell debris, isopropanol is added to the lysate to provide appropriate binding conditions for all RNA molecules 18 nucleotides and longer. The sample is then applied to a PAXgene RNA MinElute spin column where the total RNA binds to the membrane and contaminants are efficiently washed away. 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 including miRNA is eluted in a low-salt elution buffer and denatured by heating.

Total RNA purified using the PAXgene Tissue miRNA Kit is highly pure. Genomic DNA contamination is minimized, and purified RNA is ready to use in downstream applications with no detectable PCR inhibition. All RNA molecules longer than 18 nucleotides are purified.

Description of protocols

Sections of PAXgene Tissue fixed, paraffin-embedded (PFPE) tissue (page 15)

Starting material for RNA purification is freshly cut sections from tissue samples that have been fixed and stabilized in PAXgene Tissue Containers, dehydrated, and embedded in paraffin.

* Specifications for fixation and storage conditions in PAXgene Tissue Fix and PAXgene Tissue Stabilizer were determined using animal tissues.

A minimum of 2 and a maximum of 5 sections, each with a thickness of 5–10 μm and a tissue surface area of up to 100 mm^2, can be combined in one sample prep. Paraffin is removed from the tissue sections by incubation in xylene, followed by addition of ethanol and centrifugation. The resulting pellet is resuspended in a lysis buffer, diluted, and treated with proteinase K. Centrifugation through the PAXgene Shredder spin column homogenizes the cell lysate and removes residual cell debris.

PAXgene Tissue fixed samples (page 20)

Starting material for RNA purification is up to 10 mg of a tissue sample that has been fixed and stabilized in PAXgene Tissue Containers.

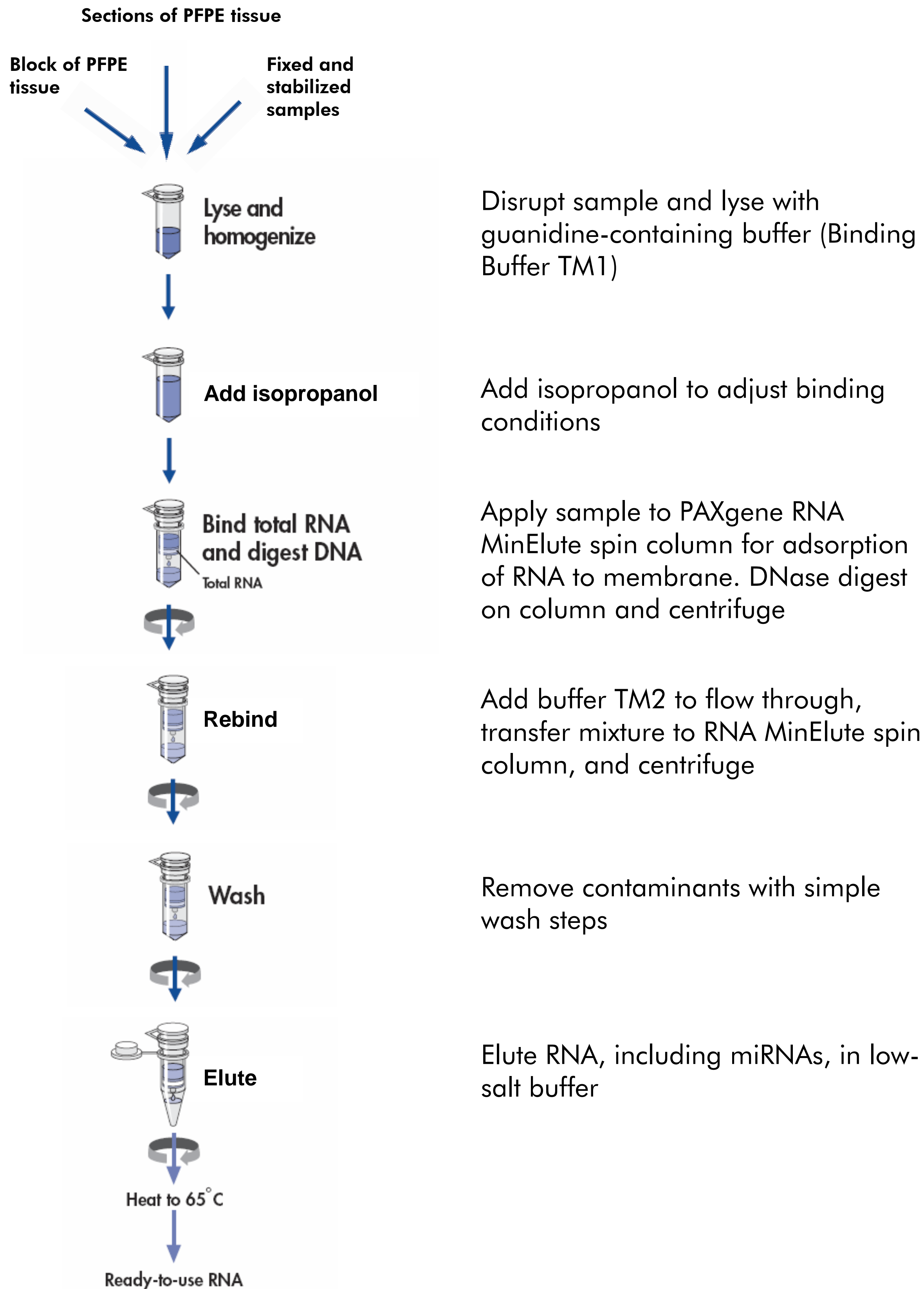
The tissue sample is removed from the tissue cassette. If necessary, the sample is cut into 2 mm cubes. Binding Buffer TM1 is added and mechanical disruption and simultaneous homogenization is performed using the TissueRuptor® or TissueLyser II (see "Disrupting and homogenizing starting materials", page 13).

Blocks of PFPE tissue (page 26)

Starting material for RNA purification is a block of tissue of up to 10 mg that has been fixed and stabilized in a PAXgene Tissue Container, dehydrated, and embedded in paraffin.

The tissue sample is cut from the paraffin block using a scalpel. After determining the amount of tissue by weight, paraffin is removed from the tissue by incubation in xylene, followed by addition of ethanol and centrifugation. Binding Buffer TM1 is added to the resulting pellet, and mechanical disruption and simultaneous homogenization is performed using the TissueRuptor or TissueLyser II (see "Disrupting and homogenizing starting materials", page 13).

The PAXgene Tissue miRNA 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 safety data sheets (SDSs), available from the product supplier.

For all protocols

- PAXgene Tissue Container
- Xylene
- Ethanol (96–100%, purity grade p.a.)
- Isopropanol
- 14.3 M β-mercaptoethanol (β-ME) (commercially available solutions are usually 14.3 M)
- Pipets* (10 μl – 1 ml)
- Sterile, aerosol-barrier, RNase-free pipet tips†
- Graduated cylinder‡
- 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 45°C and 65°C and shaking at ≥400 rpm, not exceeding 1400 rpm (e.g., Eppendorf® Thermomixer Compact, www.ependorf.com§ or equivalent)
- Vortex mixer*
- Scalpel
- Crushed ice

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

† Ensure that you are familiar with the guidelines on handling RNA (Appendix A, page 38).

‡ For the addition of isopropanol to Buffer TM2 and ethanol to Buffer TM3 concentrate.

§ This is not a complete list of suppliers and does not include many important vendors of biological supplies.

For PFPE tissue

- Equipment for tissue disruption and homogenization (see "Disrupting and homogenizing starting materials", page 13). We recommend either the TissueRuptor* with TissueRuptor Disposable Probes or the TissueLyser* system (see ordering information, page 23).
- 2 ml round-bottomed microcentrifuge tubes

For sections of PFPE tissues

- Microtome*

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

Important Notes

Disrupting and homogenizing starting materials

Efficient disruption and homogenization of the starting material is an absolute requirement for all total RNA purification procedures. Disruption and homogenization are 2 distinct steps.

- **Disruption:** Complete disruption of plasma membranes of cells and organelles is absolutely required to release all RNA contained in the sample. Incomplete disruption results in significantly reduced RNA yields.
- **Homogenization:** Homogenization is necessary to reduce the viscosity of the lysates produced by disruption. Homogenization shears high-molecular-weight genomic DNA and other high-molecular-weight cellular components to create a homogeneous lysate. Incomplete homogenization results in inefficient binding of RNA to the PAXgene RNA MinElute spin column membrane and therefore significantly reduces RNA yields.

Disruption and homogenization of tissue samples can be carried out rapidly and efficiently using either the TissueRuptor (for processing samples individually) or the TissueLyser II (for processing multiple samples simultaneously).* Table 1 gives an overview of different disruption and homogenization methods used for the different starting materials.

Table 1. Disruption and homogenization

Sample	Disruption method	Homogenization method
Sections of PFPE tissue	Sectioning; no additional disruption required	PAXgene Shredder spin column
Tissue taken directly from PAXgene Tissue Container	TissueRuptor* TissueLyser II*	TissueRuptor* TissueLyser II*
Blocks of PFPE tissue	TissueRuptor* TissueLyser II*	TissueRuptor* TissueLyser II*

* The Tissue Ruptor and TissueLyser II simultaneously disrupt and homogenize individual samples. If the TissueRuptor, TissueLyser II, or other similar instrument is not available, contact QIAGEN Technical Services for an alternative method of disruption and homogenization.

Disruption and homogenization using the TissueRuptor

The TissueRuptor is a rotor–stator homogenizer that thoroughly disrupts and simultaneously homogenizes single tissue samples in the presence of lysis buffer in 15–90 seconds, depending on the toughness and size of the sample. The blade of the TissueRuptor disposable probe rotates at a very high speed, causing the sample to be disrupted and homogenized by a combination of turbulence and mechanical shearing. For guidelines on using the TissueRuptor, refer to the *TissueRuptor Handbook*. For other rotor–stator homogenizers, refer to suppliers' guidelines.

Disruption and homogenization using the TissueLyser II

In bead-milling, tissues can be disrupted by rapid agitation in the presence of beads and lysis buffer. Disruption and simultaneous homogenization occur by the shearing and crushing action of the beads as they collide with the cells.

The TissueLyser II disrupts and homogenizes up to 48 tissue samples simultaneously when used in combination with the TissueLyser Adapter Set 2 x 24, which holds 48 x 2 ml microcentrifuge tubes containing stainless steel beads of 5 mm mean diameter. The TissueLyser II can also disrupt and homogenize up to 192 tissue samples simultaneously when used in combination with the TissueLyser Adapter Set 2 x 96, which holds 192 x 1.2 ml microtubes containing stainless steel beads of 5 mm mean diameter. For guidelines on using the TissueLyser II, refer to the *TissueLyser Handbook*. . If using other bead mills for sample disruption and homogenization, refer to suppliers' guidelines.

Note: Tungsten carbide beads react with Buffer TM1 and must not be used to disrupt and homogenize tissues.

Homogenization using PAXgene Shredder spin columns

PAXgene Shredder spin columns provide a fast and efficient way to homogenize cell and tissue lysates without cross-contamination of samples. Up to 700 µl lysate is loaded onto a PAXgene Shredder spin column placed in a 2 ml collection tube, and centrifuged for 2 minutes at maximum speed in a microcentrifuge. The lysate is homogenized as it passes through the spin column.

Protocol: Purification of Total RNA, Including miRNA, from Sections of PFPE Tissue

Starting material

Starting material for purification of total RNA, including miRNA, is a minimum of 2 and a maximum of 5 sections of PFPE tissue. Before starting, the tissue sample must be fixed and stabilized in PAXgene Tissue Containers, dehydrated, and embedded in paraffin (see the *PAXgene Tissue Container Product Circular* for information about tissue fixation, stabilization, processing, and paraffin embedding). Each section should have a thickness of 5–10 μm and a tissue surface area of up to 100 mm^2. Thicker sections may result in lower RNA yields.

Important points before starting

- **Do not overload the PAXgene RNA MinElute spin column, as this will significantly reduce RNA yield and quality.**
- If working with RNA for the first time, read "Appendix A: General Remarks on Handling RNA", page 38.
- 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.
- Spilling 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

- Tissue specimens must be fixed, stabilized, processed, and embedded in paraffin according to the *PAXgene Tissue Container Product Circular*.
- Before using the kit for the first time, prepare 80% ethanol by mixing 24 ml ethanol (96–100%, purity grade p.a.) and 6 ml RNase-free water (supplied).
- A shaker–incubator is required in steps 9 and 25. Set the temperature of the shaker–incubator to 45°C.

- Buffer TM1 and Buffer TM2 may form a precipitate upon storage. If necessary, warm to 37°C to dissolve.
- β-Mercaptoethanol (β-ME) must be added to Buffer TM1 before use. Add 10 μl β-ME per 1 ml Buffer TM1. Dispense in a fume hood and wear appropriate protective clothing. Buffer TM1 containing β-ME can be stored at room temperature (15–25°C) for up to 1 month.
- Buffer TM2 and Buffer TM3 are supplied as concentrates. Before using for the first time, add 3 volumes of isopropanol to Buffer TM2 and 4 volumes of ethanol (96–100%, purity grade p.a.) to Buffer TM3 as indicated on the bottles to obtain working solutions.
- If using the RNase-Free DNase Set for the first time, prepare a DNase I stock solution. Dissolve the solid DNase I (1500 Kunitz units)* in 550 μl 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 indicate 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 –15°C to –30°C for up to 6 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing. Ongoing studies may cause us to modify these times. Contact QIAGEN Technical Services for current details.
- When reconstituting and aliquoting DNase I, ensure that you follow the guidelines for handling RNA (see "Appendix A: General Remarks on Handling RNA", page 38).

Procedure

1. **Using a microtome, generate a minimum of 2 and a maximum of 5 tissue sections of 5–10 μm thickness from the PFPE tissue.**

   Note: If the sample surface has been exposed to air, discard the first 2 or 3 sections.
2. **Place sections in a 1.5 ml microcentrifuge tube.**
3. **Add 650 μl xylene to the sample. Vortex vigorously for 20 s, and incubate for 3 min at room temperature (15–25°C).**

* Kunitz units are the 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).

4. **Add 650 µl ethanol (96–100%, purity grade p.a.), and mix by vortexing for 20 s.**
5. **Centrifuge at maximum speed for 5 min (but do not exceed 20,000 x *g*).**

   To prevent damage to processing tubes, do not exceed 20,000 x *g*.
6. **Remove the supernatant by pipetting. Do not remove any of the pellet. Proceed immediately to step 7.**

   Note: In some cases the pellet may be loose. Remove the supernatant carefully.

   Note: The pellet might contain residual paraffin; however, the paraffin will dissolve during digestion with proteinase K and will not affect the PAXgene Tissue miRNA procedure.
7. **Add 150 µl Buffer TM1, and resuspend the pellet by vortexing for 20 s.**
8. **Add 290 µl RNase-free water to the resuspended pellet. Then add 10 µl proteinase K and mix by vortexing for 5 s.**

   Note: Do not mix Buffer TM1 and proteinase K together before adding them to the sample.
9. **Incubate for 15 min at 45°C using a shaker–incubator at 1400 rpm. After incubation, centrifuge briefly (1–2 s at 500–1000 x *g*) to remove drops from the inside of the tube lid. Set the temperature of the shaker–incubator to 65°C for use in step 25.**
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 (do not exceed 20,000 x *g*).**
11. **Carefully transfer the entire supernatant of the flow-through fraction to a new 1.5 ml microcentrifuge tube without disturbing the pellet in the processing tube.**
12. **Add 675 µl isopropanol. Mix by vortexing for 5 s, 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.

    Note: A precipitate may form after the addition of isopropanol, but this will not affect the PAXgene Tissue miRNA procedure.

13. **Pipet up to 700 µl sample, including any precipitate that may have formed, into a PAXgene RNA MinElute spin column (red) placed in a 2 ml processing tube. Centrifuge for 1 min at 8000 x *g*. Discard the flow-through.***

    Reuse the collection tube in step 14.

14. **Repeat step 13 using the remainder of the sample. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.***

    If the lysate has not completely passed through the membrane after centrifugation, centrifuge again at a higher speed until the PAXgene RNA MinElute spin column is empty.

15. **Pipet 350 µl Buffer TM2 into the PAXgene RNA MinElute spin column. Centrifuge for 20 s at 8000 x *g*. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.***

    Note: Buffer TM2 is supplied as a concentrate. Ensure that isopropanol is added to Buffer TM2 before use (see "Things to do before starting", page 15).

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.**

    For example, if processing 10 samples, add 100 µl DNase I stock solution to 700 µl DNA 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) directly onto the PAXgene RNA MinElute spin column, and incubate for 15 min at room temperature.**

    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.

18. **Centrifuge for 20 s at 8000 x *g*. Place the spin column in a new 2 ml processing tube. Keep the flow-through for rebinding.**

    Note: Keep the flow-through for rebinding in step 19.

19. **Add 350 µl Buffer TM2 to the flow-through from step 18. Mix carefully by pipetting up and down5 times.**

* Flow-through contains Buffer TM1 or Buffer TM2 and is therefore not compatible with bleach. See page 6 for safety information.

20. **Pipet the mixture from step 19 into the PAXgene RNA MinElute spin column, and centrifuge for 20 s at 8000 x *g*. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.***

21. **Pipet 500 µl Buffer TM3 into the PAXgene RNA MinElute spin column, and centrifuge for 20 s at 8000 x *g*. Discard the flow-through and reuse the 2 ml processing tube in step 22.**

    Note: Buffer TM3 is supplied as a concentrate. Ensure that ethanol is added to Buffer TM3 before use (see "Things to do before starting", page 15).

22. **Pipet 500 µl 80% ethanol into the PAXgene RNA MinElute spin column, and centrifuge for 2 min at 8000 x *g*.**

23. **Discard the processing tube containing the flow-through, and place the PAXgene RNA MinElute spin column in a new 2 ml processing tube. Open the cap of the spin column, and centrifuge for 5 min at maximum speed.**

24. **Discard the processing tube containing the flow-through. Place the PAXgene RNA MinElute spin column in a 1.5 ml microcentrifuge tube, and pipet 14–40 µl Buffer TM4 directly onto the PAXgene RNA MinElute spin column membrane. Centrifuge for 1 min at maximum speed to elute the RNA.**

    Note: It is important to wet the entire membrane with Buffer TM4 to achieve maximum elution efficiency.

    Smaller volumes of Buffer TM4 can be used to obtain a higher total RNA concentration, but this will influence the overall yield.

    Approximately 2 µl remain in the PAXgene RNA MinElute spin column; elution with 14 µl Buffer TM4 results in an eluate with a volume of 12 µl.

25. **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: Incubation at 65°C denatures the RNA for downstream applications. Do not exceed the incubation time or temperature.

26. **If the RNA samples will not be used immediately, store at –20°C or at –70°C. Since the RNA remains denatured after repeated freezing and thawing, it is not necessary to repeat the incubation at 65°C.**

    Note: For quantification in Tris buffer, use the relationship $A_{260} = 1 \Rightarrow 44$ µg/ml; see "Appendix A: General Remarks on Handling RNA", page 38.

* Flow-through contains Buffer TM1 or Buffer TM2 and is therefore not compatible with bleach. See page 6 for safety information.

Protocol: Purification of Total RNA, Including miRNA, from PAXgene Tissue Fixed Samples

Starting material

Starting material for RNA purification is up to 10 mg of a tissue sample fixed and stabilized in PAXgene Tissue Containers.

It is essential to use the correct amount of starting material to obtain optimal RNA yield and purity. A maximum amount of 10 mg tissue fixed and stabilized using the PAXgene Tissue Container can generally be processed. For most tissues, the RNA binding capacity of the PAXgene RNA MinElute spin column and the lysing capacity of Buffer TM1 will not be exceeded by these amounts.

Weighing tissue is the most accurate way to quantify the amount of starting material. As a guide, a 2 mm cube (8 mm^3) of most tissues weighs 8–12 mg.

Important points before starting

- **Do not overload the PAXgene RNA MinElute spin column, as this will significantly reduce RNA yield and quality.**
- If working with RNA for the first time, read "Appendix A: General Remarks on Handling RNA", page 38.
- 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.
- Spilling 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

- Tissue specimen must be fixed and stabilized in PAXgene Tissue Container according to the instructions in the *PAXgene Tissue Container Product Circular*.
- Before using the kit for the first time, prepare 80% ethanol by mixing 24 ml ethanol (96–100%, purity grade p.a.) and 6 ml RNase-free water (supplied).

- A shaker–incubator is required in steps 5 and 21. Set the temperature of the shaker–incubator to 45°C.
- Buffer TM1 and Buffer TM2 may form a precipitate upon storage. If necessary, warm to 37°C to dissolve.
- β-Mercaptoethanol (β-ME) must be added to Buffer TM1 before use. Add 10 µl β-ME per 1 ml Buffer TM1. Dispense in a fume hood and wear appropriate protective clothing. Buffer TM1 containing β-ME can be stored at room temperature (15–25°C) for up to 1 month.
- Buffer TM2 and Buffer TM3 are supplied as concentrates. Before using for the first time, add 3 volumes of isopropanol to Buffer TM2 and 4 volumes of ethanol (96–100%, purity grade p.a.) to Buffer TM3 as indicated on the bottles to obtain working solutions.
- If using the RNase-Free DNase Set for the first time, prepare a DNase I stock solution. Dissolve the solid DNase I (1500 Kunitz units)* in 550 µl 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 indicate 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 –15°C to –30°C for up to 6 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing. Ongoing studies may cause us to modify these times. Contact QIAGEN Technical Services for current details.
- When reconstituting and aliquoting DNase I, ensure that you follow the guidelines for handling RNA (see "Appendix A: General Remarks on Handling RNA", page 38).

* Kunitz units are the 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. **If using the TissueLyser II, add one stainless steel bead (5 mm mean diameter) to each 2 ml round-bottomed microcentrifuge tube (not provided).**
2. **Unscrew the lid of the PAXgene Tissue Container, and remove tissue cassette from lid/rack assembly. Retrieve the tissue sample from the tissue cassette using forceps and weigh the sample. Do not use more than 10 mg. Proceed immediately to step 3.**

   Weighing tissue is the most accurate way to determine the amount.

   Note: Tissue outside of the PAXgene Tissue Stabilizer is no longer protected; therefore all handling steps prior to adding Buffer TM1 should be carried out on ice and as quickly as possible.
3. **Disrupt the tissue and homogenize the lysate using either the TissueRuptor (follow step 3a) or TissueLyser II (follow step 3b).**

   See "Disrupting and homogenizing starting materials", page 13, for more details on disruption and homogenization.

   Note: Ensure that β-ME is added to Buffer TM1 before use (see "Things to do before starting", page 20).

   Note: Incomplete homogenization leads to significantly reduced RNA yields and can cause clogging of the PAXgene RNA MinElute spin column. Homogenization with the TissueRuptor or TissueLyser II generally results in higher RNA yields than with other methods.

3a. Disruption and homogenization using the TissueRuptor

- **Place the tissue in a suitably sized processing tube. Add 250 µl Buffer TM1.**

  Generally, round-bottomed tubes allow more efficient disruption and homogenization than conical-bottomed tubes.

- **Place the tip of the disposable probe into the processing tube and operate the TissueRuptor at full speed until the lysate is uniformly homogeneous (usually 20–40 s). Proceed to step 4.**

  Note: To avoid damage to the TissueRuptor and disposable probe during operation, make sure the tip of the probe remains submerged in the buffer.

3b. Disruption and homogenization using the TissueLyser II

- **Place the tissues in the tubes prepared in step 1.**
- **Immediately add 250 µl Buffer TM1 per tube.**
- **Place the tubes in the TissueLyser Adapter Set 2 x 24.**

- **Operate the TissueLyser II for 2 min at 20 Hz.**

  The time depends on the tissue being processed and can be extended until the tissue is completely homogenized.

- **Rearrange the tubes so that the outermost tubes are innermost and the innermost tubes are outermost. Operate the TissueLyser II for another 2 min at 20 Hz.**

  Rearranging the tubes allows even homogenization.

- **Proceed to step 4.**

  Do not reuse the stainless steel beads.

4. **Add 480 µl RNase-free water to the resuspension. Then add 20 µl proteinase K and mix by vortexing for 5 s.**

   Note: Do not mix Buffer TM1 and proteinase K together before adding them to the sample.

5. **Incubate for 15 min at 45°C using a shaker–incubator at 1400 rpm. After incubation, set the temperature of the shaker–incubator to 65°C (for step 19).**
6. **Centrifuge the tissue lysate for 3 min at maximum speed (but do not exceed 20,000 x *g*). Carefully transfer the supernatant fraction to a new 2 ml microcentrifuge tube (not provided) without disturbing the pellet in the processing tube.**
7. **Add 1100 µl isopropanol. Mix by vortexing for 5 s, 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.

   Note: A precipitate may form after the addition of isopropanol, but this will not affect the PAXgene Tissue miRNA procedure.

8. **Pipet up to 700 µl sample, including any precipitate that may have formed, into a PAXgene RNA MinElute spin column (red) placed in a 2 ml processing tube. Centrifuge for 1 min at 8000 x *g*. Discard the flow-through.***

   Reuse the collection tube in step 9.

* Flow-through contains Buffer TM1 or Buffer TM2 and is therefore not compatible with bleach. See page 6 for safety information.

9. **Repeat step 8 until the entire sample has passed through the PAXgene RNA MinElute spin column. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.***

   If the lysate has not completely passed through the membrane after centrifugation, centrifuge again at a higher speed until the PAXgene RNA MinElute spin column is empty.

10. **Pipet 350 µl Buffer TM2 into the PAXgene RNA MinElute spin column. Centrifuge for 20 s at 8000 x *g*. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.***

11. **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.**

    For example, if processing 10 samples, add 100 µl DNase I stock solution to 700 µl DNA 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.

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

    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.

13. **Centrifuge for 20 s at 8000 x *g*. Place the spin column in a new 2 ml processing tube. Keep the flow-through for rebinding.**

    Note: Keep the flow-through for rebinding in step 14.

14. **Add 350 µl Buffer TM2 to the flow-through from step 13. Mix carefully by pipetting 5 times up and down.**

15. **Pipet the mixture from step 14 into the PAXgene RNA MinElute spin column, and centrifuge for 20 s at 8000 x *g*. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.***

* Flow-through contains Buffer TM1 or Buffer TM2 and is therefore not compatible with bleach. See page 6 for safety information.

16. **Pipet 500 µl Buffer TM3 into the PAXgene RNA MinElute spin column, and centrifuge for 20 s at 8000 x *g*. Discard the flow-through and reuse the 2 ml processing tube in step 17.**

    Note: Buffer TM3 is supplied as a concentrate. Ensure that ethanol is added to Buffer TM3 before use (see "Things to do before starting", page 20).

17. **Pipet 500 µl 80% ethanol into the PAXgene RNA MinElute spin column, and centrifuge for 2 min at 8000 x *g*.**

    Prepare 80% ethanol with ethanol (96–100%, purity grade p.a.) and the RNase-free water supplied with the kit.

18. **Discard the processing tube containing the flow-through, and place the PAXgene RNA MinElute spin column in a new 2 ml processing tube. Open the cap of the spin column, and centrifuge for 5 min at maximum speed (do not exceed 20,000 x *g*).**

19. **Discard the processing tube containing the flow-through. Place the PAXgene RNA MinElute spin column in a 1.5 ml microcentrifuge tube, and pipet 14–40 µl Buffer TM4 directly onto the PAXgene RNA MinElute spin column membrane. Centrifuge for 1 min at maximum speed to elute the RNA.**

    Note: It is important to wet the entire membrane with Buffer TM4 to achieve maximum elution efficiency.

    Smaller volumes of Buffer TM4 can be used to obtain a higher total RNA concentration, but this will influence the overall yield.

    Approximately 2 µl remain the PAXgene RNA MinElute spin column; elution with 14 µl Buffer TM4 results in an eluate with a volume of 12 µl.

20. **Recommended: Repeat elution step (step 19) as described, using 14–40 µl Buffer TM4.**

    Omitting this step may result in reduced yields.

21. **Incubate the eluate for 5 min at 65°C in the shaker–incubator without shaking. After incubation, chill immediately on ice.**

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

22. **If the RNA samples will not be used immediately, store at –20°C or –70°C. Since the RNA remains denatured after repeated freezing and thawing, it is not necessary to repeat the incubation at 65°C.**

    Note: For quantification in Tris buffer, use the relationship $A_{260} = 1 \Rightarrow 44$ µg/ml; see "Appendix A: General Remarks on Handling RNA", page 38.

Protocol: Purification of Total RNA, Including miRNA, from Blocks of PFPE Tissue

Starting material

Starting material for RNA purification is up to 10 mg of a block of tissue fixed and stabilized in PAXgene Tissue Containers, dehydrated, and embedded in paraffin (see the *PAXgene Tissue Container Product Circular* for information about tissue fixation, stabilization, and paraffin embedding).

It is essential to use the correct amount of starting material to obtain optimal RNA yield and purity. A maximum amount of 10 mg tissue fixed and stabilized using the PAXgene Tissue Container and embedded in paraffin can generally be processed. For most tissues, the RNA binding capacity of the PAXgene RNA MinElute spin column and the lysing capacity of Buffer TM1 will not be exceeded by these amounts.

Weighing tissue is the most accurate way to quantify the amount of starting material. As a guide, a 2 mm cube (8 mm^3) of most paraffin-embedded tissues weighs 8–12 mg.

Important points before starting

- **Do not overload the PAXgene RNA MinElute spin column, as this will significantly reduce RNA yield and quality.**
- If working with RNA for the first time, read "Appendix A: General Remarks on Handling RNA", page 38.
- 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.
- Spilling 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

- Tissue specimens must be fixed, stabilized, processed, and embedded in paraffin according to the *PAXgene Tissue Container Product Circular*.
- Before using the kit for the first time, prepare 80% ethanol by mixing 24 ml ethanol (96–100%, purity grade p.a.) and 6 ml RNase-free water (supplied).
- A shaker–incubator is required in steps 11 and 27. Set the temperature of the shaker–incubator to 45°C.
- Buffer TM1 and Buffer TM2 may form a precipitate upon storage. If necessary, warm to 37°C to dissolve.
- β-Mercaptoethanol (β-ME) must be added to Buffer TM1 before use. Add 10 µl β-ME per 1 ml Buffer TM1. Dispense in a fume hood and wear appropriate protective clothing. Buffer TM1 containing β-ME can be stored at room temperature (15–25°C) for up to 1 month.
- Buffer TM2 and Buffer TM3 are supplied as concentrates. Before using for the first time, add 3 volumes of isopropanol to Buffer TM2 and 4 volumes of ethanol (96–100%, purity grade p.a.) to Buffer TM3 as indicated on the bottles to obtain working solutions.
- If using the RNase-Free DNase Set for the first time, prepare a DNase I stock solution. Dissolve the solid DNase I (1500 Kunitz units)* in 550 µl 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 indicate 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 –15°C to –30°C for up to 6 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing. Ongoing studies may cause us to modify these times. Contact QIAGEN Technical Services for current details.
- When reconstituting and aliquoting DNase I, ensure that you follow the guidelines for handling RNA (see "Appendix A: General Remarks on Handling RNA", page 38).

* Kunitz units are the 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. **Cut out a tissue sample from a block of PFPE tissue using a scalpel and weigh the sample. Do not use more than 10 mg.**

   Weighing tissue is the most accurate way to determine the amount.

2. **Cut the block into smaller pieces and place them into a 2 ml round-bottomed microcentrifuge tube (not provided).**
3. **Add 1 ml xylene to the sample. Vortex vigorously for 20 s, and incubate for 3 min on the benchtop at room temperature (15–25°C).**
4. **Centrifuge at maximum speed for 3 min (but do not exceed 20,000 x *g*).**

   To prevent damage to processing tubes, do not exceed 20,000 x *g*.

5. **Remove the supernatant by pipetting. Do not remove any of the pellet.**
6. **Add 1 ml ethanol (96–100%, purity grade p.a.) to the pellet, and mix by vortexing for 20 s.**
7. **Centrifuge at maximum speed for 3 min (but do not exceed 20,000 x *g*).**
8. **Remove the supernatant by pipetting. Do not remove any of the pellet. Continue immediately with step 9.**
9. **Disrupt the tissue and homogenize the lysate using either the TissueRuptor (follow step 9a) or TissueLyser II (follow step 9b).**

   See “Disrupting and homogenizing starting materials”, page 13, for more details on disruption and homogenization.

   Note: Ensure that β-ME is added to Buffer TM1 before use (see “Things to do before starting”, page 27).

   Note: Incomplete homogenization leads to significantly reduced RNA yields and can cause clogging of the PAXgene RNA MinElute spin column. Homogenization with the TissueRuptor or TissueLyser II generally results in higher RNA yields than with other methods.

9a. Disruption and homogenization using the TissueRuptor

- **Add 250 µl Buffer TM1 to the pellet.**
- **Place the tip of the disposable probe into the 2 ml microcentrifuge tube and operate the TissueRuptor at full speed until the lysate is uniformly homogeneous (usually 20–40 s). Proceed to step 10.**

  Note: To avoid damage to the TissueRuptor and disposable probe during operation, make sure the tip of the probe remains submerged in the buffer.

9b. Disruption and homogenization using the TissueLyser II:

- **Add one stainless steel bead (5 mm mean diameter) and 250 µl Buffer TM1 to the pellet.**
- **Place the tubes in the TissueLyser Adapter Set 2 x 24.**
- **Operate the TissueLyser II for 2 min at 20 Hz.**

  The time depends on the tissue being processed and can be extended until the tissue is completely homogenized.
- **Rearrange the tubes so that the outermost tubes are innermost and the innermost tubes are outermost. Operate the TissueLyser II for another 2 min at 20 Hz.**

  Rearranging the tubes allows even homogenization.
- **Carefully pipet the lysates into new 1.5 ml microcentrifuge tubes (not provided). Proceed to step 10.**

  Do not reuse the stainless steel beads.

10. Add 480 µl RNase-free water to the resuspension. Then add 20 µl proteinase K and mix by vortexing for 5 s.

Note: Do not mix Buffer TM1 and proteinase K together before adding them to the sample.

11. Incubate for 15 min at 45°C using a shaker–incubator at 1400 rpm. After incubation, set the temperature of the shaker–incubator to 65°C (for step 28).

12. Centrifuge the tissue lysate for 3 min at maximum speed (but do not exceed 20,000 x *g*). Carefully transfer the supernatant fraction to a new 2 ml microcentrifuge tube (not provided) without disturbing the pellet.

13. Add 1100 µl isopropanol. Mix by inverting the tube several times, 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.

Note: A precipitate may form after addition of isopropanol, but this will not affect the PAXgene Tissue miRNA procedure.

14. **Pipet up to 700 µl sample, including any precipitate that may have formed, into a PAXgene RNA MinElute spin column (red) placed in a 2 ml processing tube. Centrifuge for 1 min at 8000 x *g*. Discard the flow-through.***

    Reuse the collection tube in step 15.

15. **Repeat step 14 until the entire sample has passed through the PAXgene RNA MinElute spin column. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.***

    If the lysate has not completely passed through the membrane after centrifugation, centrifuge again at a higher speed until the PAXgene RNA MinElute spin column is empty.

16. **Pipet 350 µl Buffer TM2 into the PAXgene RNA MinElute spin column. Centrifuge for 20 s at 8000 x *g*. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.***

17. **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.**

    For example, if processing 10 samples, add 100 µl DNase I stock solution to 700 µl DNA 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.

18. **Pipet the DNase I incubation mix (80 µl) directly onto the PAXgene RNA MinElute spin column, and incubate for 15 min at room temperature.**

    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.

19. **Centrifuge for 20 s at 8000 x *g*. Place the spin column in a new 2 ml processing tube. Keep the flow-through for rebinding.**

    Note: Keep the flow-through for rebinding in step 20.

20. **Add 350 µl Buffer TM2 to the flow-through from step 19. Mix carefully by pipetting 5 times up and down.**

* Flow-through contains Buffer TM1 or Buffer TM2 and is therefore not compatible with bleach. See page 6 for safety information.

21. **Pipet the mixture from step 20 into the PAXgene RNA MinElute spin column, and centrifuge for 20 s at 8000 x *g*. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.***

22. **Pipet 500 µl Buffer TM3 into the PAXgene RNA MinElute spin column, and centrifuge for 20 s at 8000 x *g*. Discard the flow-through and reuse the 2 ml processing tube in step 23.**

    Note: Buffer TM3 is supplied as a concentrate. Ensure that ethanol is added to Buffer TM3 before use (see "Things to do before starting", page 27).

23. **Pipet 500 µl 80% ethanol into the PAXgene RNA MinElute spin column, and centrifuge for 2 min at 8000 x *g*.**

    Prepare 80% ethanol with ethanol (96–100%, purity grade p.a.) and the RNase-free water supplied with the kit.

24. **Discard the processing tube containing the flow-through, and place the PAXgene RNA MinElute spin column in a new 2 ml processing tube. Open the cap of the spin column, and centrifuge for 5 min at maximum speed (but do not exceed 20,000 x *g*).**

25. **Discard the processing tube containing the flow-through. Place the PAXgene RNA MinElute spin column in a 1.5 ml microcentrifuge tube, and pipet 14–40 µl Buffer TM4 directly onto the PAXgene RNA MinElute spin column membrane. Centrifuge for 1 min at maximum speed to elute the RNA.**

    Note: It is important to wet the entire membrane with Buffer TM4 to achieve maximum elution efficiency.

    Smaller volumes of Buffer TM4 can be used to obtain a higher total RNA concentration, but this will influence the overall yield.

    Approximately 2 µl remain the PAXgene RNA MinElute spin column; elution with 14 µl Buffer TM4 results in an eluate with a volume of 12 µl.

26. **Recommended: Repeat elution step (step 25) as described, using another 14–40 µl Buffer TM4.**

    Omitting this step may result in reduced yields.

27. **Incubate the eluate for 5 min at 65°C in the shaker–incubator (from step 11) without shaking. After incubation, chill immediately on ice.**

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

* Flow-through contains Buffer TM1 or Buffer TM2 and is therefore not compatible with bleach. See page 6 for safety information.

28. **If the RNA samples will not be used immediately, store at –20°C or –70°C. Since the RNA remains denatured after repeated freezing and thawing, it is not necessary to repeat the incubation at 65°C.**

    Note: For quantification in Tris buffer, use the relationship $A_{260} = 1 \Rightarrow 44\ \mu g/ml$; see "Appendix A: General Remarks on Handling RNA", page 38.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: 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 last page or visit www.qiagen.com).

	Comments and suggestions
RNA degraded	
a) Tissue not immediately stabilized	Tissue specimen must be fixed after resection as soon as possible, ideally within 30 min after resection.
b) Tissue specimen too large	For efficient and even fixation, the tissue specimen must not be too large. The sample should be cut to a maximum size of 4 x 10 x 10 mm for fixation in PAXgene Tissue Fix. See "Protocol: Sample Fixation and Stabilization" in the *PAXgene Tissue Container Product Circular*.
c) Fixation time exceeded	Do not fix for longer then 18 h in PAXgene Tissue Fix before transferring into PAXgene Tissue Stabilizer. Longer fixation periods may lead to RNA degradation. Fixation for 30 min per millimeter thickness of the sample is sufficient for most tissue types. See "Protocol: Sample Fixation and Stabilization" in the *PAXgene Tissue Container Product Circular*.

	Comments and suggestions
d) Sample inappropriately handled during processing	Incubating blocks of PFPE tissue specimens in water, formalin, or alcoholic solutions with less than 80% ethanol or in paraffin with a temperature above 60°C leads to degradation of RNA. Start processing with 80–100% ethanol first. Incubate specimen in liquid paraffin at 56°C for up to 3 h. Follow the protocol for "Sample Processing, Paraffin Embedding, and Sectioning" and see examples for processing protocols in the appendix "Examples of Processing Protocols Successfully Used for Specimens Treated with the PAXgene Tissue System" in the *PAXgene Tissue Container Product Circular*.
e) Paraffin-embedded tissue blocks stored inappropriately	RNA and other biomolecules in paraffin-embedded tissue undergo slow chemical degradation. Therefore store and archive blocks of PFPE tissue in a dry, dark place at 2–8°C or colder. The ideal storage temperature for preservation of nucleic acid is –20°C.
f) RNase contamination	Although all PAXgene buffers have been tested and are guaranteed RNase-free, RNases can be introduced during use. Be certain not to introduce any RNases during the PAXgene Tissue procedure or later handling. See Appendix A (page 38) for general remarks on handling RNA. Do not put RNA samples into a vacuum dryer or microcentrifuge that has been used in DNA preparations where RNases may have been used.
g) 80% ethanol not made with RNase-free water	The 80% ethanol used to wash the PAXgene RNA MinElute spin column membrane must be free of RNases. Be sure to prepare the 80% ethanol using ethanol (96–100%, purity grade p.a.) and the RNase-free water supplied with the kit, as described in "Things to do before starting" in each protocol.

	Comments and suggestions
Low RNA yield	
a) Reagents used for processing contaminated with formalin	When processing specimens fixed in PAXgene Tissue Containers, do not use reagents contaminated with formalin. Even trace amounts of formalin in the alcohol or other reagents used for sample processing can lead to a significant reduction in RNA yield.
b) Too much starting material	Reduce the amount of starting material used. Do not use more than the amount specified in "Starting material" at the beginning of each protocol.
c) RNA still bound to spin column membrane	Repeat RNA elution, but incubate the PAXgene RNA MinElute spin column on the benchtop for 10 min with Buffer TM4 before centrifuging.
d) Ethanol carryover	After the wash with 80% ethanol, be sure to centrifuge at full speed for 5 min to dry the PAXgene RNA MinElute spin column membrane. After centrifugation, carefully remove the PAXgene RNA MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.
e) RNA concentration measured in water	RNA concentration must be measured in 10 mM Tris·Cl*, pH 7.5, for accurate quantification (see Appendix B, page 39).
f) Centrifugation for more than 1–2 s after adding isopropanol to the lysate (step 12, page 17; step 7, page 23; step 13, page 29)	After adding ethanol to the lysate, the samples should only be centrifuged briefly: no more than 1–2 s. Longer centrifugation may result in pelleting of RNA and reduced yields.

* 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.

	Comments and suggestions
g) Elution Buffer TM4 incorrectly dispensed	Pipet RNase-free water into the center of the PAXgene RNA MinElute spin column membrane to ensure that the membrane is completely covered.
h) Insufficient disruption and homogenization	See "Disrupting and homogenizing starting materials", page 13, for details on disruption and homogenization methods. In subsequent preparations, reduce the amount of starting material (see the individual protocols).

PAXgene RNA MinElute spin column clogged

a) Too much starting material and/or insufficient lysis	Reduce the amount of starting material used (see "Starting material" at the beginning of each protocol). Increase *g*-force and/or duration of centrifugation steps.
b) Centrifugation temperature too low	The centrifugation temperature should be 20–25°C.

Low A_{260}/A_{280} ratio

a) RNA diluted in water before purity is measured	Use 10 mM Tris·Cl*, pH 7.5, to dilute RNA before measuring purity (see Appendix B, page 39).
b) Spectrophotometer not properly zeroed	To zero the spectrophotometer, use a blank containing the same proportion of elution buffer and dilution buffers as in the samples to be measured. Components in buffers may affect the A_{260}/A_{280} ratio.

* 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.

	Comments and suggestions
Low miRNA yield or miRNA does not perform well in downstream applications	
a) Salt carryover during elution	Ensure that Buffer TM3 has been used at room temperature (15–25°C).
b) Ethanol carryover	After the wash with 80% ethanol, be sure to centrifuge at full speed for 5 min to dry the PAXgene RNA MinElute spin column membrane. After centrifugation, carefully remove the PAXgene RNA MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

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 nondisposable 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 these 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.

Protocols for removing RNase-contamination from glassware and solutions can be found in general molecular biology guides, such as Sambrook, J. and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

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: Quantification and Determination of Quality of Total RNA

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 ($A_{260} = 1 \Rightarrow 44$ µg/ml). This relation is valid only for measurements in 10 mM Tris·Cl,* pH 7.5. Therefore, if it is necessary to dilute the RNA sample, this should be done in 10 mM Tris·Cl. As discussed below (see "Purity of RNA", page 40), the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity.

When measuring RNA samples, ensure that cuvettes are RNase-free. Use the buffer in which the RNA is diluted to zero the spectrophotometer, and make sure to add the same volume of Buffer TM4 as the volume of eluted RNA to be diluted.

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

Volume of RNA sample = 120 µl
Dilution = 10 µl RNA sample + 140 µl 10 mM Tris·Cl, pH 7.5
(1/15 dilution)
Measure absorbance of diluted sample in a cuvette (RNase-free).
$A_{260} = 0.2$

Concentration of RNA sample	= 44 x A_{260} x dilution factor
	= 44 x 0.2 x 15
	= 132 µg/ml
Total yield	= concentration x volume of sample in milliliters
	= 132 µg/ml x 0.12 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 UV light, such as protein. However, the A_{260}/A_{280} ratio is influenced considerably by pH. Lower pH results in a lower A_{260}/A_{280} ratio 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} ratio 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 TM4 as the volume of eluted RNA to be diluted. Buffer TM4 has high absorbance at 220 nm, which can lead to high background absorbance levels if the spectrophotometer is not properly zeroed.

References

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* 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.

† 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.

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Related products		
PAXgene Tissue Containers — for collection, fixation, and nucleic acid stabilization of human tissues		
PAXgene Tissue Containers (10)	For collection, fixation, and stabilization of 10 samples: 10 Prefilled Reagent Containers, containing PAXgene Tissue Fix and PAXgene Tissue Stabilizer	765112
PAXgene Tissue RNA Kit — for purification of total RNA from tissues fixed and stabilized in PAXgene Tissue Containers		
PAXgene Tissue RNA Kit (50)	For 50 RNA preps: PAXgene RNA MinElute Spin Columns, PAXgene Shredder Spin Columns, Processing Tubes, Microcentrifuge Tubes, Carrier RNA, RNase-Free DNase, and RNase-Free Buffers; to be used in conjunction with PAXgene Tissue Containers	765134
PAXgene Tissue DNA Kit — for purification of DNA from tissues fixed and stabilized in PAXgene Tissue Containers		
PAXgene Tissue DNA Kit (50)	For 50 DNA preps: PAXgene DNA Mini Spin Columns, Processing Tubes, Microcentrifuge Tubes, Carrier RNA, and Buffers; to be used in conjunction with PAXgene Tissue Containers	767134

Product	Contents	Cat. no.
TissueRuptor System — for low-throughput sample disruption using disposable probes		
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TissueRuptor Disposable Probes (25)	25 nonsterile plastic disposable probes for use with the TissueRuptor	990890
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TissueLyser II	Bead mill, 100–120/220–240 V, 50/60 Hz; requires the TissueLyser Adapter Set 2 x 24 or TissueLyser Adapter Set 2 x 96 (available separately)	85300
TissueLyser Adapter Set 2 x 24	2 sets of Adapter Plates and 2 racks for use with 2 ml microcentrifuge tubes on the TissueLyser	69982
TissueLyser Adapter Set 2 x 96	2 sets of Adapter Plates for use with Collection Microtubes (racked) on the TissueLyser II	69984
Stainless Steel Beads, 5 mm (200)	Stainless Steel Beads (5 mm diameter), suitable for use with the TissueLyser system	69989
TissueLyser Single-Bead Dispenser, 5 mm	For dispensing individual beads (5 mm diameter)	69965

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* 120 V, 60 Hz (for North America and Japan).

† 235 V, 50/60 Hz (for Europe, excluding UK and Ireland).

‡ 235 V, 50/60 Hz (for UK and Ireland).

§ 235 V, 50/60 Hz (for Australia).

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