PAXgene®

Tissue RNA Kit Handbook

For isolation and purification of total RNA from tissue samples fixed and stabilized using the PAXgene Tissue System

Important: To be used in conjunction with PAXgene Tissue Containers

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

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PreAnalytiX
A QIAGEN / BD Company

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PreAnalytiX Distributors

PreAnalytiX products are manufactured for PreAnalytiX by QIAGEN or BD and are distributed for PreAnalytiX by QIAGEN or BD. Products cannot be ordered at PreAnalytiX GmbH.

Please see the last page for contact information for your local PreAnalytiX distributor.

Contents

Kit Contents

PAXgene Tissue RNA Kit	**(50)**
Catalog no.	**765134**
Number of preps	**50**
Buffer TR1 (Binding Buffer)*	18 ml
Buffer TR2 (Wash Buffer 1)*	45 ml
Buffer TR3 (Wash Buffer 2 concentrate)†	11 ml
Buffer TR4 (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)	6 x 50
Microcentrifuge Tubes (1.5 ml)	2 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 Resupension buffer)	2 ml
Carrier RNA (red lid)§	310 µg
Handbook	1

* Contains a guanidine salt. See page 5 for safety information.

† Buffer TR3 is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%, purity grade p.a.) as indicated on the bottle to obtain a working solution.

‡ Kunitz units are the commonly used units for measuring DNase I; see page 14 for definition.

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

Shipping and Storage

The PAXgene Tissue RNA Kit is shipped at ambient temperature.

PAXgene RNA MinElute spin columns and the RNase-Free DNase Set, in the PAXgene Tissue RNA Kit, should be stored upon receipt at 2–8°C. All other components of the PAXgene Tissue RNA 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 (15–25°C). For longer storage or if ambient temperatures often exceed 25°C, we recommend storing proteinase K at 2–8°C.

Intended Use

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 RNA 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.

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 PreAnalytiX kit and kit component.

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

Buffer TR1 contains guanidine thiocyanate and Buffer TR2 contains a smaller amount of guanidine thiocyanate, which can form highly reactive compounds when combined with bleach. If liquid containing this buffer 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.

Quality Control

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

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 cannot 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 fixation reagent, a stabilization reagent, and kits for purification of RNA, DNA, or total RNA including miRNA.

The fixation reagent, PAXgene Tissue Fix, rapidly penetrates and fixes the tissue, with a fixation rate of approximately 1 mm in 30 minutes.* The reagent preserves morphology and biomolecules without destructive cross-linking and degradation found in formalin-fixed tissues. After fixation, for optimal preservation of biomolecules it is recommended to stop the fixation process by transferring the tissue specimen into the stabilization reagent, PAXgene Tissue Stabilizer. For optimal fixation and stabilization incubation times see the product circular of the PAXgene Tissue collection device used.

When the tissue is stored in PAXgene Tissue Stabilizer, nucleic acids and morphology of the tissue sample are stable for days at room temperature, weeks at 2–8°C, or years at –15 to –30°C to –80°C.* For current long-term archiving data see www.preanalytix.com.

PAXgene Tissue fixed and stabilized samples can be processed and embedded in paraffin. Sections of PAXgene Tissue fixed, paraffin-embedded (PFPE) tissue can be used for histological studies or extraction of nucleic acids or proteins. Purification of total RNA from PFPE tissue samples requires use of the PAXgene Tissue RNA Kit.

* Fixation rates and stabilization times depend on type and size of tissue. Specifications for fixation and storage conditions in PAXgene Tissue Fix and PAXgene Tissue Stabilizer were determined using animal tissues samples.

RNA purification

The PAXgene Tissue RNA Kit provides 3 protocols for purification of total RNA from tissues fixed in PAXgene Tissue Fix and stabilized in PAXgene Tissue Stabilizer (see "Description of protocols", page 7, and flowchart, page 8).

Disruption and homogenization of the tissue sample are performed in the binding buffer, Buffer TR1 (see "Disrupting and homogenizing starting materials", page 11). After a centrifugation step to remove residual cell debris, ethanol is added to the lysate to provide appropriate binding conditions for RNA. 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 is eluted in a low-salt elution buffer and denatured by heating.

Total RNA purified using the PAXgene Tissue 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. All RNA molecules longer than 200 nucleotides are purified. The procedure provides enrichment for mRNA since most RNAs <200 nucleotides (such as 5.8S rRNA, 5S rRNA, and tRNAs, which together comprise 15–20% of total RNA) are selectively excluded. The size distribution of the purified RNA is comparable to that obtained by centrifugation on a CsCl gradient, through which small RNAs do not sediment efficiently. For purification of small RNA, including microRNA (miRNA), we recommend using the PAXgene Tissue miRNA Kit (cat. no. 766134).

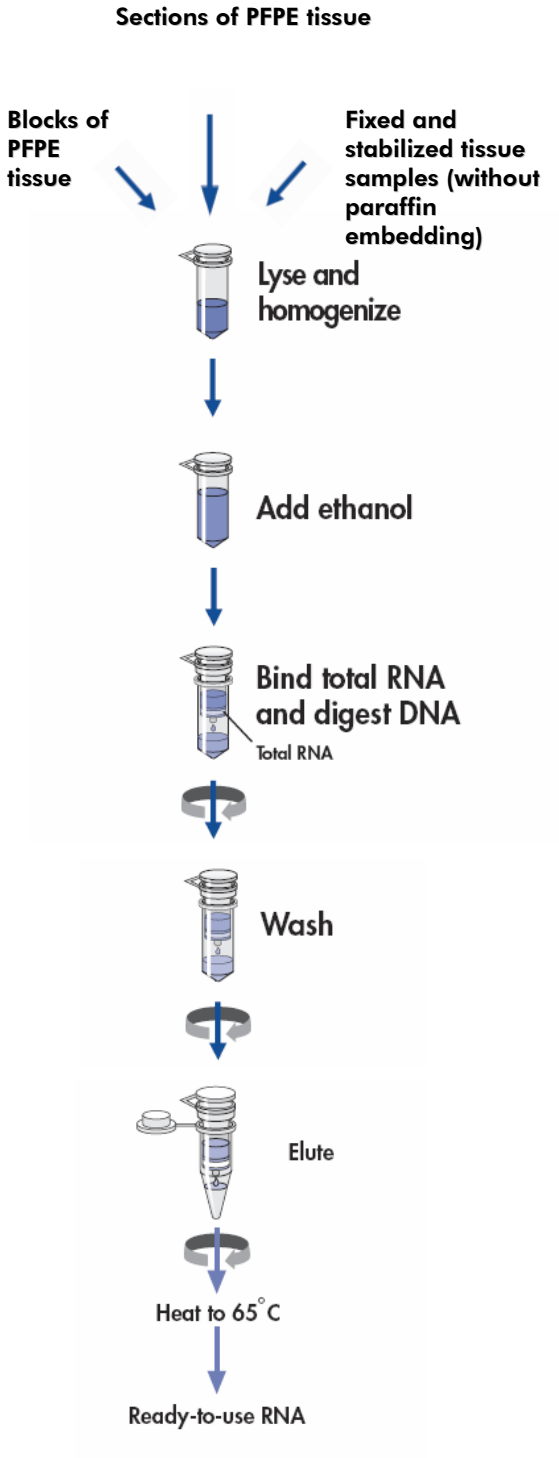
Description of protocols

Sections of PAXgene Tissue fixed, paraffin-embedded (PFPE) tissue (page 13)

Starting material for RNA purification should be freshly cut PFPE tissue samples.

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 is carried out to homogenize the cell lysate and remove residual cell debris.

The PAXgene Tissue RNA Procedure



Disrupt sample and lyse with guanidine-containing buffer (binding Buffer TR1)

Digest with Proteinase K; then add ethanol to adjust binding conditions

Apply sample to PAXgene RNA MinElute spin column for adsorption of RNA to membrane. DNase digest on column

Remove contaminants with simple wash steps

Elute RNA in low-salt buffer

PAXgene Tissue fixed samples (page 18)

Starting material for RNA purification should be up to 10 mg of a tissue sample fixed with PAXgene Tissue Fix and stabilized with PAXgene Tissue Stabilizer.

The tissue sample is removed from the PAXgene Tissue Stabilizer. If necessary, the sample is cut into 2 mm cubes. Binding Buffer TR1 is added, and mechanical disruption and simultaneous homogenization is performed using the TissueRuptor®, the TissueLyser LT, or the TissueLyser II (see "Disrupting and homogenizing starting materials", page 11).

Blocks of PAXgene Tissue fixed, paraffin-embedded (PFPE) tissue (page 24)

Starting material for RNA purification should be up to 10 mg of a block of PFPE tissue.

The tissue sample is cut out of 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 TR1 is added to the resulting pellet, and mechanical disruption and simultaneous homogenization is performed using the TissueRuptor, the TissueLyser LT, or the TissueLyser II (see "Disrupting and homogenizing starting materials", page 11).

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

- Xylene
- Ethanol (96–100%, purity grade p.a.)
- 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, or equivalent)
- Vortex mixer*
- Scalpel
- Crushed ice

For PAXgene Tissue fixed samples and blocks of PFPE tissue

- Equipment for tissue disruption and homogenization (see "Disrupting and homogenizing starting materials", page 11). We recommend either the TissueRuptor* with TissueRuptor Disposable Probes, the TissueLyser LT* system, or the TissueLyser II* system (see ordering information, page 38).
- 2 ml round-bottomed processing tubes

For sections of PFPE tissues

- Microtome

* 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 35).

‡ For the addition of ethanol to Buffer TR3 concentrate.

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 the 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), the TissueLyser LT (for low- to medium-throughput processing of up to 12 samples simultaneously), or the TissueLyser II (for medium- to high-throughput processing of up to 48 samples simultaneously).* Table 1 gives an overview of different disruption and homogenization methods used for the different starting materials.

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.

* If the TissueRuptor, TissueLyser LT, TissueLyser II, or other similar instrument is not available, contact QIAGEN Technical Services for an alternative method of disruption and homogenization.

Table 1. Disruption and homogenization

Sample	Disruption method	Homogenization method
Sections of PFPE tissue	Sectioning; no additional disruption required	PAXgene Shredder spin column
PAXgene Tissue fixed and stabilized tissue	TissueRuptor*	TissueRuptor*
	TissueLyser LT*	TissueLyser LT*
	TissueLyser II*	TissueLyser II*
Blocks of PFPE tissue	TissueRuptor*	TissueRuptor*
	TissueLyser LT*	TissueLyser LT*
	TissueLyser II*	TissueLyser II*

* Simultaneously disrupts and homogenizes individual samples.

Disruption and homogenization using the TissueLyser LT or 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 LT disrupts and homogenizes up to 12 tissue samples when used in combination with the TissueLyser LT Adapter. The adapter holds 12 x 2 ml microcentrifuge tubes containing stainless steel beads of 5 mm mean diameter.

The TissueLyser II disrupts and homogenizes up to 48 tissue samples simultaneously when used in combination with the TissueLyser Adapter Set 2 x 24. The adapter set holds 48 x 2 ml microcentrifuge tubes containing stainless steel beads of 5 mm mean diameter.

For guidelines on using TissueLyser instruments, refer to the instrument handbook. For other bead mills, refer to suppliers' guidelines.

Note: Tungsten carbide beads react with Buffer TR1 and must not be used to disrupt and homogenize tissues.

Homogenization using PAXgene Shredder spin columns

Using PAXgene Shredder spin columns is a fast and efficient way to homogenize cell and tissue lysates without cross-contamination of samples. Up to 700 µl of 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 from Sections of PFPE Tissue

Starting material

Starting material for RNA purification should be a minimum of 2 and a maximum of 5 sections of the tissue sample fixed with PAXgene Tissue Fix, stabilized with PAXgene Tissue Stabilizer, dehydrated, and embedded in paraffin (PFPE* tissue; for information about tissue fixation, stabilization, processing, and paraffin embedding see the product circular of the PAXgene Tissue collection device used). 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 35.
- 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

- Before using the kit for the first time, prepare 80% ethanol by mixing 24 ml ethanol (96–100%) and 6 ml RNase-free water (supplied).
- A shaker–incubator is required in steps 9 and 22. Set the temperature of the shaker–incubator to 45°C.

* PAXgene Tissue fixed, paraffin-embedded.

- Buffer TR1 may form a precipitate upon storage. If necessary, warm to 37°C to dissolve.
- β-Mercaptoethanol (β-ME) must be added to Buffer TR1 before use. Add 10 µl β-ME per 1 ml Buffer TR1. Dispense in a fume hood and wear appropriate protective clothing. Buffer TR1 containing β-ME can be stored at room temperature (15–25°C) for up to 1 month.
- Buffer TR3 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 550 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 –15 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 35).

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-safelock tube.**
3. **Add 650 µl xylene to the sample. Vortex vigorously for 20 s, and incubate for 3 min on the benchtop (at 15–25°C).**
4. **Add 650 µl ethanol (96–100%, purity grade p.a.), and mix by vortexing for 20 s.**

* 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).

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 RNA procedure.

7. **Add 150 µl Buffer TR1, 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 TR1 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 22.**

   Note: For purification of RNA from fibrous tissue (e.g., skin, heart or skeletal muscle, aorta), incubate for 2 h at 45°C.

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 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 225 µl ethanol (96–100%, purity grade p.a.). 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 ethanol, but this will not affect the PAXgene Tissue RNA procedure.

13. **Pipet the sample, including any precipitate that may have formed, into a PAXgene RNA MinElute spin column (red) placed in a 2 ml processing tube, and centrifuge for 1 min at 8000 x *g*. 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.

14. **Pipet 350 µl Buffer TR2 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.***

15. **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.

16. **Pipet the DNase I incubation mix (80 µl) directly onto the PAXgene RNA MinElute spin column, and incubate for 15 min at ambient temperature (20–30°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.

17. **Pipet 350 µl Buffer TR2 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.***

18. **Pipet 500 µl Buffer TR3 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.**

    Note: Buffer TR3 is supplied as a concentrate. Ensure that ethanol is added to Buffer TR3 before use (see "Things to do before starting", page 13).

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

* Flow-through contains Buffer TR1 or Buffer TR2 and is therefore not compatible with bleach. See page 5 for safety information.

20. **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.**

21. **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 TR4 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 TR4 in order to achieve maximum elution efficiency.

    Smaller volumes of Buffer TR4 can be used to obtain a higher total RNA concentration, but this will influence the overall yield.

    The dead volume of the PAXgene RNA MinElute spin column is 2 µl; elution with 14 µl of Buffer TR4 results in an eluate with a volume of 12 µl.

22. **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.

23. **If the RNA samples will not be used immediately, store at –15 to –30°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 35.

Protocol: Purification of Total RNA from PAXgene Tissue Fixed Samples

Starting material

Starting material for RNA purification should be up to 10 mg of a tissue sample fixed with PAXgene Tissue Fix and stabilized with PAXgene Tissue Stabilizer.

It is essential to use the correct amount of starting material in order to obtain optimal RNA yield and purity. A maximum amount of 10 mg tissue fixed and stabilized using the PAXgene Tissue System can generally be processed. For most tissues, the RNA binding capacity of the PAXgene RNA MinElute spin column and the lysing capacity of Buffer TR1 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 35.
- 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

- Before using the kit for the first time, prepare 80% ethanol by mixing 24 ml ethanol (96–100%) and 6 ml RNase-free water (supplied).
- A shaker–incubator is required in steps 5 and 19. Set the temperature of the shaker–incubator to 45°C.

- Buffer TR1 may form a precipitate upon storage. If necessary, warm to 37°C to dissolve.
- β-Mercaptoethanol (β-ME) must be added to Buffer TR1 before use. Add 10 µl β-ME per 1 ml Buffer TR1. Dispense in a fume hood and wear appropriate protective clothing. Buffer TR1 containing β-ME can be stored at room temperature (15–25°C) for up to 1 month.
- Buffer TR3 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 550 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 –15 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 35).

Procedure

1. **If using the TissueLyser LT or TissueLyser II, add one stainless steel bead (5 mm mean diameter) to each 2 ml round-bottomed processing tube (not supplied).**

* 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).

2. **Retrieve the tissue sample from PAXgene Tissue Stabilizer 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 TR1 should be carried out on ice and as quickly as possible.

3. **Disrupt the tissue and homogenize the lysate using either the TissueRupter (follow step 3a) or a TissueLyser instrument (follow step 3b).**

   See "Disrupting and homogenizing starting materials", page 11, for more details on disruption and homogenization.

   Note: Ensure that β-ME is added to Buffer TR1 before use (see "Things to do before starting", page 18).

   Note: Incomplete homogenization leads to significantly reduced RNA yields and can cause clogging of the PAXgene RNA MinElute spin column. Homogenization with the TissueRupter or a TissueLyser instrument 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 300 µl Buffer TR1.**

  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 LT or TissueLyser II

- **Place the tissues in the tubes prepared in step 1.**
- **Immediately add 300 µl Buffer TR1 per tube.**
- **Place the tubes in the TissueLyser LT Adapter (TissueLyser LT) or the TissueLyser Adapter Set 2 x 24 (TissueLyser II).**
- **Operate the TissueLyser instrument for 2 min at 20 Hz.**

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

- **On the TissueLyser II, rearrange the tubes so that the outermost tubes are innermost and the innermost tubes are outermost.**

  Rearranging the tubes on the TissueLyser II allows even homogenization.
- **Operate the TissueLyser instrument for another 2 min at 20 Hz.**
- **Proceed to step 4.**

  Do not reuse the stainless steel beads

4. **Add 580 µl RNase-free water to the lysate. Then add 20 µl proteinase K and mix by vortexing for 5 s.**

   Note: Do not mix RNase-free water and proteinase K together before adding them to the lysate.
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 use in step 19.**

   Note: For purification of RNA from fibrous tissue (e.g., skin, heart or skeletal muscle, aorta), incubate for 2 h at 45°C.
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 1.5 ml microcentrifuge tube without disturbing the pellet in the processing tube.**
7. **Add 450 µl of ethanol (96–100%, purity grade p.a.). 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 ethanol, but this will not affect the PAXgene Tissue RNA procedure.
8. **Pipet 700 µl of the sample, including any precipitate that may have formed, into the PAXgene RNA MinElute spin column (red) placed in a 2 ml processing tube, and centrifuge for 1 min at 8000 x *g*. Discard the flow-through.***

   Save the processing tube for re-use in step 9.

9. **Repeat step 8 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.

10. **Pipet 350 µl Buffer TR2 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 ambient temperature (20–30°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. **Pipet 350 µl Buffer TR2 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.***

14. **Pipet 500 µl Buffer TR3 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.**

    Note: Buffer TR3 is supplied as a concentrate. Ensure that ethanol is added to Buffer TR3 before use (see "Things to do before starting", page 18).

15. **Pipet 500 µl of 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%) and the RNase-free water supplied with the kit.

* Flow-through contains Buffer TR1 or Buffer TR2 and is therefore not compatible with bleach. See page 5 for safety information.

16. **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*).**

17. **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 TR4 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 TR4 in order to achieve maximum elution efficiency.

    Smaller volumes of Buffer TR4 can be used to obtain a higher total RNA concentration, but this will influence the overall yield.

    The dead volume of the PAXgene RNA MinElute spin column is 2 µl; elution with 14 µl of Buffer TR4 results in an eluate with a volume of 12 µl.

18. **Recommended: Repeat elution step (step 17) as described, using 14–40 µl Buffer TR4.**

    Omitting this step may result in reduced yields.

19. **Incubate the eluate for 5 min at 65°C in the shaker–incubator 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.

20. **If the RNA samples will not be used immediately, store at –15 to –30°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 35.

Protocol: Purification of Total RNA from Blocks of PFPE Tissue

Starting material

Starting material for RNA purification should be up to 10 mg of a block of tissue fixed with PAXgene Tissue Fix, stabilized with PAXgene Tissue Stabilizer, dehydrated, and embedded in paraffin (PFPE* tissue; for information about tissue fixation, stabilization, processing, and paraffin embedding see the product circular of the PAXgene Tissue collection device used).

It is essential to use the correct amount of starting material in order 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 TR1 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 35.
- 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).

* PAXgene Tissue fixed, paraffin-embedded.

Things to do before starting

- Before using the kit for the first time, prepare 80% ethanol by mixing 24 ml ethanol (96–100%) and 6 ml RNase-free water (supplied).
- A shaker–incubator is required in steps 11 and 25. Set the temperature of the shaker–incubator to 45°C.
- Buffer TR1 may form a precipitate upon storage. If necessary, warm to 37°C to dissolve.
- β-Mercaptoethanol (β-ME) must be added to Buffer TR1 before use. Add 10 µl β-ME per 1 ml Buffer TR1. Dispense in a fume hood and wear appropriate protective clothing. Buffer TR1 containing β-ME can be stored at room temperature (15–25°C) for up to 1 month.
- Buffer TR3 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 550 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 –15 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 35).

* 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 paraffin block using a scalpel and weigh the sample. Do not use more than 10 mg tissue.**

   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 processing tube (not supplied).**
3. **Add 1 ml xylene to the sample. Vortex vigorously for 20 s, and incubate for 3 min on the benchtop (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 of 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 TissueRupter (follow step 9a) or a TissueLyser instrument (follow step 9b).**

   See "Disrupting and homogenizing starting materials", page 11, for more details on disruption and homogenization.

   Note: Ensure that β-ME is added to Buffer TR1 before use (see "Things to do before starting", page 25).

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

9a. Disruption and homogenization using the TissueRuptor

- **Add 300 µl Buffer TR1 to the pellet.**
- **Place the tip of the disposable probe into the 2 ml processing 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 LT or TissueLyser II

- **Add one stainless steel bead (5 mm mean diameter) and 300 µl Buffer TR1 to the pellet.**
- **Place the tubes in the TissueLyser LT Adapter (TissueLyser LT) or TissueLyser Adapter Set 2 x 24 (TissueLyser II).**
- **Operate the TissueLyser instrument for 2 min at 20 Hz.**

  The time depends on the tissue being processed and can be extended until the tissue is completely homogenized.
- **On the TissueLyser II, rearrange the tubes so that the outermost tubes are innermost and the innermost tubes are outermost.**

  Rearranging the tubes on the TissueLyser II allows even homogenization.
- **Operate the TissueLyser instrument for another 2 min at 20 Hz.**
- **Carefully pipet the lysates into new 1.5 ml microcentrifuge tubes. Proceed to step 10.**

  Do not reuse the stainless steel beads

10. Add 580 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 TR1 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 use in step 25.

Note: For purification of RNA from fibrous tissue (e.g., skin, heart or skeletal muscle, aorta), incubate for 2 h at 45°C.

12. Centrifuge the tissue lysate for 3 min at maximum speed (but not to exceed 20,000 x *g*). Carefully transfer the supernatant fraction to a new 1.5 ml or 2 ml processing tube (not supplied) without disturbing the pellet.

13. Add 450 µl ethanol (96-100%, purity grade p.a.). 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 ethanol, but this will not affect the PAXgene Tissue RNA procedure.

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

    Reuse the processing tube in step 15.

15. **Repeat step 14 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.

16. **Pipet 350 µl Buffer TR2 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 ambient temperature (20–30°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.

19. **Pipet 350 µl Buffer TR2 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.***

20. **Pipet 500 µl Buffer TR3 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.**

    Note: Buffer TR3 is supplied as a concentrate. Ensure that ethanol is added to Buffer TR3 before use (see "Things to do before starting", page 25).

* Flow-through contains Buffer TR1 or Buffer TR2 and is therefore not compatible with bleach. See page 5 for safety information.

21. **Pipet 500 µl of 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%) and the RNase-free water supplied with the kit.

22. **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*).**

23. **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 TR4 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 TR4 in order to achieve maximum elution efficiency.

    Smaller volumes of Buffer TR4 can be used to obtain a higher total RNA concentration, but this will influence the overall yield.

    The dead volume of the PAXgene RNA MinElute spin column is 2 µl; elution with 14 µl of Buffer TR4 results in an eluate with a volume of 12 µl.

24. **Recommended: Repeat elution step (step 23) as described, using another 14–40 µl Buffer TR4.**

    Omitting this step may result in reduced yields.

25. **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: This 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 –15 to –30°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 35.

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 not properly fixed	PAXgene Tissue Fix rapidly penetrates and fixes the tissue with a fixation rate, depending on tissue type, of approximately 1 mm in 30 min. Therefore a tissue sample with dimensions of 4 x 10 x 10 mm has to be fixed for at least 2 h.
c) Extended fixation or stabilization times	Fixation rates and stabilization times depend on type and size of tissue. For optimal fixation and stabilization incubation times refer to the product circular of the PAXgene Tissue collection device used.
d) Sample inappropriately handled during processing	Incubation of PAXgene Tissue treated specimens in water, formalin, alcoholic solutions with less than 70% ethanol, or paraffin with a temperature above 60°C leads to degradation of RNA. Start processing with 70–100% ethanol. Use a low-melting-point paraffin (≤60°C), and make sure that incubation in liquid paraffin does not exceed 3 h. Do not use reagents contaminated with formalin.

	Comments and suggestions
e) Paraffin-embedded tissue blocks stored inappropriately	RNA and other biomolecules in paraffin-embedded tissue undergo slow chemical degradation. Therefore store and archive PAXgene treated and paraffin-embedded blocks of tissue in a dry and dark place at 2–8°C or colder. The ideal storage temperature for preservation of nucleic acid is –15 to –30°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 35) 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%) and the RNase-free water supplied with the kit, as described in "Things to do before starting" in each protocol.
Low RNA yield	
a) Tissue sample processed with reagents contaminated with formalin	Do not use reagents contaminated with formalin. Even trace amounts of formalin in the alcohol or other reagents used for sample processing or embedding can lead to significant reduction of RNA yield. In case of a known contamination with formalin, increase lysate digestion with proteinase K from 15 min at 45°C to 6 hours at 45°C (step 9 in "Protocol: Purification of Total RNA from Sections of PFPE Tissue"; step 11 of "Protocol: Purification of Total RNA from Blocks of PFPE Tissue").
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.

	Comments and suggestions
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 TR4 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 36).
f) Centrifugation for more than 1–2 s after adding ethanol to the lysate (step 12, page 15; step 7, page 21; step 13, page 27)	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.
g) Elution Buffer TR4 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 11, for details on disruption and homogenization methods. In subsequent preparations, reduce the amount of starting material (see the individual protocols).

* 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
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 36).
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.
RNA does not perform well in downstream applications	
a) Salt carryover during elution	Ensure that Buffer TR3 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.

	Comments and suggestions
c) Reverse transcription with too small an amount of RNA	When performing reverse transcription with very small amounts of RNA, we recommend using the Sensiscript® RT Kit, which is specially designed for cDNA synthesis from <50 ng RNA. If synthesizing cDNA for use in real-time PCR, we recommend the QuantiTect® Reverse Transcription Kit, which is compatible with a wide range of RNA amounts (10 pg to 1 µg), or the QuantiTect Whole Transcriptome Kit, which provides whole transcriptome amplification from as little as 1 ng RNA. See page 39 for ordering information.

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 ⇒ 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 37), 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 TR4 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 of 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 TR4 as the volume of eluted RNA to be diluted. Buffer TR4 has high absorbance at 220 nm, which can lead to high background absorbance levels if the spectrophotometer is not properly zeroed.

References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN and PreAnalytiX products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc.

For a complete list of references, visit the QIAGEN Reference Database online at www.qiagen.com/RefDB/search.asp or contact QIAGEN Technical Services or your local distributor.

* 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.

Ordering Information

Product	Contents	Cat. no.
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
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 miRNA Kit — for purification of microRNA and total RNA from tissues fixed and stabilized in PAXgene Tissue Containers		
PAXgene Tissue miRNA 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	766134
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
TissueRuptor System — for low-throughput sample disruption using disposable probes		
TissueRuptor	Handheld rotor–stator homogenizer, 5 TissueRuptor Disposable Probes	Varies*

* Visit www.qiagen.com/automation to find out more about the TissueRuptor and to order.

Product	Contents	Cat. no.
TissueRuptor Disposable Probes (25)	25 nonsterile plastic disposable probes for use with the TissueRuptor	990890
TissueLyser LT System — for low- to medium-throughput sample disruption for molecular analysis		
TissueLyser LT*	Compact bead mill, 100–240 V AC, 50–60 Hz; requires the TissueLyser LT Adapter, 12-Tube (available separately)	85600
TissueLyser LT Adapter, 12-Tube	Adapter for disruption of up to 12 samples in 2 ml microcentrifuge tubes on the TissueLyser LT	69980
TissueLyser II System — for medium- to high-throughput sample disruption for molecular analysis		
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	For disrupting up to 48 samples: 2 sets of adapter plates and 2 racks for use with 2 ml microcentrifuge tubes on the TissueLyser II	69982
TissueLyser Accessories		
Stainless Steel Beads, 5 mm (200)	200 stainless steel beads (5 mm diameter), suitable for use with TissueLyser systems	69989
TissueLyser Single-Bead Dispenser, 5 mm	For dispensing individual beads (5 mm diameter)	69965
Sensiscript RT Kit — for reverse transcription of less than 50 ng RNA per reaction		
Sensiscript RT Kit (50)†	For 50 reverse-transcription reactions: Sensiscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix (contains 5 mM each dNTP), RNase-free water	205211

* Visit www.qiagen.com/automation to find out more about the TissueLyser LT and TissueLyser II.

† Larger kit sizes available; please inquire.

Product	Contents	Cat. no.
QuantiTect Rev. Transcription Kit — for fast cDNA synthesis for sensitive real-time two-step RT-PCR		
QuantiTect Reverse Transcription Kit (50)	For 50 x 20 µl reactions: gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-Free Water	205311
QuantiTect Whole Transcriptome Kit — for unlimited real-time PCR analysis from precious RNA samples		
QuantiTect Whole Transcriptome Kit (25)*	For 25 x 50 µl reactions: T-Script Enzyme and Buffer, Ligation Enzymes, Reagent, and Buffer, and REPLI-g® DNA Polymerase and Buffer	207043

* Larger kit sizes available; please inquire.

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Notes

Notes

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