RNeasy® Micro Handbook

For isolation of total RNA from

microdissected tissues
small amounts of tissues (e.g., fine needle aspirates)
small amounts of fibrous tissues (e.g., heart, muscle)
small numbers of cells (e.g., FACS sorted cells)
and for RNA cleanup and concentration

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QIAGEN Worldwide

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www.qiagen.com

QIAGEN Distributors

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

Contents

Kit Contents

RNeasy® Micro Kit (50)	
Catalog no.	**74004**
Preps per kit	**50**
RNeasy MinElute™ Spin Columns in collection tubes	50
Collection tubes (1.5 ml)	50
Collection tubes (2 ml)	100
Buffer RLT*	45 ml
Buffer RW1*	45 ml
Buffer RPE†	11 ml
RNase-free water (bottle)	3 x 10 ml
RNase-free water (tube)	1.5 ml
Buffer RDD	2 x 2 ml
RNase-free DNase	1 tube (1500 units)
Carrier RNA, poly-A	1 vial (310 µg)
Handbook	1

** Not compatible with disinfecting reagents containing bleach. Contains a guanidine salt. See page 5 for safety information.*

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

Shipping and Storage

The RNeasy Micro Kit is shipped at room temperature (15–25°C). RNase-Free DNase Set box, containing RNase-free DNase, Buffer RDD, and RNase-free water, should be stored immediately upon receipt at 2–8°C. The remaining components of the RNeasy Micro Kit should be stored dry at room temperature. All components are stable for at least 9 months under these conditions.

Product Use Limitations

The RNeasy Micro Kit is intended as a general-purpose device. No claim or representation is intended for its use to identify any specific organism or for clinical use (diagnostic, prognostic, therapeutic, or blood banking). It is the user's responsibility to validate the performance of the RNeasy Micro Kit for any particular use, since the performance characteristics of this kit have not been validated for any specific organism. The RNeasy Micro Kit may be used in clinical diagnostic laboratory systems after the laboratory has validated their complete system as required by CLIA '88 regulations in the U.S. or equivalents in other countries.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Product Warranty and Satisfaction Guarantee

QIAGEN 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, QIAGEN 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 QIAGEN 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 QIAGEN 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 inside front cover).

Quality Control

As part of the stringent QIAGEN quality assurance program, the performance of RNeasy Micro Kits is monitored routinely on a lot-to-lot basis. All components are tested separately to ensure highest performance and reliability.

Safety Information

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

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

Buffer RLT contains guanidine thiocyanate and Buffer RW1 contains a small amount of guanidine thiocyanate, which can form highly reactive compounds when combined with bleach.

If liquid containing these buffers is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

The following risk and safety phrases apply to the components of the RNeasy Micro Kit.

Buffer RLT

Contains guanidine thiocyanate: harmful. Risk and safety phrases:*
R20/21/22-32, S13-26-36-46

Buffer RW1

Contains ethanol: flammable. Risk phrase:* R10

RNase-free DNase

Contains deoxyribonuclease: sensitizer. Risk and safety phrases:*
R42/43, S22-24-26-36/37

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

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 molecular biology and the use of QIAGEN products. If you have any questions or experience any difficulties regarding the RNeasy Micro Kit or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN 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 QIAGEN. 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 call one of the QIAGEN Technical Service Departments or local distributors (see inside front cover).

* *R10: Flammable; R20/21/22: Harmful by inhalation, in contact with skin and if swallowed; R32: Contact with acids liberates very toxic gas; R42/43: May cause sensitization by inhalation and skin contact; S13: Keep away from food, drink and animal feedingstuffs; S22: Do not breathe dust; S24: Avoid contact with skin; S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice; S36: Wear suitable protective clothing; S36/37: Wear suitable protective clothing and gloves; S46: If swallowed, seek medical advice immediately and show the container or label.*

Introduction

The RNeasy Micro procedure uses a novel technology to purify RNA (maximum 45 µg) from small amounts of tissues or cells (as little as 1 cell), such as laser-microdissected (LMD) samples, fine needle aspirates (FNA), and FACS® sorted cells. QIAGEN provides a wide range of other kits for isolation of total RNA from different sample sources (see pages 68–70 for ordering information).

Principle and procedure

RNeasy Micro technology combines the selective binding properties of a silica-gel–based membrane with the speed of microspin technology. Guanidine-isothiocyanate–containing lysis buffer and ethanol are added to the sample to create conditions that promote selective binding of RNA to the RNeasy MinElute membrane. The sample is then applied to the RNeasy MinElute Spin Column. RNA binds to the silica-gel membrane. Traces of DNA that may copurify are removed by a DNase treatment on the RNeasy Spin Column. DNase and any contaminants are washed away, and high-quality total RNA is eluted in RNase-free water (see flowchart, page 8).

With the RNeasy Micro procedure, all RNA molecules longer than 200 nucleotides are isolated. The procedure enriches for mRNA since most RNAs <200 nucleotides (such as 5.8S rRNA, 5S rRNA, and tRNAs, which together make up 15–20% of total RNA) are selectively excluded. The size distribution of purified RNA is comparable to that obtained by centrifugation through a CsCl cushion, where small RNAs do not sediment efficiently.

In this handbook, different protocols are provided for different starting materials. The protocols differ primarily in the lysis and homogenization of the sample and in the adjustment of the conditions for binding RNA to the RNeasy MinElute membrane. Once the sample is bound to the membrane, the protocols are similar.

RNeasy Micro Procedure

❶ Disrupt sample and lyse with GITC-containing buffer (Buffer RLT).

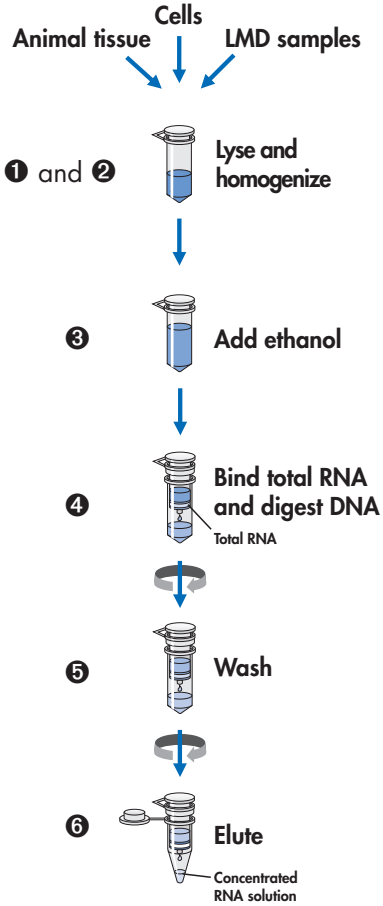
❷ Homogenize sample to shear genomic DNA and reduce viscosity of lysate.

❸ Add ethanol to adjust binding conditions.

❹ Apply sample to RNeasy MinElute Spin Column for adsorption of RNA to membrane. DNase digest on column.

❺ Remove contaminants with simple wash steps.

❻ Elute ready-to-use RNA in water.



Equipment and Reagents to Be Supplied by User

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

For all protocols

- 14.3 M β-mercaptoethanol (β-ME)* (commercially available solutions are usually 14.3 M)
- Sterile, RNase-free pipet tips
- Microcentrifuge (with rotor for 2 ml tubes)
- Ethanol (70% and 96–100%)†
- 80% ethanol, prepared using the RNase-free water supplied with the kit†
- Disposable gloves
- Equipment for disruption and homogenization (see pages 14–17)

For total RNA isolation from microdissected formalin-fixed tissues or from heart, muscle, and skin tissue

- QIAGEN Proteinase K, >600 mAU/ml (cat. no. 19131 or 19133)‡
- Heating block or water bath

For RNA cleanup after purification using the PAXgene™ Blood RNA Kit

- Buffer BR5 (included in the PAXgene Blood RNA Kit)
- Heating block or water bath

Supplier of bead-mill homogenizers§

- QIAGEN (Mixer Mill MM 300)

Glass, stainless steel, and tungsten carbide beads can be purchased from:

- Retsch, Haan, Germany

** β-ME must be added to Buffer RLT before use. Dispense in a fume hood and wear appropriate protective clothing. Add 10 µl of β-ME per 1 ml of Buffer RLT. The solution is stable for 1 month at room temperature after the addition of β-ME.*

† Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone. Note: 70% ethanol is not required for the RNA cleanup protocols.

‡ If using proteinase K from another supplier, use a 20 mg/ml solution in water.

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

Suppliers of rotor–stator homogenizers*

- Bio-Spec Products, Bartlesville, OK, USA (Tissue-Tearor™)
- Charles Ross & Son Company, Hauppauge, NY, USA
- Craven Laboratories, Austin, TX, USA
- IKA Analysentechnik GmbH, Germany (Ultra Turrax®)
- IKA Works, Cincinnati, OH, USA
- Kinematica AG; sold by Brinkmann Instruments, Westbury, NY, USA (Polytron® Homogenizers)
- OMNI International Inc., Waterbury, CT, USA (Omni Homogenizers)
- Silverson Machines, Bay Village, OH, USA
- Tekmar Inc., Cincinnati, OH, USA (Tissuemizer®)
- VirTis Company, Gardiner, NY, USA

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

Important Notes

Determining the amount of starting material

Using the correct amount of starting material is essential in order to obtain high yields and pure RNA with RNeasy MinElute Spin Columns. The maximum amount that can be used is limited by the volume of Buffer RLT required for efficient lysis and the maximum loading volume of the RNeasy MinElute Spin Column.

Even though the RNA binding capacity of the column may not be reached, the maximum amount of starting material must not be exceeded or lysis will be incomplete, resulting in lower yield and purity. The maximum amount of a specific biological sample that can be processed depends on the type of tissue or cells being processed and the corresponding RNA content. More information for calculating starting amounts of material is given in each protocol. Specifications of RNeasy MinElute Spin Columns are shown in Table 1. Table 2 gives examples of expected RNA yields from various sources.

Table 1. RNeasy MinElute Spin Column specifications

Maximum binding capacity	45 µg RNA
Maximum loading volume	700 µl
RNA size distribution	RNA >200 nucleotides
Minimum elution volume	10 µl
Maximum amount of starting material	
Animal cells	5×10^5
Animal tissue	5 mg

Note: If the binding capacity of the RNeasy MinElute Spin Column is exceeded, yields of total RNA will not be consistent and less than 45 µg of total RNA may be recovered. If lysis of the starting material is incomplete, yields of total RNA will be lower than expected, even if the binding capacity of the RNeasy MinElute Spin Column is not exceeded.

Table 2. Yields of total RNA with RNeasy Micro Kits

Source	Average yield of total RNA* (µg)
Mouse/rat tissues (5 mg)	
Embryo (13 day)	10
Brain	4
Heart	5†
Kidney	15
Liver	15
Spleen	15
Thymus	20
Lung	5
Cell cultures (5 x 10^5 cells)	
NIH/3T3	5
HeLa	7.5
COS-7	17.5
LMH	6
Huh	7.5

** Amounts can vary due to species, developmental stage, growth conditions used, etc. Since the RNeasy Micro procedure enriches for mRNA and other RNA species >200 nucleotides, the total RNA yield does not include 5S rRNA, tRNA, and other low-molecular-weight RNAs, which make up 15–20% of total cellular RNA.*

† Using the specialized RNeasy Micro protocol for total RNA isolation from fibrous tissues, page 33.

Weighing tissue or counting cells are the most accurate ways to quantify the amount of starting material. However, the following may be used as a guide.

Animal tissue

A 1.5 mm cube (approximately this size: ■; volume, approximately 3.4 mm^3) of most animal tissues weighs 3.5–4.5 mg.

Animal cells

The number of HeLa cells obtained in various culture dishes after confluent growth is given in Table 3.

Table 3. Growth area and number of HeLa cells in various culture dishes

Cell culture vessel	Growth area (cm^2)*	Number of cells†
Multiwell plates		
96-well	0.32–0.6	4–5 x 10^4
48-well	1	1 x 10^5
24-well	2	2.5 x 10^5
12-well	4	5 x 10^5
6-well	9.5	1 x $10^{6‡}$
Dishes		
35 mm	8	1 x $10^{6‡}$
Flasks		
40–50 ml	25	3 x $10^{6‡}$

* *Per well, if multiwell plates are used; varies slightly depending on the supplier.*

† *Cell numbers are given for HeLa cells (approximate length = 15 µm), assuming confluent growth. Numbers will vary for different kinds of animal cells, which vary in length from 10 to 30 µm.*

‡ *This number of cells exceeds the maximum binding capacity of the RNeasy MinElute Spin Columns. To process this many cells, split the lysate into appropriate aliquots (≤5 x 10^5 cells each) and load them onto separate RNeasy MinElute Spin Columns.*

Handling and storage of starting material

RNA in tissues is not protected after harvesting until the sample is treated with RNA*later*™ RNA Stabilization Reagent, flash frozen, or disrupted and homogenized in the presence of RNase-inhibiting or denaturing reagents. See the *RNA*later *Handbook* for information about RNA*later* RNA Stabilization Reagent and about stabilizing RNA in tissues.

Samples can be immediately flash frozen in liquid nitrogen§ and stored at –70°C as soon as they are harvested or excised. Frozen tissue should not be allowed to thaw during handling or weighing, but cell pellets can partially thaw enough to allow them to be dislodged by flicking. The relevant procedures should be carried out as quickly as possible.

Samples can also be stored at –70°C in lysis buffer (Buffer RLT) after disruption and homogenization. Frozen samples are stable for months.

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

Disruption and homogenization of starting materials

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

Disruption: Complete disruption of plasma membranes of cells and organelles is absolutely required to release all the RNA contained in the sample. Different samples require different methods to achieve complete disruption. Incomplete disruption results in significantly reduced yields.

Homogenization: Homogenization is necessary to reduce the viscosity of the cell lysates produced by disruption. Homogenization shears the 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 RNeasy MinElute membrane and therefore significantly reduced yields.

Some disruption methods simultaneously homogenize the sample while others require an additional homogenization step. Table 4 gives an overview of different disruption and homogenization methods suitable for various starting materials. It can be used as a guide to choose the appropriate method for your starting material. The different disruption and homogenization methods listed in Table 4 are described in more detail below.

Table 4. Disruption and homogenization methods

Starting material	Disruption method	Homogenization method	Comments
Microdissected samples	Addition of lysis buffer	Vortexing	No additional homogenization is necessary.
Animal tissue	Rotor–stator homogenization	Rotor–stator homogenization	Simultaneously disrupts and homogenizes
	Mortar and pestle	Syringe and needle QIAshredder™ homogenizer	Homogenization using rotor–stator homogenizers or the Mixer Mill MM 300 usually gives higher yields than other homogenization methods.
	Mixer Mill MM 300	Mixer Mill MM 300	The Mixer Mill MM 300 gives results comparable to using a rotor–stator homogenizer.
Fine needle aspirates (FNA) and cultured animal cells	Addition of lysis buffer	Rotor–stator homogenization QIAshredder homogenizer Syringe and needle	If ≤1 x 10^5 cells are processed, the lysate can be homogenized by vortexing.

Disruption and homogenization using rotor–stator homogenizers

Rotor–stator homogenizers thoroughly disrupt and simultaneously homogenize, in the presence of lysis buffer, animal tissues in 15–90 seconds depending on the toughness of the sample. Rotor–stator homogenizers can also be used to homogenize cell lysates. The rotor turns at a very high speed causing the sample to be disrupted and homogenized by a combination of turbulence and mechanical shearing. Foaming of the sample should be kept to a minimum by using properly sized vessels, by keeping the tip of the homogenizer submerged, and by holding the immersed tip to one side of the tube. Rotor–stator homogenizers are available in different sizes and operate with differently sized probes. Probes with diameters of 5 mm and 7 mm are suitable for volumes up to 300 µl and can be used for homogenization in microcentrifuge tubes. Probes with a diameter of 10 mm or above require larger tubes. See page 10 for a list of suppliers of rotor–stator homogenizers.

Disruption and homogenization using the Mixer Mill MM 300 and other bead mills

In bead-milling, cells and 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. Disruption efficiency is influenced by:

- size and composition of beads
- ratio of buffer to beads
- amount of starting material
- speed and configuration of agitator
- disintegration time

Stainless steel beads with a 3–7 mm diameter are optimal to use for animal tissues. All other disruption parameters must be determined empirically for each application. Appendix D (page 67) contains guidelines for disruption and homogenization of tissues using the Mixer Mill MM 300 and stainless steel beads. Refer to suppliers' guidelines for further details.

Disruption using a mortar and pestle

For disruption using a mortar and pestle, freeze the sample immediately in liquid nitrogen* and grind to a fine powder under liquid nitrogen. Transfer the suspension (tissue powder and liquid nitrogen) into a liquid-nitrogen–cooled, appropriately sized tube and allow the liquid nitrogen to evaporate without allowing the sample to thaw. Add lysis buffer and continue as quickly as possible with the homogenization according to one of the methods below.

Note: Grinding the sample using a mortar and pestle will disrupt the sample, but it will not homogenize it. Homogenization must be performed separately before proceeding with the RNeasy Micro protocol.

Homogenization using QIAshredder homogenizers

Use of QIAshredder modules is a fast and efficient way to homogenize cell and tissue lysates without cross contamination of the samples. The lysate (maximum volume 700 µl) is loaded onto the QIAshredder Spin Column in a 2 ml collection tube, centrifuged for 2 minutes at maximum speed in a microcentrifuge and the homogenized lysate collected. QIAshredder Spin Columns can be purchased separately for use with RNeasy Micro Kits (see page 71 for ordering information). Call our Technical Service Group for further details.

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

Homogenization using a syringe and needle

Cell and tissue lysates can be homogenized using a syringe and needle. High-molecular-weight DNA can be sheared by passing the lysate through a 20-gauge (0.9 mm) needle, attached to a sterile plastic syringe, at least 5–10 times or until a homogeneous lysate is achieved. Increasing the volume of lysis buffer may be required to facilitate handling and minimize loss.

Carrier RNA

The RNeasy Micro Kit contains poly-A RNA to be added as carrier RNA to the lysate when processing <10 µg tissue or <5000 cells. As demonstrated in many different RT-PCR systems, the small amounts of poly-A RNA used as carrier RNA do not interfere with subsequent RT-PCR, even when oligo-dT is used as a primer for reverse transcription. Reverse-transcription reactions typically contain a large excess of oligo-dT, and the small amounts of poly-A used as carrier RNA are insignificant in comparison.

As an alternative, other types of RNA can be purchased separately for use as carrier RNA. Note, however, that tRNA and other RNAs <200 nt will not bind to the RNeasy membrane and cannot be used as carrier RNA. For most applications, bacterial ribosomal RNA (e.g., from Roche, cat. no. 206938)* gives good results and can be used as an alternative to poly-A RNA supplied.

Other types of carrier RNA, such as poly-C RNA, can give poor results and are not recommended.

* *This is not a complete list of suppliers and does not include many important vendors of biological supplies. When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.*

Protocol: Total RNA Isolation from Microdissected Cryosections

This protocol is designed for the isolation of RNA from frozen, microdissected animal tissue samples. For formalin-fixed samples, see "Protocol: Total RNA Isolation from Microdissected Formalin-Fixed Tissues" (page 22).

Laser-microdissected tissue specimens present a particular challenge for molecular analysis, as nucleic acids must be purified from very small amounts of starting material. In addition, fixation and staining steps may compromise the integrity of RNA, and it may be necessary either to modify fixation protocols or to use cryosections from flash-frozen specimens to minimize this problem.

A wide range of equipment and consumables for sectioning, staining, and microdissection of specimens is available from Leica (**www.leica-microsystems.com**).

Important points before starting

- If using RNeasy Micro Kits for the first time, read "Important Notes" (page 11).
- If working with RNA for the first time, read Appendix A (page 58).
- To minimize RNA degradation, avoid prolonged storage of unstabilized samples at room temperature. RNA in tissues is not protected before fixation, stabilization in RNA*later* RNA Stabilization Reagent,* or flash-freezing in liquid nitrogen.†
- Homogenized tissue lysates (in Buffer RLT, step 4) can be stored at –70°C for several months. To process frozen lysates, thaw samples at room temperature or at 37°C in a water bath until they are completely thawed and salts in the lysis buffer are dissolved. Avoid extended treatment at 37°C, which can cause chemical degradation of the RNA. Continue with step 5.
- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature.
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 5 for safety information. Take appropriate safety measures and wear gloves when handling.
- All steps of the protocol, including centrifugation, should be performed at room temperature. During the procedure, work quickly.
- Blue (marked with a ▲) denotes reduced volumes that can be used with the Leica AS LMD System; red (marked with a ●) denotes amounts when using other laser microdissection systems.

* *A modified protocol for preparation of RNA*later *preserved tissues for histological studies is available from QIAGEN Technical Services; please inquire.*

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

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 with the kit).
- β-Mercaptoethanol (β-ME) must be added to Buffer RLT before use. Add 10 µl β-ME per 1 ml Buffer RLT. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT is stable at room temperature for 1 month after addition of β-ME.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.
- Prepare DNase I stock solution before using the RNase-free DNase for the first time. Dissolve the solid DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided. Take care that no DNase I is lost when opening the vial. Mix gently by inverting the tube. **Do not vortex**.

  For long-term storage of reconstituted DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.
- When processing <5000 cells, carrier RNA should be added to the lysate before homogenization. The RNeasy Micro Kit contains poly-A RNA to be added as carrier RNA (see "Carrier RNA", page 17). Before using for the first time, dissolve the carrier RNA (310 µg) in 1 ml RNase-free water. Store this stock solution at –20°C, and use it to make fresh dilutions for each set of RNA preps.

  The concentration of this stock solution is 310 µg/ml (= 310 ng/µl). To make a working solution (4 ng/µl) for 10 preps, add 5 µl of the dissolved RNA to 34 µl of Buffer RLT and mix by pipetting. Take 6 µl of this diluted solution, and add it to 54 µl Buffer RLT. The final concentration is 4 ng/µl. Add 5 µl of this solution to the lysate in step 3.

Procedure

1. **Collect the sample directly into an appropriate volume of Buffer RLT (the volume depends on the collection vessel used for the microdissection, but should not be greater than ▲ 65 µl or ● 300 µl).**
2. **If necessary, transfer the sample and Buffer RLT into a larger reaction vessel (such as a 1.5 ml or 2.0 ml microcentrifuge tube).**

   This step is generally not necessary when using the Leica AS LMD System.
3. **Adjust the sample volume to ▲ 75 µl or ● 350 µl with Buffer RLT.**

   Note: When processing <5000 cells, add 20 ng of carrier RNA (5 µl of a 4 ng/µl solution) to the lysate before homogenization. Prepare a dilution of the carrier RNA provided, as described in "Things to do before starting".
4. **Vortex the sample for 30 s.**

   No further homogenization steps are necessary.

5. **Add 1 volume (▲ 75 µl or ● 350 µl) of 70% ethanol to the homogenized lysate, and mix well by pipetting. Do not centrifuge. Continue immediately with step 6.**

   If some lysate is lost during homogenization, reduce the volume of 70% ethanol accordingly.

   A precipitate may form after the addition of ethanol, but this will not affect the RNeasy procedure.

6. **Apply the sample, including any precipitate that may have formed, to an RNeasy MinElute Spin Column in a 2 ml collection tube (supplied). Close the tube gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.***

   Reuse the collection tube in step 7.

7. **Add 350 µl Buffer RW1 to the RNeasy MinElute Spin Column. Close the tube gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the column. Discard the flow-through.***

   Reuse the collection tube in step 9.

   Optional: If on-column DNase treatment using the RNase-free DNase and Buffer RDD is not desired, increase the amount of Buffer RW1 in this step to 700 µl, centrifuge for 15 s at ≥8000 x *g* to wash, and discard flow-through and collection tube.* Continue the protocol with step 11.

8. **Add 10 µl DNase I stock solution to 70 µl Buffer RDD. Mix by gently inverting the tube.**

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

9. **Pipet the DNase I incubation mix (80 µl) directly onto the RNeasy MinElute silica-gel membrane, and place on the benchtop at room temperature for 15 min.**

   Note: Make sure to pipet the DNase I incubation mix directly onto the RNeasy MinElute silica-gel membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the RNeasy MinElute Spin Column.

10. **Pipet 350 µl Buffer RW1 into the RNeasy MinElute Spin Column, and centrifuge for 15 s at ≥8000 x *g*. Discard flow-through and collection tube.***

11. **Transfer the RNeasy MinElute Spin Column into a new 2 ml collection tube (supplied). Pipet 500 µl Buffer RPE onto the RNeasy MinElute Spin Column. Close the tube gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the column. Discard the flow-through.**

    Reuse the collection tube in step 12.

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

** Flow-through contains Buffer RLT or Buffer RW1 and is therefore not compatible with bleach. See page 5 for safety information.*

12. **Add 500 µl of 80% ethanol to the RNeasy MinElute Spin Column. Close the tube gently and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to dry the silica-gel membrane. Discard the flow-through and collection tube.**

    Prepare the 80% ethanol with ethanol (96–100%) and the RNase-free water supplied with the kit.

    Note: Following the centrifugation, remove the RNeasy MinElute Spin Column from the collection tube carefully so the column does not come into contact with the flow-through as this will result in carryover of ethanol.

13. **Transfer the RNeasy MinElute Spin Column into a new 2 ml collection tube (supplied). Open the cap of the spin column, and centrifuge in a microcentrifuge at full speed for 5 min. Discard the flow-through and collection tube.**

    To avoid damage to the caps, place the columns into the centrifuge with at least one empty position between each column. Place the caps so that they point in the opposite direction to the rotation of the rotor (i.e., if the rotor rotates in a clockwise direction, orient the caps in a counter-clockwise direction).

    It is important to dry the silica-gel membrane since residual ethanol may interfere with downstream reactions. Centrifuging with the caps open ensures that no ethanol is carried over during elution.

14. **To elute, transfer the spin column to a new 1.5 ml collection tube (supplied). Pipet 14 µl RNase-free water directly onto the center of the silica-gel membrane. Close the tube gently, and centrifuge for 1 min at maximum speed to elute.**

    Smaller volumes of RNase-free water can be used to obtain a higher total RNA concentration, but this will influence the overall yield. The yield will be approximately 20% less when using 10 µl RNase-free water for elution. Elution with less than 10 µl may not be enough to sufficiently hydrate the silica-gel membrane and is therefore not recommended.

    The dead volume of the RNeasy MinElute Spin Column is 2 µl; elution with 14 µl of RNase-free water results in an eluate with a volume of 12 µl.

    Note: When performing RT-PCR with RNA isolated using this procedure we recommend using the QIAGEN OneStep RT-PCR Kit. This kit contains a specially formulated blend of Omniscript™ Reverse Transcriptase, designed for RNA amounts greater than 50 ng, and Sensiscript® Reverse Transcriptase, for very small amounts of RNA (<50 ng). See page 70 for ordering information. For quantitative real-time RT-PCR we recommend QIAGEN QuantiTect™ RT-PCR Kits.

Protocol: Total RNA Isolation from Microdissected Formalin-Fixed Tissues

This protocol is designed for the isolation of RNA from microdissected, formalin-fixed animal tissue samples. For frozen samples, see "Protocol: Total RNA Isolation from Microdissected Cryosections" (page 18).

Laser-microdissected tissue specimens present a particular challenge for molecular analysis, as nucleic acids must be purified from very small amounts of starting material. In addition, fixation and staining steps may compromise the integrity of RNA, and it may be necessary either to modify fixation protocols or to use cryosections from flash-frozen specimens to minimize this problem.

In order to remove proteins, which can interfere with the procedure, the standard RNeasy Micro protocol for total RNA isolation from animal tissues has been adapted to include a proteinase K digest. Samples are lysed in a guanidine-isothiocyanate–containing lysis buffer (Buffer RLT). After dilution of the lysate, the sample is treated with proteinase K. Debris is pelleted by centrifugation. Ethanol is then added to the cleared lysate and RNA is bound to the RNeasy MinElute silica-gel membrane. Traces of DNA that may copurify are removed by a DNase treatment on the RNeasy MinElute Spin Column. DNase and any contaminants are washed away, and total RNA is eluted in RNase-free water.

Depending on the fixation protocol, the age of the samples, the staining procedure, and the storage conditions used, RNA can be highly fragmented into pieces smaller than 300 nucleotides, thus limiting the size of RNA fragments isolated. Furthermore, as the RNeasy procedure removes RNA smaller than 200 nucleotides, this can lead to an overall loss in yield if the RNA is highly degraded.

A wide range of equipment and consumables for sectioning, staining, and microdissection of specimens is available from Leica (**www.leica-microsystems.com**).

Additional reagents to be supplied by user

- QIAGEN Proteinase K, >600 mAU/ml (cat. no. 19131 or 19133). Proteinase K must be used in the procedure. If using proteinase K from another supplier, use a 20 mg/ml solution in water.

Important points before starting

- If using RNeasy Micro Kits for the first time, read "Important Notes" (page 11).
- If working with RNA for the first time, read Appendix A (page 58).

- To minimize RNA degradation, avoid prolonged storage of unstabilized samples at room temperature. RNA in tissues is not protected before fixation, stabilization in RNA*later* RNA Stabilization Reagent,* or flash-freezing in liquid nitrogen.†
- Homogenized tissue lysates (in Buffer RLT, step 4) can be stored at –70°C for several months. To process frozen lysates, thaw samples at room temperature or at 37°C in a water bath until they are completely thawed and salts in the lysis buffer are dissolved. Avoid extended treatment at 37°C, which can cause chemical degradation of the RNA. Continue with step 5.
- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature.
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 5 for safety information. Take appropriate safety measures and wear gloves when handling.
- Unless otherwise indicated, all protocol steps, including centrifugation, should be performed at room temperature. During the procedure, work quickly.

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 with the kit).
- β-Mercaptoethanol (β-ME) must be added to Buffer RLT before use. Add 10 µl β-ME per 1 ml Buffer RLT. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT is stable at room temperature for 1 month after addition of β-ME.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.
- Prepare DNase I stock solution before using the RNase-free DNase for the first time. Dissolve the solid DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided. Take care that no DNase I is lost when opening the vial. Mix gently by inverting the tube. **Do not vortex**.

  For long-term storage of reconstituted DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.

** A modified protocol for preparation of RNAlater preserved tissues for histological studies is available from QIAGEN Technical Services; please inquire.*

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

- When processing <5000 cells, carrier RNA should be added to the lysate before the proteinase K digest. The RNeasy Micro Kit contains poly-A RNA to be added as carrier RNA (see "Carrier RNA", page 17). Before using for the first time, dissolve the carrier RNA (310 µg) in 1 ml RNase-free water. Store this stock solution at –20°C, and use it to make fresh dilutions for each set of RNA preps.

  The concentration of this stock solution is 310 µg/ml (= 310 ng/µl). To make a working solution (4 ng/µl) for 10 preps, add 5 µl of the dissolved RNA to 34 µl of Buffer RLT and mix by pipetting. Take 6 µl of this diluted solution, and add it to 54 µl Buffer RLT. The final concentration is 4 ng/µl. Add 5 µl of this solution to the lysate in step 4.

Procedure

1. **Heat a water bath or heating block to 55°C for proteinase K digestion in step 6.**
2. **Collect the sample directly into an appropriate volume of Buffer RLT (the volume depends on the collection vessel used for the microdissection, but should not be greater than 140 µl).**
3. **If necessary, transfer the sample and Buffer RLT into a larger reaction vessel (such as a 1.5 ml or 2.0 ml microcentrifuge tube).**
4. **Adjust the sample volume to 150 µl with Buffer RLT.**

   Note: When processing <5000 cells, add 20 ng of carrier RNA (5 µl of a 4 ng/µl solution) to the lysate before proceeding to step 5. Prepare a dilution of the carrier RNA provided, as described in "Things to do before starting".
5. **Add 295 µl RNase-free water to the lysate. Then add 5 µl QIAGEN Proteinase K solution and mix thoroughly by pipetting.**
6. **Incubate at 55°C for 10 min.**
7. **Centrifuge for 3 min at 10,000 x *g* at room temperature.**

   A small pellet of tissue debris will form, sometimes accompanied by a thin layer or film on top of the supernatant.
8. **Pipet the supernatant (approximately 450 µl) into a new tube (not provided).**

   Avoid transferring any of the pellet. If unavoidable, however, a small amount of pelleted debris may be carried over without affecting the RNeasy Micro procedure. Hold the pipet tip under the thin layer or film on top of the supernatant, if present. This layer will usually adhere to the outside of the pipet tip and should not be transferred.

9. **Add 0.5 volumes (usually 225 µl) of ethanol (96–100%) to the cleared lysate. Mix well by pipetting. Do not centrifuge.**

   A precipitate may form after the addition of ethanol, but this will not affect the RNeasy procedure.

10. **Pipet the sample, including any precipitate that may have formed, into an RNeasy MinElute Spin Column in a 2 ml collection tube. Centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.***

    Reuse the collection tube in step 11.

11. **Pipet 350 µl Buffer RW1 into the RNeasy MinElute Spin Column, and centrifuge for 15 s at ≥8000 x *g* to wash. Discard flow-through.***

    Reuse the collection tube in step 13.

    Optional: If on-column DNase treatment using the RNase-free DNase and Buffer RDD is not desired, increase the amount of Buffer RW1 in this step to 700 µl, centrifuge for 15 s at ≥8000 x *g* to wash, and discard flow-through* and collection tube. Continue the protocol with step 15.

12. **Add 10 µl DNase I stock solution to 70 µl Buffer RDD. Mix by gently inverting the tube.**

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

13. **Pipet the DNase I incubation mix (80 µl) directly onto the RNeasy MinElute silica-gel membrane, and place on the benchtop at room temperature for 15 min.**

    Note: Make sure to pipet the DNase I incubation mix directly onto the RNeasy MinElute silica-gel membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the RNeasy MinElute Spin Column.

14. **Pipet 350 µl Buffer RW1 into the RNeasy MinElute Spin Column, and centrifuge for 15 s at ≥8000 x *g*. Discard flow-through and collection tube.***

15. **Transfer the RNeasy MinElute Spin Column into a new 2 ml collection tube (supplied). Pipet 500 µl of Buffer RPE onto the RNeasy MinElute Spin Column. Close the tube gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the column. Discard the flow-through.**

    Reuse the collection tube in step 16.

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

** Flow-through contains Buffer RLT or Buffer RW1 and is therefore not compatible with bleach. See page 5 for safety information.*

16. **Add 500 µl of 80% ethanol to the RNeasy MinElute Spin Column. Close the tube gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to dry the RNeasy MinElute silica-gel membrane.**

    Prepare the 80% ethanol with ethanol (96–100%) and the RNase-free water supplied with the kit.

    Note: Following the centrifugation, remove the RNeasy MinElute Spin Column from the collection tube carefully so the column does not come into contact with the flow-through as this will result in carryover of ethanol.

17. **Transfer the RNeasy MinElute Spin Column into a new 2 ml collection tube (supplied). Open the cap of the spin column, and centrifuge in a microcentrifuge at maximum speed for 5 min. Discard the flow-through and collection tube.**

    To avoid damage to the caps, place the columns into the centrifuge with at least one empty position between each column. Place the caps so that they point in the opposite direction to the rotation of the rotor (i.e., if the rotor rotates in a clockwise direction, orient the caps in a counter-clockwise direction).

    It is important to dry the silica-gel membrane since residual ethanol may interfere with downstream reactions. Centrifuging with the caps open ensures that no ethanol is carried over during elution.

18. **To elute, transfer the spin column to a new 1.5 ml collection tube (supplied). Pipet 14 µl RNase-free water directly onto the center of the silica-gel membrane. Close the tube gently, and centrifuge for 1 min at maximum speed to elute.**

    Smaller volumes of RNase-free water can be used to obtain a higher total RNA concentration, but this will influence the overall yield. The yield will be approximately 20% less when using 10 µl RNase-free water for elution. Elution with less than 10 µl may not be enough to sufficiently hydrate the silica-gel membrane and is therefore not recommended.

    The dead volume of the RNeasy MinElute Spin Column is 2 µl; elution with 14 µl of RNase-free water results in an eluate with a volume of 12 µl.

    Note: When performing RT-PCR with RNA isolated using this procedure we recommend using the QIAGEN OneStep RT-PCR Kit. This kit contains a specially formulated blend of Omniscript Reverse Transcriptase, designed for RNA amounts greater than 50 ng, and Sensiscript Reverse Transcriptase, for very small amounts of RNA (<50 ng). See page 70 for ordering information. For quantitative real-time RT-PCR we recommend QIAGEN QuantiTect RT-PCR Kits.

Protocol: Total RNA Isolation from Animal Tissues

This protocol is for total RNA isolation from most animal tissues. For total RNA isolation from fibrous tissues, follow the specialized protocol on page 33. For laser-microdissected samples, follow the protocols on page 18 (for cryosections) or page 22 (for formalin-fixed samples).

Determining the correct amount of starting material

It is essential to begin with the correct amount of tissue in order to obtain optimal RNA yield and purity with RNeasy MinElute Spin Columns. The maximum amount of tissue that can be used depends on the specific RNA content of the tissue used, which varies greatly between tissue types. Average RNA yields from various sources are given in Table 2 (page 12). Two main criteria limit the maximum amount of tissue to use:

- The binding capacity of the RNeasy MinElute Spin Column (45 µg RNA)
- The volume of Buffer RLT required for efficient lysis. The maximum volume of Buffer RLT that can be used in the RNeasy Micro procedure generally limits the amount of starting material to a maximum of 5 mg tissue.

Some tissues such as spleen, parts of brain, lung, and thymus tend to form precipitates during the procedure. However, this does not affect the RNA isolation.

We recommend starting with no more than 5 mg of tissue. Do not overload the column. Overloading will significantly reduce yield and quality.

Important points before starting

- If using RNeasy Micro Kits for the first time, read "Important Notes" (page 11).
- If working with RNA for the first time, read Appendix A (page 58).
- For best results, stabilize animal tissues immediately in RNA*later* RNA Stabilization Reagent. Tissues can be stored in RNA*later* TissueProtect Tubes for up to 1 day at 37°C, 7 days at 18–25°C, 4 weeks at 2–8°C, or for archival storage at –20°C or –80°C. See the *RNA*later *Handbook* for more information about RNA*later* RNA Stabilization Reagent and about stabilizing RNA in tissues.

- Fresh, frozen, or RNA*later* stabilized tissue can be used. To freeze tissue for long-term storage, flash-freeze in liquid nitrogen,* and immediately transfer to –70°C. Tissue can be stored for several months at –70°C. To process, do not allow tissue to thaw during weighing or handling before disruption in Buffer RLT. Homogenized tissue lysates (in Buffer RLT, step 4) can also be stored at –70°C for several months. To process frozen lysates, thaw samples at room temperature or at 37°C in a water bath until they are completely thawed and salts in the lysis buffer are dissolved. Avoid extended treatment at 37°C, which can cause chemical degradation of the RNA. Continue with step 5.
- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming and then place at room temperature.
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 5 for safety information. Take appropriate safety measures and wear gloves when handling.
- All steps of the protocol, including centrifugation, should be performed at room temperature. During the procedure, work quickly.

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 with the kit).
- β-Mercaptoethanol (β-ME) must be added to Buffer RLT before use. Add 10 µl β-ME per 1 ml Buffer RLT. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT is stable at room temperature for 1 month after addition of β-ME.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.
- Prepare DNase I stock solution before using the RNase-free DNase for the first time. Dissolve the solid DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided. Take care that no DNase I is lost when opening the vial. Mix gently by inverting the tube. **Do not vortex**.

  For long-term storage of reconstituted DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.

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

- When processing <10 µg tissue, carrier RNA should be added to the lysate before homogenization. The RNeasy Micro Kit contains poly-A RNA to be added as carrier RNA (see "Carrier RNA", page 17). Before using for the first time, dissolve the carrier RNA (310 µg) in 1 ml RNase-free water. Store this stock solution at –20°C, and use it to make fresh dilutions for each set of RNA preps.

  The concentration of this stock solution is 310 µg/ml (= 310 ng/µl). To make a working solution (4 ng/µl) for 10 preps, add 5 µl of the dissolved RNA to 34 µl of Buffer RLT and mix by pipetting. Take 6 µl of this diluted solution, and add it to 54 µl Buffer RLT. The final concentration is 4 ng/µl. Add 5 µl of this solution to the lysate in step 2.

Procedure

1. **Determine the amount of tissue. Do not use more than 5 mg. Proceed immediately with step 2.**

   Weighing tissue is the most accurate way to determine the amount. See page 11 for guidelines to determine the amount of starting material.

   RNA in tissues is not protected after harvesting until the sample is treated with RNA*later* RNA Stabilization Reagent, flash frozen, or disrupted and homogenized in protocol step 2. Frozen animal tissue should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible.

2. **Disrupt tissue and homogenize lysate in Buffer RLT according to steps 2a, 2b, or 2c. (Do not use more than 5 mg tissue.)**

   Disruption and homogenization of animal tissue can be performed by one of three methods. See pages 14–17 for a more detailed description of disruption and homogenization methods.

   Note: When processing <10 µg tissue, add 20 ng of carrier RNA (5 µl of a 4 ng/µl solution) to the lysate before homogenization. Prepare a dilution of the carrier RNA provided, as described in "Things to do before starting".

   Note: Incomplete homogenization will lead to significantly reduced yields and can cause clogging of the RNeasy MinElute Spin Column. Homogenization with rotor–stator homogenizers or the Mixer Mill MM 300 generally results in higher total RNA yields than with other homogenization methods.

2a. Rotor–stator homogenization:
Place the weighed (fresh, frozen, or RNA*later* stabilized) tissue in a suitably sized vessel for the homogenizer. Add 350 µl Buffer RLT. Homogenize immediately using a conventional rotor–stator homogenizer until the sample is uniformly homogeneous (usually 20–40 s). Continue the protocol with step 3.

Rotor–stator homogenization simultaneously disrupts and homogenizes the sample.

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

2b. Mixer Mill MM 300:
See "Appendix D: Disruption and Homogenization of Tissues Using the Mixer Mill MM 300" (page 67) for guidelines.

The Mixer Mill MM 300 simultaneously disrupts and homogenizes the sample.

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

2c. Mortar and pestle with QIAshredder or needle-and-syringe homogenization:
Immediately place the weighed (fresh, frozen, or RNA*later* stabilized) tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled, 2 ml microcentrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw.

Add 350 µl Buffer RLT. Pipet the lysate directly onto a QIAshredder Spin Column (not supplied; see page 71 for ordering information) placed in 2 ml collection tube, and centrifuge for 2 min at maximum speed to homogenize. Alternatively, homogenize by passing lysate at least 5 times through a 20-gauge needle fitted to an RNase-free syringe. Continue the protocol with step 3.

Grinding the sample using a mortar and pestle will disrupt the sample, but it will not homogenize it. Homogenization is carried out by centrifugation through the QIAshredder Spin Column or by passage through the 20-gauge needle.

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

3. Centrifuge the tissue lysate for 3 min at maximum speed in a microcentrifuge. Carefully transfer the supernatant to a new microcentrifuge tube (not supplied) by pipetting. Use only this supernatant (lysate) in subsequent steps.

In some preparations, very small amounts of insoluble material will be present, making the pellet invisible.

4. Add 1 volume (350 µl) of 70% ethanol to the homogenized lysate, and mix well by pipetting. Do not centrifuge. Continue immediately with step 5.

If some lysate is lost during homogenization, reduce the volume of 70% ethanol accordingly.

A precipitate may form after the addition of ethanol, but this will not affect the RNeasy procedure.

5. Apply the sample, including any precipitate that may have formed, to an RNeasy MinElute Spin Column in a 2 ml collection tube (supplied). Close the tube gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.*

Reuse the collection tube in step 6.

** Flow-through contains Buffer RLT or Buffer RW1 and is therefore not compatible with bleach. See page 5 for safety information.*

6. **Add 350 µl Buffer RW1 to the RNeasy MinElute Spin Column. Close the tube gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the column. Discard the flow-through.***

   Reuse the collection tube in step 8.

   Optional: If on-column DNase treatment using the RNase-free DNase and Buffer RDD is not desired, increase the amount of Buffer RW1 in this step to 700 µl, centrifuge for 15 s at ≥8000 x *g* to wash, and discard flow-through and collection tube.* Continue the protocol with step 10.

7. **Add 10 µl DNase I stock solution to 70 µl Buffer RDD. Mix by gently inverting the tube.**

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

8. **Pipet the DNase I incubation mix (80 µl) directly onto the RNeasy MinElute silica-gel membrane, and place on the benchtop at room temperature for 15 min.**

   Note: Make sure to pipet the DNase I incubation mix directly onto the RNeasy MinElute silica-gel membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the RNeasy MinElute Spin Column.

9. **Pipet 350 µl Buffer RW1 into the RNeasy MinElute Spin Column, and centrifuge for 15 s at ≥8000 x *g*. Discard flow-through* and collection tube.**

10. **Transfer the RNeasy MinElute Spin Column into a new 2 ml collection tube (supplied). Pipet 500 µl Buffer RPE onto the RNeasy MinElute Spin Column. Close the tube gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the column. Discard the flow-through.**

    Reuse the collection tube in step 11.

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

11. **Add 500 µl of 80% ethanol to the RNeasy MinElute Spin Column. Close the tube gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to dry the silica-gel membrane. Discard the flow-through and collection tube.**

    Prepare the 80% ethanol with ethanol (96–100%) and the RNase-free water supplied with the kit.

    Note: Following the centrifugation, remove the RNeasy MinElute Spin Column from the collection tube carefully so the column does not come into contact with the flow-through as this will result in carryover of ethanol.

** Flow-through contains Buffer RLT or Buffer RW1 and is therefore not compatible with bleach. See page 5 for safety information.*

12. **Transfer the RNeasy MinElute Spin Column into a new 2 ml collection tube (supplied). Open the cap of the spin column, and centrifuge in a microcentrifuge at full speed for 5 min. Discard the flow-through and collection tube.**

    To avoid damage to the caps, place the columns into the centrifuge with at least one empty position between each column. Place the caps so that they point in the opposite direction to the rotation of the rotor (i.e., if the rotor rotates in a clockwise direction, orient the caps in a counter-clockwise direction).

    It is important to dry the silica-gel membrane since residual ethanol may interfere with downstream reactions. Centrifuging with the caps open ensures that no ethanol is carried over during elution.

13. **To elute, transfer the spin column to a new 1.5 ml collection tube (supplied). Pipet 14 µl RNase-free water directly onto the center of the silica-gel membrane. Close the tube gently, and centrifuge for 1 min at maximum speed to elute.**

    Smaller volumes of RNase-free water can be used to obtain a higher total RNA concentration, but this will influence the overall yield. The yield will be approximately 20% less when using 10 µl RNase-free water for elution. Elution with less than 10 µl may not be enough to sufficiently hydrate the silica-gel membrane and is therefore not recommended.

    The dead volume of the RNeasy MinElute Spin Column is 2 µl; elution with 14 µl of RNase-free water results in an eluate with a volume of 12 µl.

    Note: When performing RT-PCR with RNA isolated using this procedure we recommend using the QIAGEN OneStep RT-PCR Kit. This kit contains a specially formulated blend of Omniscript Reverse Transcriptase, designed for RNA amounts greater than 50 ng, and Sensiscript Reverse Transcriptase, for very small amounts of RNA (<50 ng). See page 70 for ordering information. For quantitative real-time RT-PCR we recommend QIAGEN QuantiTect RT-PCR Kits.

Protocol: Total RNA Isolation from Fibrous Tissues

Total RNA isolation from fibrous tissues, such as skeletal muscle, heart, and skin tissue, can be difficult due to the abundance of contractile proteins, connective tissue, and collagen. In order to remove these proteins, which can interfere with the procedure, the standard RNeasy Micro protocol for total RNA isolation from animal tissues has been adapted to include a proteinase K digest. Samples are lysed in Buffer RLT. After dilution of the lysate, the sample is treated with proteinase K. Debris is pelleted by centrifugation. Ethanol is then added to the cleared lysate and RNA is bound to the RNeasy MinElute silica-gel membrane. Traces of DNA that may copurify are removed by a DNase treatment on the RNeasy MinElute Spin Column. DNase and any contaminants are washed away, and total RNA is eluted in RNase-free water.

The procedure has been used successfully for isolation of total RNA from heart, muscle, and skin tissue. For other tissues, the standard RNeasy Micro protocol is generally the method of choice. If working with other tissues rich in proteins where a proteinase K digestion might be desired, we recommend performing a comparison of the two protocols. Since the RNase-inactivating Buffer RLT must be diluted to permit proteinase K digestion, this protocol should not be used for tissues rich in RNases, such as spleen or intestine.

Additional reagents to be supplied by user

- QIAGEN Proteinase K, >600 mAU/ml (cat. no. 19131 or 19133). Proteinase K must be used in the procedure. If using proteinase K from another supplier, use a 20 mg/ml solution in water.

Determining the correct amount of starting material

It is essential to begin with the correct amount of tissue in order to obtain optimal RNA yield and purity with RNeasy MinElute Spin Columns. The maximum amount of tissue that can be used depends on the specific RNA content of the tissue used, which varies greatly between tissue types. Average RNA yields from various sources are given in Table 2 (page 12). Two main criteria limit the maximum amount of tissue to use:

- The binding capacity of the RNeasy MinElute Spin Column (45 µg RNA)
- The volume of Buffer RLT required for efficient lysis. The maximum volume of Buffer RLT that can be used in the RNeasy Micro procedure generally limits the amount of starting material to a maximum of 5 mg tissue.

We recommend starting with no more than 5 mg of tissue. Do not overload the column. Overloading will significantly reduce yield and quality.

Important points before starting

- If using RNeasy Micro Kits for the first time, read "Important Notes" (page 11).
- If working with RNA for the first time, read Appendix A (page 58).
- For best results, stabilize animal tissues immediately in RNA*later* RNA Stabilization Reagent. Tissues can be stored in RNA*later* TissueProtect Tubes for up to 1 day at 37°C, 7 days at 18–25°C, 4 weeks at 2–8°C, or for archival storage at –20°C or –80°C. See the *RNA*later *Handbook* for more information about RNA*later* RNA Stabilization Reagent and about stabilizing RNA in tissues.
- Fresh, frozen, or RNA*later* stabilized tissue can be used. To freeze tissue for long-term storage, flash-freeze in liquid nitrogen,* and immediately transfer to –70°C. Tissue can be stored for several months at –70°C. To process, do not allow tissue to thaw during weighing or handling before disruption in Buffer RLT. Homogenized tissue lysates (in Buffer RLT, step 5) can also be stored at –70°C for several months. To process frozen lysates, thaw samples at room temperature or at 37°C in a water bath until they are completely thawed and salts in the lysis buffer are dissolved. Avoid extended treatment at 37°C, which can cause chemical degradation of the RNA. Continue with step 6.
- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature.
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 5 for safety information. Take appropriate safety measures and wear gloves when handling.
- Unless otherwise indicated, all protocol steps, including centrifugation, should be performed at room temperature. During the procedure, work quickly.

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 with the kit).
- β-Mercaptoethanol (β-ME) must be added to Buffer RLT before use. Add 10 µl β-ME per 1 ml Buffer RLT. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT is stable at room temperature for 1 month after addition of β-ME.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.

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

- Prepare DNase I stock solution before using the RNase-free DNase for the first time. Dissolve the solid DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided. Take care that no DNase I is lost when opening the vial. Mix gently by inverting the tube. **Do not vortex**.

  For long-term storage of reconstituted DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.

- When processing <10 µg tissue, carrier RNA should be added to the lysate before homogenization. The RNeasy Micro Kit contains poly-A RNA to be added as carrier RNA (see "Carrier RNA", page 17). Before using for the first time, dissolve the carrier RNA (310 µg) in 1 ml RNase-free water. Store this stock solution at –20°C, and use it to make fresh dilutions for each set of RNA preps.

  The concentration of this stock solution is 310 µg/ml (= 310 ng/µl). To make a working solution (4 ng/µl) for 10 preps, add 5 µl of the dissolved RNA to 34 µl of Buffer RLT and mix by pipetting. Take 6 µl of this diluted solution, and add it to 54 µl Buffer RLT. The final concentration is 4 ng/µl. Add 5 µl of this solution to the lysate in step 3.

Procedure

1. **Heat a water bath or heating block to 55°C for proteinase K digestion in step 5.**
2. **Determine the amount of tissue. Do not use more than 5 mg. Proceed immediately with step 3.**

   Weighing tissue is the most accurate way to determine the amount. See page 11 for guidelines to determine the amount of starting material.

   RNA in tissues is not protected after harvesting until the sample is treated with RNA*later* RNA Stabilization Reagent, flash frozen, or disrupted and homogenized in protocol step 3. Frozen animal tissue should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible.
3. **Disrupt tissue and homogenize lysate in Buffer RLT according to steps 3a or 3b.**

   See pages 14–17 for a more detailed description of disruption and homogenization methods.

   Note: When processing <10 µg tissue, add 20 ng of carrier RNA (5 µl of a 4 ng/µl solution) to the lysate before homogenization. Prepare a dilution of the carrier RNA provided, as described in "Things to do before starting".

   Note: Incomplete homogenization will lead to significantly reduced yields and can cause clogging of the RNeasy MinElute Spin Column. Homogenization with rotor–stator homogenizers or the Mixer Mill MM 300 generally results in higher total RNA yields than with other homogenization methods.

3a. Rotor–stator homogenization:

Place the weighed (fresh, frozen, or RNA*later* stabilized) tissue in a suitably sized vessel for the homogenizer. Add 150 µl Buffer RLT, and homogenize immediately using a conventional rotor–stator homogenizer until the sample is uniformly homogeneous (usually 20–40 s). Continue the protocol with step 4.

Rotor–stator homogenization simultaneously disrupts and homogenizes the sample.

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

3b. Mixer Mill MM 300:

See "Appendix D: Disruption and Homogenization of Tissues Using the Mixer Mill MM 300" (page 67) for guidelines.

The Mixer Mill MM 300 simultaneously disrupts and homogenizes the sample.

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

Alternative method for disruption and homogenization: The sample can alternatively be disrupted using a mortar and pestle and homogenized using QIAshredder homogenizers (not supplied; see page 71 for ordering information) or a needle and syringe. These methods, however, generally result in lower RNA yields. Homogenization with rotor–stator homogenizers is the method of choice for heart, muscle, or skin tissue.

- Immediately place the weighed (fresh, frozen, or RNA*later* stabilized) tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into a liquid-nitrogen–cooled tube. Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw.
- Add 150 µl Buffer RLT.

  Note: Ensure that β-ME is added to Buffer RLT before use (see "Things to do before starting).
- Pipet the lysate directly onto a QIAshredder Spin Column placed in a 2 ml collection tube, and centrifuge for 2 min at maximum speed. Alternatively, homogenize by passing the lysate at least 5–10 times through a 20-gauge needle (0.9 mm diameter) fitted to a syringe. Continue the protocol with step 4.

4. Add 295 µl RNase-free water to the homogenate. Then add 5 µl QIAGEN Proteinase K solution and mix thoroughly by pipetting.

5. Incubate at 55°C for 10 min.

6. Centrifuge for 3 min at 10,000 x *g* at room temperature.

A small pellet of tissue debris will form, sometimes accompanied by a thin layer or film on top of the supernatant.

7. **Pipet the supernatant (approximately 450 µl) into a new tube (not provided).**

   Avoid transferring any of the pellet. If unavoidable, however, a small amount of pelleted debris may be carried over without affecting the RNeasy Micro procedure. Hold the pipet tip under the thin layer or film on top of the supernatant, if present. This layer will usually adhere to the outside of the pipet tip and should not be transferred.

8. **Add 0.5 volumes (usually 225 µl) of ethanol (96–100%) to the cleared lysate. Mix well by pipetting. Do not centrifuge.**

   A precipitate may form after the addition of ethanol, but this will not affect the RNeasy procedure.

9. **Pipet the sample, including any precipitate that may have formed, into an RNeasy MinElute Spin Column in a 2 ml collection tube. Centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.***

   Reuse the collection tube in step 10.

10. **Pipet 350 µl Buffer RW1 into the RNeasy MinElute Spin Column, and centrifuge for 15 s at ≥8000 x *g* to wash. Discard flow-through.***

    Reuse the collection tube in step 12.

    Optional: If on-column DNase treatment using the RNase-free DNase and Buffer RDD is not desired, increase the amount of Buffer RW1 in this step to 700 µl, centrifuge for 15 s at ≥8000 x *g* to wash, and discard flow-through and collection tube.* Continue the protocol with step 14.

11. **Add 10 µl DNase I stock solution to 70 µl Buffer RDD. Mix by gently inverting the tube.**

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

12. **Pipet the DNase I incubation mix (80 µl) directly onto the RNeasy MinElute silica-gel membrane, and place on the benchtop at room temperature for 15 min.**

    Note: Make sure to pipet the DNase I incubation mix directly onto the RNeasy MinElute silica-gel membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the RNeasy MinElute Spin Column.

13. **Pipet 350 µl Buffer RW1 into the RNeasy MinElute Spin Column, and centrifuge for 15 s at ≥8000 x *g*. Discard flow-through* and collection tube.**

** Flow-through contains Buffer RLT or Buffer RW1 and is therefore not compatible with bleach. See page 5 for safety information.*

14. **Transfer the RNeasy MinElute Spin Column into a new 2 ml collection tube (supplied). Pipet 500 µl Buffer RPE onto the RNeasy MinElute Spin Column. Close the tube gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the column. Discard the flow-through.**

    Reuse the collection tube in step 15.

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

15. **Add 500 µl of 80% ethanol to the RNeasy MinElute Spin Column. Close the tube gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to dry the RNeasy MinElute silica-gel membrane.**

    Prepare the 80% ethanol with ethanol (96–100%) and the RNase-free water supplied with the kit.

    Note: Following the centrifugation, remove the RNeasy MinElute Spin Column from the collection tube carefully so the column does not come into contact with the flow-through as this will result in carryover of ethanol.

16. **Transfer the RNeasy MinElute Spin Column into a new 2 ml collection tube (supplied). Open the cap of the spin column, and centrifuge in a microcentrifuge at maximum speed for 5 min. Discard the flow-through and collection tube.**

    To avoid damage to the caps, place the columns into the centrifuge with at least one empty position between each column. Place the caps so that they point in the opposite direction to the rotation of the rotor (i.e., if the rotor rotates in a clockwise direction, orient the caps in a counter-clockwise direction).

    It is important to dry the silica-gel membrane since residual ethanol may interfere with downstream reactions. Centrifuging with the caps open ensures that no ethanol is carried over during elution.

17. **To elute, transfer the spin column to a new 1.5 ml collection tube (supplied). Pipet 14 µl RNase-free water directly onto the center of the silica-gel membrane. Close the tube gently, and centrifuge for 1 min at maximum speed to elute.**

    Smaller volumes of RNase-free water can be used to obtain a higher total RNA concentration, but this will influence the overall yield. The yield will be approximately 20% less when using 10 µl RNase-free water for elution. Elution with less than 10 µl may not be enough to sufficiently hydrate the silica-gel membrane and is therefore not recommended.

    The dead volume of the RNeasy MinElute Spin Column is 2 µl; elution with 14 µl of RNase-free water results in an eluate with a volume of 12 µl.

    Note: When performing RT-PCR with RNA isolated using this procedure we recommend using the QIAGEN OneStep RT-PCR Kit. This kit contains a specially formulated blend of Omniscript Reverse Transcriptase, designed for RNA amounts greater than 50 ng, and Sensiscript Reverse Transcriptase, for very small amounts of RNA (<50 ng). See page 70 for ordering information. For quantitative real-time RT-PCR we recommend QIAGEN QuantiTect RT-PCR Kits.

Protocol: Total RNA Isolation from Animal Cells

Determining the correct amount of starting material

It is essential to use the correct number of cells in order to obtain optimal RNA yield and purity with RNeasy MinElute Spin Columns. The maximum number of cells that can be used depends on the specific RNA content of the cell line used, which varies greatly between cell types. Two main criteria limit the maximum number of cells to use:

- The binding capacity of the RNeasy MinElute Spin Column (45 µg RNA)
- The volume of Buffer RLT required for efficient lysis. The maximum volume of Buffer RLT that can be used in the RNeasy Micro procedure generally limits the amount of starting material to a maximum of 5 x 10^5 cells.

If the cell type used is not shown in Table 2 (page 12) and you have no information about the RNA content of your starting material, we recommend starting with no more than 5 x 10^5 cells.

Do not overload the column. Overloading will significantly reduce yield and purity.

The numbers of HeLa cells expected in certain cell culture vessels are shown in Table 3 (page 13).

Important points before starting

- If using RNeasy Micro Kits for the first time, read "Important Notes" (page 11).
- If working with RNA for the first time, read Appendix A (page 58).
- Cell pellets can be stored at –70°C for later use or used directly in the procedure. Determine the number of cells before freezing. Frozen cell pellets should be thawed slightly so that cell pellets can be dislodged by flicking in step 2. Homogenized cell lysates (in Buffer RLT, step 3) can be stored at –70°C for several months. To process frozen lysates, thaw samples at room temperature or at 37°C in a water bath until they are completely thawed and salts in the lysis buffer are dissolved. Avoid extended treatment at 37°C, which can cause chemical degradation of the RNA. If any insoluble material is visible, centrifuge for 5 minutes at 3000–5000 x *g*. Transfer supernatant to a new RNase-free glass or polypropylene tube, and continue with step 4.
- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature.
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 5 for safety information. Take appropriate safety measures and wear gloves when handling.
- All steps of the protocol, including centrifugation, should be performed at room temperature. During the procedure, work quickly.

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 with the kit).
- β-Mercaptoethanol (β-ME) must be added to Buffer RLT before use. Add 10 µl β-ME per 1 ml Buffer RLT. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT is stable at room temperature for 1 month after addition of β-ME.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.
- Prepare DNase I stock solution before using the RNase-free DNase for the first time. Dissolve the solid DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided. Take care that no DNase I is lost when opening the vial. Mix gently by inverting the tube. **Do not vortex**.

  For long-term storage of reconstituted DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.
- When processing <5000 cells, carrier RNA should be added to the lysate before homogenization. The RNeasy Micro Kit contains poly-A RNA to be added as carrier RNA (see "Carrier RNA", page 17). Before using for the first time, dissolve the carrier RNA (310 µg) in 1 ml RNase-free water. Store this stock solution at –20°C, and use it to make fresh dilutions for each set of RNA preps.

  The concentration of this stock solution is 310 µg/ml (= 310 ng/µl). To make a working solution (4 ng/µl) for 10 preps, add 5 µl of the dissolved RNA to 34 µl of Buffer RLT and mix by pipetting. Take 6 µl of this diluted solution, and add it to 54 µl Buffer RLT. The final concentration is 4 ng/µl. Add 5 µl of this solution to the lysate in step 3.

Procedure

1. Harvest cells according to steps 1a (for cells grown in suspension) or 1b (for cells grown in a monolayer).

1a. Cells grown in suspension (do not use more than 5 x 10^5 cells):
Determine the number of cells. Pellet the appropriate number of cells by centrifuging for 5 min at 300 x *g* in a centrifuge tube (not supplied). Carefully remove all supernatant by aspiration, and continue with step 2 of the protocol.

Note: Incomplete removal of the cell-culture medium will inhibit lysis and dilute the lysate, affecting the conditions for binding of RNA to the RNeasy MinElute silica-gel membrane. Both effects may reduce RNA yield.

1b. Cells grown in a monolayer (do not use more than 5 x 10^5 cells):
Cells grown in a monolayer in cell-culture vessels can either be lysed directly in the culture vessel (up to 10 cm diameter) or trypsinized and collected as a cell pellet before lysis. Cells grown in a monolayer in cell-culture flasks should always be trypsinized.

To lyse cells directly in culture dish:
Determine the number of cells. (Table 3 on page 13 gives approximate cell numbers for various cell-culture vessels.) Completely aspirate cell-culture medium,* and continue immediately with step 2 of the protocol.

Note: Incomplete removal of the cell-culture medium will inhibit lysis and dilute the lysate, affecting the conditions for binding of RNA to the RNeasy MinElute silica-gel membrane. Both effects may reduce RNA yield.

To trypsinize cells:
Determine the number of cells. (Table 3 on page 13 gives approximate cell numbers for various cell-culture vessels.) Aspirate medium,* and wash cells with PBS.* Aspirate PBS and add 0.10–0.25% trypsin* in PBS to trypsinize the cells. After cells detach from the dish or flask, add medium (containing serum* to inactivate the trypsin), transfer cells to an RNase-free glass or polypropylene centrifuge tube (not supplied), and pellet by centrifugation at 300 x *g* for 5 min. Completely aspirate supernatant, and continue with step 2 of the protocol.

Note: Incomplete removal of the cell-culture medium will inhibit lysis and dilute the lysate, affecting the conditions for binding of RNA to the RNeasy MinElute silica-gel membrane. Both effects may reduce RNA yield.

2. **Disrupt cells by addition of Buffer RLT. For pelleted cells, loosen the cell pellet thoroughly by flicking the tube. Add 350 µl Buffer RLT. Vortex or pipet to mix, and proceed to step 3.**

   When processing ≤1 x 10^5 cells, the amount of Buffer RLT can be reduced to 75 µl. This allows use of smaller tubes for cell pelleting. Vortex for 1 min to homogenize and proceed with step 4.

   Note: Incomplete loosening of the cell pellet may lead to inefficient lysis and reduced yields. Ensure that β-ME is added to Buffer RLT before use (see "Things to do before starting").

   For direct lysis of cells grown in a monolayer, add 350 µl Buffer RLT to the cell-culture dish. Collect cell lysate with a rubber policeman. Pipet lysate into a microcentrifuge tube (not supplied). Vortex or pipet to mix, and ensure that no cell clumps are visible before proceeding to step 3.

   When processing ≤1 x 10^5 cells, the amount of Buffer RLT can be reduced to 75 µl. This may be necessary for multiwell plates or cell-culture dishes.

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

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

3. Homogenize the sample according to steps 3a, 3b, or 3c.

One of three methods may be used to homogenize the sample. After homogenization, proceed with step 4. See "Disruption and homogenization of starting materials", pages 14–17, for a more detailed description of homogenization methods.

Note: When processing <5000 cells, add 20 ng of carrier RNA (5 µl of a 4 ng/µl solution) to the lysate before homogenization. Prepare a dilution of the carrier RNA provided, as described in "Things to do before starting".

Note: Incomplete homogenization will lead to significantly reduced yields and can cause clogging of the RNeasy MinElute Spin Column. Homogenization with rotor–stator or QIAshredder homogenizers generally results in higher RNA yields than with a syringe and needle.

3a. Pipet the lysate directly onto a QIAshredder Spin Column (not supplied; see page 71 for ordering information) placed in a 2 ml collection tube, and centrifuge for 2 min at maximum speed.

3b. Homogenize cells for 30 s using a rotor–stator homogenizer.

3c. Pass the lysate at least 5 times through a 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe.

4. Add 1 volume (usually 350 µl) of 70% ethanol to the homogenized lysate, and mix well by pipetting. Do not centrifuge.

If some lysate is lost during homogenization, reduce the volume of 70% ethanol accordingly. If only 75 µl of Buffer RLT was used in step 2, then add only 75 µl of 70% ethanol in this step.

Note: Visible precipitates may form after addition of ethanol when preparing RNA from certain cell lines; this will not affect the procedure.

5. Apply the sample, including any precipitate that may have formed, to an RNeasy MinElute Spin Column in a 2 ml collection tube (supplied). Close the tube gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.*

Reuse the collection tube in step 6.

** Flow-through contains Buffer RLT or Buffer RW1 and is therefore not compatible with bleach. See page 5 for safety information.*

6. **Add 350 µl Buffer RW1 to the RNeasy MinElute Spin Column. Close the tube gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the column. Discard the flow-through.***

   Reuse the collection tube in step 8.

   Optional: If on-column DNase treatment using the RNase-free DNase and Buffer RDD is not desired, increase the amount of Buffer RW1 in this step to 700 µl, centrifuge for 15 s at ≥8000 x *g* to wash, and discard flow-through and collection tube.* Continue the protocol with step 10.

7. **Add 10 µl DNase I stock solution to 70 µl Buffer RDD. Mix by gently inverting the tube.**

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

8. **Pipet the DNase I incubation mix (80 µl) directly onto the RNeasy MinElute silica-gel membrane, and place on the benchtop at room temperature for 15 min.**

   Note: Make sure to pipet the DNase I incubation mix directly onto the RNeasy MinElute silica-gel membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the RNeasy MinElute Spin Column.

9. **Pipet 350 µl Buffer RW1 into the RNeasy MinElute Spin Column, and centrifuge for 15 s at ≥8000 x *g*. Discard flow-through* and collection tube.**

10. **Transfer the RNeasy MinElute Spin Column into a new 2 ml collection tube (supplied). Pipet 500 µl Buffer RPE onto the RNeasy MinElute Spin Column. Close the tube gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the column. Discard the flow-through.**

    Reuse the collection tube in step 11.

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

11. **Add 500 µl of 80% ethanol to the RNeasy MinElute Spin Column. Close the tube gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to dry the silica-gel membrane. Discard the flow-through and collection tube.**

    Prepare the 80% ethanol with ethanol (96–100%) and the RNase-free water supplied with the kit.

    Note: Following the centrifugation, remove the RNeasy MinElute Spin Column from the collection tube carefully so the column does not come into contact with the flow-through as this will result in carryover of ethanol.

** Flow-through contains Buffer RLT or Buffer RW1 and is therefore not compatible with bleach. See page 5 for safety information.*

12. **Transfer the RNeasy MinElute Spin Column into a new 2 ml collection tube (supplied). Open the cap of the spin column, and centrifuge in a microcentrifuge at maximum speed for 5 min. Discard the flow-through and collection tube.**

    To avoid damage to the caps, place the columns into the centrifuge with at least one empty position between each column. Place the caps so that they point in the opposite direction to the rotation of the rotor (i.e., if the rotor rotates in a clockwise direction, orient the caps in a counter-clockwise direction).

    It is important to dry the silica-gel membrane since residual ethanol may interfere with downstream reactions. Centrifuging with the caps open ensures that no ethanol is carried over during elution.

13. **To elute, transfer the spin column to a new 1.5 ml collection tube (supplied). Pipet 14 µl RNase-free water directly onto the center of the silica-gel membrane. Close the tube gently, and centrifuge for 1 min at maximum speed to elute.**

    Smaller volumes of RNase-free water can be used to obtain a higher total RNA concentration, but this will influence the overall yield. The yield will be approximately 20% less when using 10 µl RNase-free water for elution. Elution with less than 10 µl may not be enough to sufficiently hydrate the silica-gel membrane and is therefore not recommended.

    The dead volume of the RNeasy MinElute Spin Column is 2 µl; elution with 14 µl of RNase-free water results in an eluate with a volume of 12 µl.

    Note: When performing RT-PCR with RNA isolated using this procedure we recommend using the QIAGEN OneStep RT-PCR Kit. This kit contains a specially formulated blend of Omniscript Reverse Transcriptase, designed for RNA amounts greater than 50 ng, and Sensiscript Reverse Transcriptase, for very small amounts of RNA (<50 ng). See page 70 for ordering information. For quantitative real-time RT-PCR we recommend QIAGEN QuantiTect RT-PCR Kits.

Protocol: RNA Cleanup and Concentration

RNeasy Micro Kits can be used to clean up and concentrate RNA previously isolated by different methods or after enzymatic reactions, such as labeling or DNase digestion. For cleanup and concentration of total cellular RNA isolated using the PAXgene Blood RNA Kit, see "Protocol: RNA Cleanup and Concentration after Isolation Using the PAXgene Blood RNA Kit" on page 49.

Important points before starting

- A maximum of 45 µg RNA in a maximum volume of 200 µl can be used. This amount corresponds to the binding capacity of the RNeasy MinElute Spin Columns.
- If working with RNA for the first time, read Appendix A (page 58).
- Generally, DNase digestion is not required since the RNeasy MinElute silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan® or LightCycler® RT-PCR analysis with a low-abundance target). We recommend using the RNase-free DNase and Buffer RDD, included in this kit for optional on-column DNase digestion, as described in the protocol. Alternatively, the RNA solution can be digested with DNase before starting the procedure. The DNase is then removed during the cleanup procedure.
- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature.
- Buffer RLT contains a guanidine salt and is therefore not compatible with disinfecting reagents containing bleach. See page 5 for safety information. Take appropriate safety measures and wear gloves when handling.
- All steps of the protocol, including centrifugation, should be performed at room temperature. During the procedure, work quickly.
- Blue (marked with a ▲) denotes amounts for starting volumes ≤100 µl; red (marked with a ●) denotes amounts for starting volumes of 100–200 µl.

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 with the kit).
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.
- Recommended: For on-column DNase digestion, prepare DNase I stock solution before using the RNase-free DNase for the first time. Dissolve the solid DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided. Take care that no DNase I is lost when opening the vial. Mix gently by inverting the tube. **Do not vortex**.

For long-term storage of reconstituted DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.

- Optional: Adding β-Mercaptoethanol (β-ME) to Buffer RLT may be helpful when cleaning up crude preps of RNA (e.g., after salting-out methods) or samples that contain large amounts of RNases. Add 10 µl β-ME per 1 ml Buffer RLT. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT is stable at room temperature for 1 month after addition of β-ME.

Procedure

1. **Adjust sample to a volume of ▲ 100 µl or ● 200 µl with RNase-free water. Add ▲ 350 µl or ● 700 µl Buffer RLT, and mix thoroughly.**

   If starting with an RNA pellet, be sure that the pellet is dissolved in the RNase-free water (supplied) before adding Buffer RLT.

   Optional: Add β-ME to Buffer RLT before use (see "Things to do before starting").

2. **Add ▲ 250 µl or ● 500 µl of 96–100% ethanol to the diluted RNA, and mix thoroughly by pipetting. Do not centrifuge. Continue immediately with step 3.**

3. **Apply 700 µl of the sample to an RNeasy MinElute Spin Column in a 2 ml collection tube (supplied). Close the tube gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.***

   For ● samples >700 µl, apply the remaining sample (up to 700 µl) and repeat the centrifugation. Discard the flow-through and collection tube.*

4. **For on-column DNase digestion (recommended), continue immediately with steps 5–8. If on-column DNase treatment using the RNase-free DNase and Buffer RDD is not desired, proceed directly with step 9.**

5. **Recommended: Pipet 350 µl Buffer RW1 into the RNeasy MinElute Spin Column, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash. Discard the flow-through.**

   Reuse the collection tube in step 7.

6. **Recommended: Add 10 µl DNase I stock solution (see above) to 70 µl Buffer RDD. Mix by gently inverting the tube.**

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

** Flow-through contains Buffer RLT or Buffer RW1 and is therefore not compatible with bleach. See page 5 for safety information.*

7. **Recommended: Pipet the DNase I incubation mix (80 µl) directly onto the RNeasy MinElute silica-gel membrane, and place on the benchtop at room temperature for 15 min.**

   Note: Make sure to pipet the DNase I incubation mix directly onto the RNeasy MinElute silica-gel membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the RNeasy MinElute Spin Column.

8. **Recommended: Pipet 350 µl Buffer RW1 into the RNeasy MinElute Spin Column, and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.***

9. **Transfer the spin column into a new 2 ml collection tube (supplied). Pipet 500 µl Buffer RPE onto the spin column. Close the tube gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the column. Discard the flow-through.**

   Reuse the collection tube in step 10.

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

10. **Add 500 µl of 80% ethanol to the RNeasy MinElute Spin Column. Close the tube gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to dry the silica-gel membrane. Discard the flow-through and collection tube.**

    Prepare the 80% ethanol with ethanol (96–100%) and the RNase-free water supplied with the kit.

    Note: Following the centrifugation, remove the RNeasy MinElute Spin Column from the collection tube carefully so the column does not come into contact with the flow-through as this will result in carryover of ethanol.

11. **Transfer the RNeasy MinElute Spin Column into a new 2 ml collection tube (supplied). Open the cap of the spin column, and centrifuge in a microcentrifuge at full speed for 5 min. Discard the flow-through and collection tube.**

    To avoid damage to the caps, place the columns into the centrifuge with at least one empty position between each column. Place the caps so that they point in the opposite direction to the rotation of the rotor (i.e., if the rotor rotates in a clockwise direction, orient the caps in a counter-clockwise direction).

    It is important to dry the silica-gel membrane since residual ethanol may interfere with downstream reactions. Centrifuging with the caps open ensures that no ethanol is carried over during elution.

** Flow-through contains Buffer RLT or Buffer RW1 and is therefore not compatible with bleach. See page 5 for safety information.*

12. **To elute, transfer the spin column to a new 1.5 ml collection tube (supplied). Pipet 14 µl RNase-free water directly onto the center of the silica-gel membrane. Close the tube gently, and centrifuge for 1 min at maximum speed to elute.**

    Smaller volumes of RNase-free water can be used to obtain a higher total RNA concentration, but this will influence the overall yield. The yield will be approximately 20% less when using 10 µl RNase-free water for elution. Elution with less than 10 µl may not be enough to sufficiently hydrate the silica-gel membrane and is therefore not recommended.

    The dead volume of the RNeasy MinElute Spin Column is 2 µl; elution with 14 µl of RNase-free water results in an eluate with a volume of 12 µl.

    Note: When performing RT-PCR with RNA isolated using this procedure we recommend using the QIAGEN OneStep RT-PCR Kit. This kit contains a specially formulated blend of Omniscript Reverse Transcriptase, designed for RNA amounts greater than 50 ng, and Sensiscript Reverse Transcriptase, for very small amounts of RNA (<50 ng). See page 70 for ordering information. For quantitative real-time RT-PCR we recommend QIAGEN QuantiTect RT-PCR Kits.

Protocol: RNA Cleanup and Concentration after Isolation Using the PAXgene Blood RNA Kit

This protocol is designed to clean up and concentrate total cellular RNA isolated using the PAXgene Blood RNA Kit.

Important points before starting

- A maximum of 45 µg RNA in a maximum volume of 200 µl can be used. This amount corresponds to the binding capacity of the RNeasy MinElute Spin Columns.
- PAXgene Blood RNA eluates can be pooled before cleanup as long as the total amount of RNA is ≤45 µg and the total volume is ≤200 µl. In order to keep the pooled volume ≤200 µl, perform the second elution step in the PAXgene purification protocol by re-applying the first eluate to the PAXgene Column (instead of using another 40 µl of Buffer BR5).
- The final denaturation step of the PAXgene Blood RNA Kit protocol is not necessary before cleanup. Denaturation of the RNA is included as a step at the end of this protocol.
- Buffer BR5 (included in the PAXgene Blood RNA Kit) is required for elution in this protocol.
- If working with RNA for the first time, read Appendix A (page 58).
- Generally, DNase digestion is not required since the RNeasy MinElute silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan or LightCycler RT-PCR analysis with a low-abundance target). We recommend using the RNase-free DNase and Buffer RDD, included in this kit for optional on-column DNase digestion, as described in the protocol. Alternatively, on-column DNase digestion can be carried out during the PAXgene procedure, or the RNA solution can be digested with DNase before starting the procedure. The DNase is then removed during the cleanup procedure.
- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature.
- Buffer RLT contains a guanidine salt and is therefore not compatible with disinfecting reagents containing bleach. See page 5 for safety information. Take appropriate safety measures and wear gloves when handling.
- Unless otherwise indicated, all protocol steps, including centrifugation, should be performed at room temperature. During the procedure, work quickly.
- Blue (marked with a ▲) denotes amounts for starting volumes ≤100 µl; red (marked with a ●) denotes amounts for starting volumes of 100–200 µl.

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 with the kit).
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.
- Recommended: For on-column DNase digestion, prepare DNase I stock solution before using the RNase-free DNase for the first time. Dissolve the solid DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided. Take care that no DNase I is lost when opening the vial. Mix gently by inverting the tube. **Do not vortex**.

  For long-term storage of reconstituted DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.

Procedure

1. **Heat a heating block or water bath to 65°C for use in step 14.**
2. **Adjust sample to a volume of ▲ 100 µl or ● 200 µl with RNase-free water. Add ▲ 350 µl or ● 700 µl Buffer RLT, and mix thoroughly.**
3. **Add ▲ 250 µl or ● 500 µl of 96–100% ethanol to the diluted RNA, and mix thoroughly by pipetting. Do not centrifuge. Continue immediately with step 4.**
4. **Apply 700 µl of the sample to an RNeasy MinElute Spin Column in a 2 ml collection tube (supplied). Close the tube gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.***

   For ● samples >700 µl, apply the remaining sample (up to 700 µl) and repeat the centrifugation. Discard the flow-through and collection tube.*
5. **For on-column DNase digestion (recommended), continue immediately with steps 6–9. If on-column DNase treatment using the RNase-free DNase and Buffer RDD is not desired, proceed directly with step 10.**
6. **Recommended: Pipet 350 µl Buffer RW1 into the RNeasy MinElute Spin Column, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash. Discard the flow-through.***

   Reuse the collection tube in step 8.
7. **Recommended: Add 10 µl DNase I stock solution (see above) to 70 µl Buffer RDD. Mix by gently inverting the tube.**

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

** Flow-through contains Buffer RLT or Buffer RW1 and is therefore not compatible with bleach. See page 5 for safety information.*

8. **Recommended: Pipet the DNase I incubation mix (80 µl) directly onto the RNeasy MinElute silica-gel membrane, and place on the benchtop at room temperature for 15 min.**

   Note: Make sure to pipet the DNase I incubation mix directly onto the RNeasy MinElute silica-gel membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the RNeasy MinElute Spin Column.

9. **Recommended: Pipet 350 µl Buffer RW1 into the RNeasy MinElute Spin Column, and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.***

10. **Transfer the spin column into a new 2 ml collection tube (supplied). Pipet 500 µl Buffer RPE onto the spin column. Close the tube gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the column. Discard the flow-through.**

    Reuse the collection tube in step 11.

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

11. **Add 500 µl of 80% ethanol to the RNeasy MinElute Spin Column. Close the tube gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to dry the silica-gel membrane. Discard the flow-through and collection tube.**

    Prepare the 80% ethanol with ethanol (96–100%) and the RNase-free water supplied with the kit.

    Note: Following the centrifugation, remove the RNeasy MinElute Spin Column from the collection tube carefully so the column does not come into contact with the flow-through as this will result in carryover of ethanol.

12. **Transfer the RNeasy MinElute Spin Column into a new 2 ml collection tube (supplied). Open the cap of the spin column, and centrifuge in a microcentrifuge at full speed for 5 min. Discard the flow-through and collection tube.**

    To avoid damage to the caps, place the columns into the centrifuge with at least one empty position between each column. Place the caps so that they point in the opposite direction to the rotation of the rotor (i.e., if the rotor rotates in a clockwise direction, orient the caps in a counter-clockwise direction).

    It is important to dry the silica-gel membrane since residual ethanol may interfere with downstream reactions. Centrifuging with the caps open ensures that no ethanol is carried over during elution.

** Flow-through contains Buffer RW1 and is therefore not compatible with bleach. See page 5 for safety information.*

13. **To elute, transfer the spin column to a new 1.5 ml collection tube (supplied). Pipet 14 µl Buffer BR5 (from the PAXgene Blood RNA Kit) directly onto the center of the silica-gel membrane. Close the tube gently, and centrifuge for 1 min at maximum speed to elute.**

    Smaller volumes of Buffer BR5 can be used to obtain a higher total RNA concentration, but this will influence the overall yield. The yield will be approximately 20% less when using 10 µl Buffer BR5 for elution. Elution with less than 10 µl may not be enough to sufficiently hydrate the silica-gel membrane and is therefore not recommended.

    The dead volume of the RNeasy MinElute Spin Column is 2 µl; elution with 14 µl Buffer BR5 results in an eluate with a volume of 12 µl.

14. **Incubate the eluate for 5 min at 65°C in a heating block or water bath. Following incubation, chill immediately on ice.**

    Denaturation of the eluate is essential for maximum efficiency in downstream applications such as RT-PCR, other amplification reactions, or cDNA synthesis. It is not necessary to denature samples more than once, and samples remain denatured after freezing and thawing.

    Note: When performing RT-PCR with RNA isolated using this procedure we recommend using the QIAGEN OneStep RT-PCR Kit. This kit contains a specially formulated blend of Omniscript Reverse Transcriptase, designed for RNA amounts greater than 50 ng, and Sensiscript Reverse Transcriptase, for very small amounts of RNA (<50 ng). See page 70 for ordering information. For quantitative real-time RT-PCR we recommend QIAGEN QuantiTect RT-PCR Kits.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. 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 molecular biology applications (see inside front cover for contact information).

	Comments and suggestions
Clogged column	
a) Inefficient disruption and/or homogenization	See "Disruption and homogenization of starting materials" (pages 14–17) for a detailed description of homogenization methods. Increase *g*-force and centrifugation time if necessary. In subsequent preparations, reduce the amount of starting material (see pages 11–13 and protocols) and/or increase the homogenization time. If working with tissues rich in proteins, the RNeasy Micro protocol for isolation of total RNA from fibrous tissues (page 33) may provide better results than the standard tissue protocol.
a) Too much starting material	In subsequent preparations, reduce amounts of starting material. It is essential to use the correct amount of starting material (see pages 11–13 and protocols).
b) Centrifugation temperature too low	Centrifugation at low temperatures (e.g., under refrigeration) can cause precipitates to form that can clog the RNeasy MinElute Spin Column. Unless otherwise indicated, all steps of the protocols, including centrifugation, should be performed at room temperature.

	Comments and suggestions
Low RNA yield	
a) Insufficient disruption and homogenization	See "Disruption and homogenization of starting materials" See "Disruption and homogenization of starting materials" (pages 14–17) for a detailed description of homogenization methods. Increase *g*-force and centrifugation time if necessary. In subsequent preparations, reduce the amount of starting material (see pages 11–13 and protocols) and/or increase the volume of lysis buffer and the homogenization time. If working with tissues rich in proteins, the RNeasy Micro protocol for isolation of total RNA from fibrous tissues (page 33) may provide better results than the standard tissue protocol.
b) Too much starting material	In subsequent preparations, reduce amounts of starting material. It is essential to use the correct amount of starting material (see pages 11–13 and protocols).
c) RNA still bound to the membrane	Repeat elution, but incubate the RNeasy MinElute Spin Column on the benchtop for 10 min with elution buffer before centrifuging.
d) Ethanol carryover	After the 80% ethanol wash step, be sure to dry the RNeasy MinElute silica-gel membrane by centrifugation at full speed for 5 min, as described in the protocol. Following centrifugation, remove the RNeasy MinElute Spin Column from the centrifuge tube carefully so the column does not contact the flow-through, as this will result in carryover of the ethanol.
e) 80% ethanol not made with RNase-free water	RNases can be introduced if the water used to dilute the ethanol is not RNase-free. Prepare the 80% ethanol for the wash steps using ethanol (96–100%) and the RNase-free water supplied with the kit, as described in "Things to do before starting".

	Comments and suggestions
Low or no recovery	
a) RNase-free water/Buffer BR5 buffer incorrectly dispensed	Pipet RNase-free water/Buffer BR5 to the center of the RNeasy MinElute membrane to ensure that the buffer completely covers the membrane.
b) Ethanol carryover	After the 80% ethanol wash step, be sure to dry the RNeasy MinElute silica-gel membrane by centrifugation at full speed for 5 min, as described in the protocol. Following centrifugation, remove the RNeasy MinElute Spin Column from the centrifuge tube carefully so the column does not contact the flow-through, as this will result in carryover of the ethanol.
c) 80% ethanol not made with RNase-free water	RNases can be introduced if the water used to dilute the ethanol is not RNase-free. Prepare the 80% ethanol for the wash steps using ethanol (96–100%) and the RNase-free water supplied with the kit, as described in "Things to do before starting".
Low A_{260}/A_{280} value	Use 10 mM Tris·Cl, pH 7.5,* not RNase-free water to dilute the sample before measuring purity (see Appendix B, page 60)
RNA degraded	
a) Sample inappropriately handled	For best results, ensure that samples are properly stabilized and stored in RNA*later* RNA Stabilization Reagent (see the *RNA*later *Handbook* for information about RNA*later* RNA Stabilization Reagent and about stabilizing RNA in tissues). For frozen cell pellets or tissue samples, ensure that they were flash-frozen immediately in liquid nitrogen* and properly stored at –70°C. Perform the protocol quickly, especially the first few steps. See Appendix A (page 58) and "Handling and storage of starting material" (page 13).

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

	Comments and suggestions
b) RNase contamination	Although all buffers have been tested and guaranteed RNase-free, RNases can be introduced during use. Be certain not to introduce any RNases during the procedure or later handling. See Appendix A (page 58). Do not put RNA samples into a vacuum dryer or microcentrifuge that has been used in DNA preparation where RNases may have been used.
c) 80% ethanol not made with RNase-free water	RNases can be introduced if the water used to dilute the ethanol is not RNase-free. Prepare the 80% ethanol for the wash steps using ethanol (96–100%) and the RNase-free water supplied with the kit, as described in "Things to do before starting".
DNA contamination in downstream experiments	
No DNase treatment	Follow the recommended on-column DNase digestion using RNase-free DNase and Buffer RDD at the point indicated in the individual protocols.
RNA does not perform well in downstream experiments	
a) Ethanol carryover	After the 80% ethanol wash step, be sure to dry the RNeasy MinElute silica-gel membrane by centrifugation at full speed for 5 min, as described in the protocol. Following centrifugation, remove the RNeasy MinElute Spin Column from the centrifuge tube carefully so the column does not contact the flow-through, as this will result in carryover of the ethanol. To eliminate any chance of possible ethanol carryover, transfer the RNeasy MinElute Spin Column to a new 2 ml collection tube, and centrifuge for an additional 1 min.
b) Salt carryover during elution	Ensure that Buffer RPE is at room temperature.

c) Reverse transcription with too small an amount of RNA	Most reverse transcriptases are intended for use with approximately 1 µg RNA. When performing reverse transcription with very small amounts of RNA, we recommend using the QIAGEN Sensiscript RT Kit, which is specially designed for highly sensitive reverse transcription using <50 ng RNA. For one-step RT-PCR and quantitative, real-time RT-PCR, we recommend the QIAGEN OneStep RT-PCR Kit and QuantiTect RT-PCR Kits, respectively. These kits contain a specially formulated blend of Omniscript and Sensiscript Reverse Transcriptases for amplification of a wide range of RNA amounts, from as little as 1 pg per reaction.

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 isolation procedure. In order to create and maintain an RNase-free environment, the following precautions must be taken during pretreatment and use of disposable and non-disposable vessels and solutions while working with RNA.

General handling

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

Disposable plasticware

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

Non-disposable plasticware

Non-disposable plasticware should be treated before use to ensure that it is RNase-free. Plasticware should be thoroughly rinsed with 0.1 M NaOH, 1 mM EDTA* followed by RNase-free water (see "Solutions", page 59). Alternatively, chloroform-resistant plasticware can be rinsed with chloroform* to inactivate RNases.

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

Glassware

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

Electrophoresis tanks

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

Solutions

Solutions (water and other solutions)* should be treated with 0.1% DEPC. DEPC is a strong, but not absolute, inhibitor of RNases. It is commonly used at a concentration of 0.1% to inactivate RNases on glass or plasticware or to create RNase-free solutions and water. DEPC inactivates RNases by covalent modification. Add 0.1 ml DEPC to 100 ml of the solution to be treated and shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 hours at 37°C. Autoclave for 15 minutes to remove any trace of DEPC. DEPC will react with primary amines and cannot be used directly to treat Tris buffers. DEPC is highly unstable in the presence of Tris buffers and decomposes rapidly into ethanol and CO_2. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris* to make the appropriate buffer. Trace amounts of DEPC will modify purine residues in RNA by carboxymethylation. Carboxymethylated RNA is translated with very low efficiency in cell-free systems. However, its ability to form DNA:RNA or RNA:RNA hybrids is not seriously affected unless a large fraction of the purine residues have been modified. Residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 minutes.

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

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

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

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

Storage of RNA

Purified RNA may be stored at –20°C or –70°C in RNase-free water. Under these conditions, no degradation of RNA is detectable after 1 year.

Quantification of RNA

The concentration of RNA should be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer (see "Spectrophotometric quantification of RNA" below). For small amounts of RNA, however, it may be difficult to determine amounts photometrically. Small amounts of RNA can be accurately quantified using an Agilent 2100 Bioanalyzer, quantitative RT-PCR, or fluorometric quantification (see "Fluorometric quantification of small amounts of RNA" on page 61).

Spectrophotometric quantification of RNA

To ensure significance, A_{260} readings should be greater than 0.15. 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 at a neutral pH. Therefore, if it is necessary to dilute the RNA sample, this should be done in a buffer with neutral pH.* As discussed below (see "Purity of RNA", page 62), the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity.

When measuring RNA samples, be certain that cuvettes are RNase-free, especially if the RNA is to be recovered after spectrophotometry. This can be accomplished by washing cuvettes with 0.1M NaOH, 1 mM EDTA* followed by washing with RNase-free water (see "Solutions", page 59). Use the buffer in which the RNA is diluted to zero the spectrophotometer.

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

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

Volume of RNA sample = 10 µl

Dilution = 1 µl of RNA sample + 499 µl of 10 mM Tris·Cl,* pH 7.0 (1/500 dilution).

Measure absorbance of diluted sample in a 1 ml cuvette (RNase-free).

A_{260} = 0.20

Concentration of RNA sample	= 44 µg/ml x A_{260} x dilution factor
	= 44 µg/ml x 0.20 x 500
	= 4400 µg/ml
Total amount	= concentration x volume in milliliters
	= 4400 µg/ml x 0.01 ml
	= 44 µg of RNA

Fluorometric quantification of small amounts of RNA

Small amounts of RNA may be difficult to measure photometrically. Fluorometric determination or quantitative RT-PCR are more sensitive and accurate methods for low amounts of RNA. Fluorometric measurements are carried out using an RNA-binding dye, such as RiboGreen® RNA quantitation reagent* (Molecular Probes, Inc.), Hoechst 33258,*[†] or ethidium bromide.*

In the absence of a fluorometer, the following simplified method can be used.

Procedure

1. **Make a series of RNA reference solutions by diluting a stock solution containing a known amount of RNA (e.g., total RNA).**

   We recommend using a series of dilutions in RNase-free water with concentrations of 0, 2, 4, 6, 10, and 12 ng/µl (1 ng/µl = 1 µg/ml).
2. **Prepare a solution of 1.5 µg/ml ethidium bromide in 0.1 M ammonium acetate.***
3. **Mix 2 µl of each RNA reference with 8 µl of the ethidium bromide solution.**
4. **Prepare 3 different dilutions of the RNA solution to be measured. These dilutions should be chosen so that at least one lies within the range of the series of RNA references.**
5. **Mix 2 µl of each RNA dilution with 8 µl of the ethidium bromide solution.**

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

[†] *Ausubel, F.M. et al., eds. (1991)* Current Protocols in Molecular Biology. *New York: John Wiley & Sons, p. A.3D.3.*

6. **Pipet all mixtures (10 µl each) onto a UV transilluminator.* Keep the UV lamp turned off while pipetting.**

   To avoid contaminating the UV transilluminator surface with ethidium bromide, cover it first with disposable, transparent plastic wrap.

7. **Turn on the UV lamp and photograph the samples, taking care to adjust the timing and aperture settings so that the differences in signal intensity of the different RNA standards are clearly distinguishable.**

8. **Compare the signal intensities of the RNA dilutions with the series of RNA reference solutions. Determine which RNA reference(s) is nearest in signal intensity to the RNA dilution(s).**

   The original concentration of this RNA reference then corresponds to the RNA concentration in the RNA dilution. Multiply by the dilution factor to find the RNA concentration in the original, undiluted sample.

   For example, if a tenfold dilution of the RNA gives the same signal intensity as the 6 ng/µl RNA reference, then the original, undiluted RNA sample has a concentration of 10 x 6 ng/µl = 60 ng/µl. In this case, a fivefold dilution of the RNA should give the same signal intensity as the 12 ng/µl RNA reference (5 x 12 ng/µl = 60 ng/µl).

Purity of RNA

The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV, such as protein. However, the A_{260}/A_{280} ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting A_{260}/A_{280} ratio can vary greatly. 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 1.9–2.1‡ in 10 mM Tris·Cl, pH 7.5. Always be sure to calibrate the spectrophotometer with the same solution.

For determination of RNA concentration, however, we recommend dilution of the sample in a buffer with neutral pH since the relationship between absorbance and concentration (A_{260} reading of 1 = 40 µg/ml RNA) is based on an extinction coefficient calculated for RNA at neutral pH (see "Quantification of RNA").

** Make sure that the UV source is appropriately shielded. Wear a face shield that blocks UV radiation while the transilluminator is switched on. For more information, consult the product supplier.*

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

‡ Values up to 2.3 are routinely obtained for pure RNA (in 10 mM Tris·Cl, pH 7.5) with some spectrophotometers.

DNA contamination

No currently available purification method can guarantee that RNA is completely free of DNA, even when it is not visible on an agarose gel. To prevent any interference by DNA in RT-PCR applications, such as TaqMan and LightCycler RT-PCR analyses, we recommend working with intron-spanning primers so that genomic DNA will not be amplified. Alternatively, DNA contamination can be detected on agarose gels following RT-PCR by performing control experiments in which no reverse transcriptase is added before the PCR step. For sensitive applications, such as differential display, or if it is not practical to use intron-spanning primers in TaqMan RT-PCR analysis, on-column DNase digestion, as described in the protocols, is recommended.

Instructions for on-column DNase digestion using RNase-free DNase and Buffer RDD are provided in the individual protocols. The DNase is efficiently washed away in the subsequent wash steps.

Integrity of RNA

The integrity and size distribution of total RNA purified with RNeasy Kits can be checked by denaturing agarose* gel electrophoresis and ethidium bromide staining or using an Agilent 2100 Bioanalyzer. The respective ribosomal RNAs should appear as sharp bands or peaks. The apparent ratio of 28S rRNA to 18S RNA should be approximately 2:1. If the ribosomal bands or peaks of a specific sample are not sharp, but appear as a smear towards smaller sized RNAs, it is likely that the RNA sample suffered major degradation during preparation.

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

Appendix C: RNA Cleanup after Lysis and Homogenization with QIAzol Lysis Reagent

QIAzol Lysis Reagent is a monophasic solution of phenol and guanidine thiocyanate, which can be used for lysis and partial purification of total RNA (see page 68 for ordering information). Cleanup using the RNeasy Micro Cleanup Kit is recommended to remove any contaminating phenol.*

Important points before starting

- A maximum of 45 µg RNA can be used. This amount corresponds to the binding capacity of the RNeasy MinElute Spin Columns. If the expected RNA yield is >45 µg, use an appropriate proportion of the QIAzol lysate per RNeasy MinElute Spin Column.
- This protocol is designed for QIAzol preps with a maximum starting volume of 1 ml QIAzol Lysis Reagent. This corresponds to approximately 600 µl final volume (aqueous phase) for RNA cleanup.
- If working with RNA for the first time, read Appendix A (page 58).
- Generally, DNase digestion is not required since the combination of QIAzol and RNeasy MinElute technologies efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan or LightCycler RT-PCR analysis with a low-abundance target). We recommend using the RNase-free DNase and Buffer RDD, included in this kit for optional on-column DNase digestion, as described in the protocol.
- QIAzol Lysis Reagent contains a guanidine salt and is therefore not compatible with disinfecting reagents containing bleach. See the *QIAzol Handbook* for safety information. Take appropriate safety measures and wear gloves when handling.
- Unless otherwise indicated, all steps of the protocol, including centrifugation, should be performed at room temperature. During the procedure, work quickly.

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 with the kit).
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.
- Recommended: For on-column DNase digestion, prepare DNase I stock solution before using the RNase-free DNase for the first time. Dissolve the solid DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided. Take care that no DNase I is lost when opening the vial. Mix gently by inverting the tube. **Do not vortex**.

** This protocol also works well with some other reagents containing phenol and guanidine thiocyanate. Please contact QIAGEN Technical Services for more details (see inside front cover for contact information).*

For long-term storage of reconstituted DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.

Procedure

C1. Carry out homogenization of the sample in QIAzol Lysis Reagent, followed by phase separation, as described in the *QIAzol Handbook* (steps 1–7 of the QIAzol protocol or lysis and homogenization).

C2. Transfer the upper aqueous phase to a new collection tube. Add 1 volume of 70% ethanol, and mix thoroughly by vortexing. Do not centrifuge. Continue immediately with step C3.

C3. Apply up to 700 µl of the sample to an RNeasy MinElute Spin Column in a 2 ml collection tube (supplied). Close the tube gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.*

If the sample is >700 µl, apply another aliquot of the sample (up to 700 µl) and repeat the centrifugation. Discard the flow-through.*

C4. For on-column DNase digestion (recommended), continue immediately with steps C5–C8. If on-column DNase treatment using the RNase-free DNase and Buffer RDD is not desired, proceed directly with step C9.

C5. Recommended: Pipet 350 µl Buffer RW1 into the RNeasy MinElute Spin Column, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash. Discard the flow-through.†

Reuse the collection tube in step C7.

C6. Recommended: Add 10 µl DNase I stock solution (see above) to 70 µl Buffer RDD. Mix by gently inverting the tube.

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

C7. Recommended: Pipet the DNase I incubation mix (80 µl) directly onto the RNeasy MinElute silica-gel membrane, and place on the benchtop at room temperature for 15 min.

Note: Make sure to pipet the DNase I incubation mix directly onto the RNeasy MinElute silica-gel membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the RNeasy MinElute Spin Column.

C8. Recommended: Pipet 350 µl Buffer RW1 into the RNeasy MinElute Spin Column, and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.†

* *Flow-through contains QIAzol Lysis Reagent and is therefore not compatible with bleach. See the* QIAzol Handbook *for safety information.*

† *Flow-through contains Buffer RW1 and is therefore not compatible with bleach. See page 5 for safety information.*

C9. Transfer the spin column into a new 2 ml collection tube (supplied). Pipet 500 µl Buffer RPE onto the spin column. Close the tube gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the column. Discard the flow-through.

Reuse the collection tube in step C10.

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

C10. Add 500 µl of 80% ethanol to the RNeasy MinElute Spin Column. Close the tube gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to dry the silica-gel membrane. Discard the flow-through and collection tube.

Prepare the 80% ethanol with ethanol (96–100%) and the RNase-free water supplied with the kit.

Note: Following the centrifugation, remove the RNeasy MinElute Spin Column from the collection tube carefully so the column does not come into contact with the flow-through as this will result in carryover of ethanol.

C11. Transfer the RNeasy MinElute Spin Column into a new 2 ml collection tube (supplied). Open the cap of the spin column, and centrifuge in a microcentrifuge at full speed for 5 min. Discard the flow-through and collection tube.

To avoid damage to the caps, place the columns into the centrifuge with at least one empty position between each column. Place the caps so that they point in the opposite direction to the rotation of the rotor (i.e., if the rotor rotates in a clockwise direction, orient the caps in a counter-clockwise direction).

It is important to dry the silica-gel membrane since residual ethanol may interfere with downstream reactions. Centrifuging with the caps open ensures that no ethanol is carried over during elution.

C12. To elute, transfer the spin column to a new 1.5 ml collection tube (supplied). Pipet 14 µl RNase-free water directly onto the center of the silica-gel membrane. Close the tube gently, and centrifuge for 1 min at maximum speed to elute.

Smaller volumes of RNase-free water can be used to obtain a higher total RNA concentration, but this will influence the overall yield. The yield will be approximately 20% less when using 10 µl RNase-free water for elution. Elution with less than 10 µl may not be enough to sufficiently hydrate the silica-gel membrane and is therefore not recommended.

The dead volume of the RNeasy MinElute Spin Column is 2 µl; elution with 14 µl of RNase-free water results in an eluate with a volume of 12 µl.

Note: When performing RT-PCR with RNA isolated using this procedure we recommend using the QIAGEN OneStep RT-PCR Kit. This kit contains a specially formulated blend of Omniscript Reverse Transcriptase, designed for RNA amounts greater than 50 ng, and Sensiscript Reverse Transcriptase, for very small amounts of RNA (<50 ng). See page 70 for ordering information. For quantitative real-time RT-PCR we recommend QIAGEN QuantiTect RT-PCR Kits.

Appendix D: Disruption and Homogenization of Tissues Using the Mixer Mill MM 300

The Mixer Mill MM 300 allows high-throughput, rapid, and effective disruption of 48 biological samples in 2–4 minutes. Homogenization and disruption with the Mixer Mill MM 300 gives results comparable to using rotor–stator homogenization.

The following guidelines can be used for disruption and homogenization of tissues using the Mixer Mill MM 300. If the tissues are not stabilized in RNA*later* RNA Stabilization Reagent, be sure to work quickly in order to prevent RNA degradation. See the *RNA*later *Handbook* for information about RNA*later* RNA Stabilization Reagent and about stabilizing RNA in tissues.

Procedure

D1. For best results, stabilize tissue in RNA*later* RNA Stabilization Reagent.

D2. Pipet 350 µl Buffer RLT into a 2 ml microcentrifuge tube (not supplied).

Note: For isolation of total RNA from small amounts of fibrous tissues (page 33), use only 150 µl Buffer RLT in this step.

D3. Add one stainless steel bead* to each tube. For best results, we recommend using a 5 mm (mean diameter) stainless steel bead.

D4. Add up to 5 mg tissue per tube.

D5. Homogenize on the Mixer Mill MM 300 for 2 min at 20 Hz. Homogenization time depends on the tissue used and can be extended until the tissue is completely homogenized.

D6. Rotate the Mixer Mill rack to allow even homogenization, and homogenize for another 2 min at 20 Hz.

D7. Centrifuge the tissue sample (including the bead) for 3 min at maximum speed in a microcentrifuge. Carefully transfer the supernatant to a new microcentrifuge tube (not supplied) by pipetting. Use only this supernatant (lysate) in subsequent steps. Do not reuse the stainless steel bead.

D8. Proceed with step 4 of "Protocol: Total RNA Isolation from Animal Tissues" (page 30) or step 4 of "Protocol: Total RNA Isolation from Fibrous Tissues" (page 36).

** Stainless steel beads for the Mixer Mill MM 300 are available from F. Kurt Retsch GmbH & Co. KG, Haan, Germany or their local distributors (see www.retsch.de).*

Ordering Information

Product	Contents	Cat. No.
RNeasy Micro Kit (50)	50 RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free DNase I, Carrier RNA, RNase-free Reagents and Buffers	74004
Related products		
RNeasy MinElute Cleanup Kit — for RNA cleanup and concentration with small elution volumes		
RNeasy MinElute Cleanup Kit (50)	50 RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	74204
RNeasy Fibrous Tissue Kits — for isolation of total RNA from fiber-rich tissues		
RNeasy Fibrous Tissue Mini Kit (50)	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), Proteinase K, RNase-free DNase I, RNase-free Reagents and Buffers	74704
RNeasy Fibrous Tissue Midi Kit (10)	10 RNeasy Midi Spin Columns, Collection Tubes (15 ml), Proteinase K, RNase-free DNase I, RNase-free Reagents and Buffers	75742
RNeasy Lipid Tissue Kits — for isolation of total RNA from fatty tissues		
RNeasy Lipid Tissue Mini Kit (50)	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), QIAzol Lysis Reagent, RNase-free Reagents and Buffers	74804
RNeasy Lipid Tissue Midi Kit (10)	10 RNeasy Midi Spin Columns, Collection Tubes (15 ml), QIAzol Lysis Reagent, RNase-free Reagents and Buffers	75842
QIAzol Lysis Reagent — for efficient lysis of fatty tissues before RNA isolation		
QIAzol Lysis Reagent (200 ml)	200 ml QIAzol Lysis Reagent	79306

Ordering Information

Product	Contents	Cat. No.
RNeasy Protect Kits — for stabilization and isolation of RNA from tissues		
RNeasy Protect Mini Kit (50)*	RNA*later* RNA Stabilization Reagent† (50 ml), 50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	74124
RNeasy Protect Bacteria Kits — for stabilization and isolation of RNA from bacteria		
RNeasy Protect Bacteria Mini Kit (50)*	RNeasy Mini Kit (50) and RNAprotect™ Bacteria Reagent† (2 x 100 ml)	74524
RNA*later* TissueProtect Tubes — for stabilization of RNA in animal tissues and storage of stabilized tissues		
RNA*later* TissueProtect Tubes (50 x 1.5 ml)*	For stabilization of RNA in 50 x 150 mg tissue samples: 50 screw-top tubes containing 1.5 ml RNA*later* RNA Stabilization Reagent each	76154
RNeasy Kits — for isolation of RNA from a wide variety of samples		
RNeasy Mini Kit (50)*	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	74104
PAXgene Blood RNA System — for integrated collection of blood samples with stabilization and purification of their RNA		
PAXgene Blood RNA Kit (50)	50 PAXgene RNA Spin Columns, Processing Tubes, RNase-free Reagents and Buffers. To be used in conjunction with PAXgene Blood RNA Tubes	762134
PAXgene Blood RNA Tubes (100)	100 blood collection tubes. To be used in conjunction with the PAXgene Blood RNA Kit (50)	762115‡ 762125§

* *Larger kit sizes and/or formats available; please inquire.*

† *Also available separately; please inquire.*

‡ *US and Canada*

§ *Other countries*

Ordering Information

Product	Contents	Cat. No.
RNeasy 96 Kit — for high-throughput manual isolation of RNA from animal cells and tissues		
RNeasy 96 Kit (4)*†	For 4 x 96 total RNA preps: 4 RNeasy 96 Plates, Elution Microtubes CL, Caps, RNase-free Reagents and Buffers	74181
RNeasy 96 BioRobot® 8000 Kit — for fully automated, high-throughput isolation of RNA from animal cells and tissues		
RNeasy 96 BioRobot 8000 Kit (12)	For 12 x 96 fully automated total RNA preps on the BioRobot 8000 or Gene Expression workstation: 12 RNeasy 96 Plates, Elution Microtubes CL, Caps, Square-Well Blocks, RNase-Free Reagents and Buffers	967152
RNase-Free DNase Set — for DNase digestion during RNA purification		
RNase-Free DNase Set (50)	1500 units RNase-free DNase I, RNase-free Buffer RDD, and RNase-free water for 50 RNA minipreps	79254
Omniscript Reverse Transcriptase — for standard reverse transcription using ≥50 ng RNA		
Omniscript RT Kit (10)*	For 10 reverse-transcription reactions: 40 units Omniscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix (containing 5 mM each dNTP), RNase-free water	205110
Sensiscript Reverse Transcriptase — for reverse transcription using small amounts of RNA (<50 ng)		
Sensiscript RT Kit (50)*	For 50 reverse-transcription reactions: Sensiscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix (containing 5 mM each dNTP), RNase-free water	205211
QIAGEN OneStep RT-PCR Kit — for fast and efficient one-step RT-PCR		
QIAGEN OneStep RT-PCR Kit (25)*	For 25 reactions: QIAGEN OneStep RT-PCR Enzyme Mix, 5x QIAGEN OneStep RT-PCR Buffer (containing 12.5 mM $MgCl_2$), dNTP Mix (containing 10 mM each dNTP), 5x Q-Solution, RNase-free water	210210

** Larger kit sizes and/or formats available; please inquire.*

† Requires use of either QIAvac 96 or the QIAGEN 96-Well-Plate Centrifugation System.

Ordering Information

Product	Contents	Cat. No.
QIAshredder Homogenizers — for simple and rapid homogenization of cell and tissue lysates		
QIAshredder (50)	50 disposable cell-lysate homogenizers for use in nucleic acid minipreps, caps	79654
QIAshredder (250)	250 disposable cell-lysate homogenizers for use in nucleic acid minipreps, caps	79656
Mixer Mill MM 300 — for simultaneous, rapid, and effective disruption of up to 192 biological samples		
Mixer Mill MM 300	Universal laboratory mixer mill	Inquire

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Notes

Notes

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