Third Edition December 2014

RNeasy® Micro Handbook

For purification of total RNA from small samples, including

- animal and human cells (≤5 x 10^5)
- animal and human tissues (≤5 mg)
- fibrous tissues (≤5 mg)
- microdissected cryosections

and for RNA cleanup and concentration

QIAGEN®

Sample & Assay Technologies

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Kit Contents

RNeasy Micro Kit	**(50)**
Catalog no.	**74004**
Number of preps	**50**
RNeasy MinElute® Spin Columns (each in a 2 ml Collection Tube)	50
Collection Tubes (1.5 ml)	50
Collection Tubes (2 ml)	100
Buffer RLT*	45 ml
Buffer RW1*	45 ml
Buffer RPE† (concentrate)	11 ml
RNase-Free Water	3 x 10 ml
Carrier RNA, poly-A	310 µg
RNase-Free DNase Set	
■ RNase-Free DNase I (lyophilized)	1500 units
■ Buffer RDD	2 x 2 ml
■ RNase-Free Water	1.5 ml
Handbook	1

* Contains a guanidine salt. Not compatible with disinfectants containing bleach. See page 6 for safety information.

† 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 ambient temperature. Store the RNeasy MinElute spin columns and the RNase-Free DNase Set (i.e., the box containing RNase-free DNase, Buffer RDD, and RNase-free water) immediately upon receipt at 2–8°C. Store the remaining components of the kit dry at room temperature (15–25°C). All kit components are stable for at least 9 months under these conditions.

Quality Control

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

Product Use Limitations

The RNeasy Micro Kit is intended for research use. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

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. Separate conditions apply to QIAGEN scientific instruments, service products, and to products shipped on dry ice. Please inquire for more information.

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 back cover or visit www.qiagen.com).

Technical Assistance

At QIAGEN, we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of 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 see our Technical Support Center at www.qiagen.com/goto/TechSupportCenter or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at www.qiagen.com/safety where you can find, view, and print the SDS for each QIAGEN 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. Guanidine salts can form highly reactive compounds when combined with bleach. If liquid containing these buffers is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

Introduction

The RNeasy Micro Kit 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 purification of total RNA from different sample sources: for details, visit www.qiagen.com/RNA .

Principle and procedure

RNeasy Micro technology combines the selective binding properties of a silica-based membrane with the speed of microspin technology. Guanidine-thiocyanate–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 membrane. Traces of DNA that may copurify are removed by DNase treatment on the RNeasy MinElute spin column. DNase and any contaminants are washed away, and high-quality total RNA is eluted in RNase-free water (see flowchart, next page).

With the RNeasy Micro procedure, all RNA molecules longer than 200 nucleotides are purified. 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 the purified RNA is comparable to that obtained by centrifugation through a CsCl cushion, where small RNAs do not sediment efficiently.*

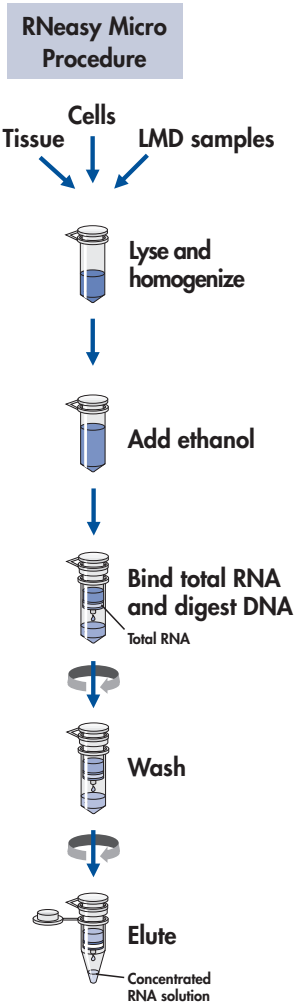
In this handbook, different protocols are provided for different starting materials. The protocols differ primarily in the lysis and homogenization of the sample and in the adjustment of the conditions for binding RNA to the RNeasy MinElute membrane. Once the sample is bound to the membrane, the protocols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

Automated purification

Purification of RNA can be fully automated on the QIAcube®. The innovative QIAcube uses advanced technology to process QIAGEN spin columns, enabling seamless integration of automated, low-throughput sample prep into your laboratory workflow. Sample preparation using the QIAcube follows the same steps as the manual procedure (i.e., lyse, bind, wash, and elute) enabling you to continue using the RNeasy Micro Kit for purification of high-quality RNA. For more information about the automated procedure, see the relevant protocol sheet available at www.qiagen.com/MyQIAcube .

* For purification of miRNA and total RNA from a wide range of cells and tissues, we recommend using miRNeasy Kits. For details, visit www.qiagen.com/miRNA .

The QIAcube is preinstalled with protocols for purification of plasmid DNA, genomic DNA, RNA, viral nucleic acids, and proteins, plus DNA and RNA cleanup. The range of protocols available is continually expanding, and additional QIAGEN protocols can be downloaded free of charge at www.qiagen.com/MyQIAcube .



Equipment and Reagents to Be Supplied by User

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

For all protocols

- 14.3 M β-mercaptoethanol (β-ME) (commercially available solutions are usually 14.3 M) or, alternatively, 2 M dithiothreitol (DTT) in water
- Ethanol (70% and 96–100%)*
- Sterile, RNase-free pipet tips
- Microcentrifuge (with rotor for 2 ml tubes)
- Vortexer
- Disposable gloves
- Reagent for RNA stabilization (see pages 11–12):
  - For cell samples: RNAprotect® Cell Reagent† or liquid nitrogen
  - For tissue samples: RNA*later*® RNA Stabilization Reagent† (stabilizes RNA only), Allprotect Tissue Reagent† (stabilizes DNA, RNA, and protein), or liquid nitrogen
- Equipment for sample disruption and homogenization (see pages 12–14). Depending on the method chosen, one or more of the following are required:
  - Trypsin and PBS
  - QIAshredder homogenizer†
  - Blunt-ended needle and syringe
  - Mortar and pestle
  - TissueRuptor® with TissueRuptor Disposable Probes†
  - TissueLyser†

For RNA purification from heart, muscle, and skin tissue

- QIAGEN Proteinase K (>600 mAU/ml, solution)†‡
- Heating block or water bath capable of reaching 55°C

* Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone.

† For ordering information, see page 61.

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

Important Notes

Determining the amount of starting material

It is essential to use the correct amount of starting material in order to obtain optimal RNA yield and purity. The maximum amount that can be used is determined by:

- The type of sample and its RNA content
- The volume of Buffer RLT required for efficient lysis
- The RNA binding capacity of the RNeasy MinElute spin column

When processing samples containing high amounts of RNA, less than the maximum amount of starting material shown in Table 1 should be used, so that the RNA binding capacity of the RNeasy MinElute spin column is not exceeded.

When processing samples containing average or low amounts of RNA, the maximum amount of starting material shown in Table 1 can be used. However, even though the RNA binding capacity of the RNeasy MinElute spin column is not reached, the maximum amount of starting material must not be exceeded. Otherwise, lysis will be incomplete and cellular debris may interfere with the binding of RNA to the RNeasy spin column membrane, resulting in lower RNA yield and purity.

More information on using the correct amount of starting material is given in each protocol. Table 2 shows typical RNA yields from various cells and tissues.

Note: If the binding capacity of the RNeasy MinElute spin column is exceeded, RNA yields will not be consistent and may be reduced. If lysis of the starting material is incomplete, RNA yields will be lower than expected, even if the binding capacity of the RNeasy MinElute spin column is not exceeded.

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 and human cells	5×10^5
■ Animal and human tissues	5 mg

Table 2. Typical yields of total RNA with the RNeasy Micro Kit

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

* Amounts can vary due to factors such as species, developmental stage, and growth conditions. 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 protocol for purification of total RNA from fibrous tissues (page 30).

Handling and storing starting material

Cells

After harvesting, cells should be immediately lysed in Buffer RLT to prevent unwanted changes in the gene expression profile. This highly denaturing lysis buffer inactivates RNases and other proteins to prevent RNA degradation as well as downregulation or upregulation of transcripts.

If the cells are to be shipped to another lab for RNA purification, they should be pelleted, frozen in liquid nitrogen, and transported on dry ice. Alternatively, the cells can be mixed with RNAprotect Cell Reagent at room temperature and then shipped at ambient temperature.

Tissues

RNA in harvested tissue is not protected 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. Otherwise, unwanted changes in the gene expression profile will occur. It is therefore important that tissue samples are immediately frozen in liquid nitrogen and stored at –70°C, or immediately immersed in RNA*later* RNA Stabilization Reagent. An alternative to RNA*later* RNA Stabilization Reagent is Allprotect Tissue Reagent, which provides immediate stabilization of DNA, RNA, and protein in tissue samples at room temperature.

The procedures for tissue harvesting and RNA protection should be carried out as quickly as possible. Frozen tissue samples should not be allowed to thaw during handling or weighing. After disruption and homogenization in Buffer RLT (lysis buffer), samples can be stored at –70°C for months.

Disrupting and homogenizing starting material

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

- **Disruption**: Complete disruption of plasma membranes of cells and organelles is absolutely required to release all the RNA contained in the sample. Different samples require different methods to achieve complete disruption. Incomplete disruption results in significantly reduced RNA yields.
- **Homogenization**: Homogenization is necessary to reduce the viscosity of the 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 spin column membrane and therefore significantly reduced RNA yields.

Some disruption methods simultaneously homogenize the sample, while others require an additional homogenization step. Table 3 gives an overview of different disruption and homogenization methods, and is followed by a detailed description of each method.

Table 3. Disruption and homogenization methods

Sample	Disruption method	Homogenization method
Microdissected samples	Addition of lysis buffer	Vortexing
Cells and fine-needle aspirates (FNA)	Addition of lysis buffer	TissueRuptor or QIAshredder homogenizer or syringe and needle*
Tissues	TissueRuptor†	TissueRuptor†
	TissueLyser‡	TissueLyser‡
	Mortar and pestle§	QIAshredder homogenizer or syringe and needle

* If processing ≤1 x 10^5 cells, the lysate can be homogenized by vortexing.

† Simultaneously disrupts and homogenizes individual samples.

‡ Simultaneously disrupts and homogenizes up to 192 samples in parallel. Results are comparable to those obtained using the TissueRuptor or other rotor–stator homogenizer.

§ The TissueRuptor and TissueLyser usually give higher RNA yields than mortar and pestle.

Disruption and homogenization using the TissueRuptor

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

Disruption and homogenization using the TissueLyser

In bead-milling, tissues can be disrupted by rapid agitation in the presence of beads and lysis buffer. Disruption and simultaneous homogenization occur by the shearing and crushing action of the beads as they collide with the cells. The TissueLyser disrupts and homogenizes up to 48 tissue samples simultaneously when used in combination with the TissueLyser Adapter Set 2 x 24, which holds 48 x 2 ml microcentrifuge tubes containing stainless steel beads of 5 mm mean diameter. The TissueLyser can also disrupt and homogenize up to 192 tissue samples simultaneously when used in combination with the TissueLyser Adapter Set 2 x 96, which holds 192 x 1.2 ml microtubes containing stainless steel beads of 5 mm mean diameter. For guidelines on using the TissueLyser, refer to the *TissueLyser Handbook*. For other bead mills, refer to suppliers' guidelines.

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

Disruption using a mortar and pestle

For disruption using a mortar and pestle, freeze the tissue 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 2 methods below.

Note: Grinding the sample using a mortar and pestle will disrupt the sample, but will not homogenize it. Homogenization must be performed afterwards.

Homogenization using QIAshredder homogenizers

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

Homogenization using a syringe and needle

Cell and tissue lysates can be homogenized using a syringe and needle. Lysate is passed 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 for use as carrier RNA. When added to lysates from very small samples, the carrier RNA may in some cases improve the recovery of total RNA. Carrier RNA is not required when processing more than 500 cells or more than about 2 µg tissue.

As demonstrated in many different RT-PCR systems, the small amounts of poly-A RNA used as carrier RNA in total RNA purification do not interfere with subsequent RT-PCR, even when oligo-dT is used as a primer for reverse transcription. Reverse-transcription reactions typically contain an excess of oligo-dT primers, and the small amounts of poly-A used as carrier RNA are insignificant in comparison.

Total RNA purified using poly-A RNA as carrier RNA can be amplified with the QuantiTect® Whole Transcriptome Kit, which uses a mix of random and oligo-dT primers. However, total RNA purified using poly-A RNA as carrier RNA is not compatible with protocols that amplify mRNA transcripts using oligo-dT primers, such as the Eberwine method and certain Affymetrix® protocols.* For these protocols, other types of RNA can be purchased separately for use as carrier RNA. Note, however, that tRNA and other RNAs <200 nucleotides will not bind to the RNeasy MinElute 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 the poly-A RNA supplied with this kit.

Limitations of small samples

When purifying RNA from particularly small samples (e.g., laser-microdissected samples), the amounts of RNA may be too small for quantification by spectrophotometry or even fluorometric assays. In this case, quantitative, real-time RT-PCR should be used for quantification.

When purifying RNA from less than 100 cells, stochastic problems with respect to copy number can occur. This is because some RNA transcripts may be present at very low copy numbers per cell, or only in a fraction of all cells in the sample of interest. For example, if a particular RNA transcript is present at an abundance of 1 copy per cell, and 10 cells are processed with RNA eluted in the recommended volume of 14 µl, there will be less than 1 copy of the transcript per microliter.

Whole transcriptome amplification can be carried out to generate sufficient amounts of cDNA if several real-time RT-PCR assays need to be performed from a single small sample. However, care should be taken to include a sufficient amount of starting material in the amplification reaction to avoid stochastic problems. The QuantiTect Whole Transcriptome Kit provides highly uniform amplification of the transcriptome. For details, visit www.qiagen.com/goto/WTA .

* RNA purified using poly-A RNA as carrier RNA is not compatible with Affymetrix kits for 3' in vitro transcription, such as One-Cycle Target Labeling and Control Reagents; Two-Cycle Target Labeling and Control Reagents; and GeneChip® HT One-Cycle Target Labeling and Controls Kit.

† 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 safety data sheets (SDSs), available from the product supplier.

Protocol: Purification of Total RNA from Animal and Human Cells

Determining the correct amount of starting material

It is essential to use the correct amount of starting material in order to obtain optimal RNA yield and purity. The maximum amount depends on:

- The RNA content of the cell type
- The RNA binding capacity of the RNeasy MinElute spin column (45 µg RNA)
- The volume of Buffer RLT required for efficient lysis

In addition, cellular debris can reduce the binding capacity of the RNeasy MinElute spin column. If processing a cell type not listed in Table 2 (page 11) and if there is no information about its RNA content, we recommend starting with no more than 5×10^5 cells.

Table 4. Growth area and number of HeLa cells in various culture vessels

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

* 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 and human cells, which vary in length from 10 to 30 µm.

‡ This number of cells exceeds the binding capacity of the RNeasy MinElute spin columns. To process this many cells, split the lysate into appropriate aliquots ($\leq 5 \times 10^5$ cells each) and load them onto separate RNeasy MinElute spin columns.

Do not overload the RNeasy MinElute spin column, as this will significantly reduce RNA yield and purity.

Counting cells is the most accurate way to quantitate the amount of starting material. As a guide, the number of HeLa cells obtained in various culture vessels after confluent growth is given in Table 4.

Important points before starting

- If using the RNeasy Micro Kit for the first time, read "Important Notes" (page 10).
- If preparing RNA for the first time, read Appendix A (page 50).
- If using the TissueRuptor, ensure that you are familiar with operating it by referring to the *TissueRuptor User Manual* and *TissueRuptor Handbook*.
- 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 they can be dislodged by flicking the tube in step 2. Homogenized cell lysates from step 3 can be stored at –70°C for several months. Frozen lysates should be incubated at 37°C in a water bath until completely thawed and salts are dissolved. Avoid prolonged incubation, which may compromise RNA integrity. If any insoluble material is visible, centrifuge for 5 min at 3000–5000 x *g*. Transfer the supernatant to a new RNase-free glass or polypropylene tube, and continue with step 4.
- Cells stored in RNAprotect Cell Reagent can also be used in the procedure. Transfer the entire sample, including any material deposited at the bottom of the storage vessel, to a centrifuge tube. Pellet the cells by centrifuging for 5 min at 5000 x *g*, and remove the supernatant by pipetting (if necessary, thaw the sample before centrifuging). Proceed immediately to step 2.
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 6 for safety information.
- Perform all steps of the procedure at room temperature (15–25°C). During the procedure, work quickly.
- Perform all centrifugation steps at 20–25°C in a standard microcentrifuge. Ensure that the centrifuge does not cool below 20°C.

Things to do before starting

- If purifying RNA from cell lines rich in RNases, we recommend adding β-mercaptoethanol (β-ME) 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 containing β-ME can be stored at room temperature (15–25°C) for up to 1 month. Alternatively, add 20 µl of 2 M dithiothreitol (DTT) per 1 ml Buffer RLT. The stock solution of 2 M DTT in water should be prepared fresh, or frozen in single-use aliquots. Buffer RLT containing DTT can be stored at room temperature for up to 1 month.
- When processing <500 cells, carrier RNA may be added to the lysate before homogenization (see "Carrier RNA", page 14). Before using for the first time, dissolve the carrier RNA (310 µg) in 1 ml RNase-free water. Store this stock solution at –30 to –15°, and use it to make fresh dilutions for each set of RNA preps. The concentration of this stock solution is 310 µg/ml (i.e., 310 ng/µl). To make a working solution (4 ng/µl) for 10 preps, add 5 µl stock solution to 34 µl Buffer RLT and mix by pipetting. Add 6 µl of this diluted solution to 54 µl Buffer RLT to give a working solution of 4 ng/µl. Add 5 µl of this solution to the lysate in step 3. **Do not add the carrier RNA to the lysate if purifying RNA for use in oligo-dT–based amplification.**
- 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.
- 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). The procedure also requires 70% ethanol, which can be prepared by diluting ethanol (96–100%) with distilled water (not supplied).
- Buffer RLT may form a precipitate during storage. If necessary, redissolve by warming, and then place at room temperature.
- Prepare DNase I stock solution before using the RNase-Free DNase Set for the first time. Dissolve the lyophilized DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided. To avoid loss of DNase I, do not open the vial. Inject RNase-free water into the vial using an RNase-free needle and syringe. Mix gently by inverting the vial. **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 –30 to –15° 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. **Harvest cells according to step 1a or 1b.**

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 proceed to step 2.

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

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

To lyse cells directly:
Determine the number of cells. Completely aspirate the cell-culture medium, and proceed immediately to step 2.

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

To trypsinize and collect cells:
Determine the number of cells. Aspirate the medium, and wash the cells with PBS. Aspirate the PBS, and add 0.1–0.25% trypsin in PBS. After the cells detach from the dish or flask, add medium (containing serum to inactivate the trypsin), transfer the cells to an RNase-free glass or polypropylene centrifuge tube (not supplied), and centrifuge at 300 x *g* for 5 min. Completely aspirate the supernatant, and proceed to step 2.

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

2. **Disrupt the cells by adding Buffer RLT.**

For pelleted cells, loosen the cell pellet thoroughly by flicking the tube. Add 350 µl Buffer RLT (if processing $\leq$1 x 10^5 cells, add 75 µl Buffer RLT instead). Vortex or pipet to mix, and proceed to step 3.

Note: Incomplete loosening of the cell pellet may lead to inefficient lysis and reduced RNA yields.

For direct lysis of cells grown in a monolayer, add 350 µl Buffer RLT to the cell-culture dish (if processing ≤1 x 10^5 cells, especially in multiwell plates or cell-culture dishes, 75 µl Buffer RLT can be added instead). Collect the lysate with a rubber policeman. Pipet the 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.

3. **Homogenize the lysate according to step 3a, 3b, or 3c.**

   See "Disrupting and homogenizing starting material", page 12, for more details on homogenization. If processing ≤1 x 10^5 cells, homogenize by vortexing for 1 min. After homogenization, proceed to step 4.

   Note: If processing <500 cells, 20 ng carrier RNA (5 µl of a 4 ng/µl solution) may be added to the lysate before homogenization. Prepare the carrier RNA as described in "Things to do before starting".

   Note: Incomplete homogenization leads to significantly reduced RNA yields and can cause clogging of the RNeasy MinElute spin column. Homogenization with the TissueRuptor or QIAshredder homogenizer generally results in higher RNA yields than with a syringe and needle.

3a. **Pipet the lysate directly into a QIAshredder spin column (not supplied) placed in a 2 ml collection tube, and centrifuge for 2 min at full speed. Proceed to step 4.**

3b. **Place the tip of the TissueRuptor disposable probe into the lysate and operate the TissueRuptor at full speed until the lysate is homogenous (usually 30 s). Proceed to step 4.**

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

3c. **Pass the lysate at least 5 times through a blunt 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe. Proceed to step 4.**

4. **Add 1 volume of 70% ethanol to the lysate, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 5.**

   Note: The volume of lysate may be less than 350 µl due to loss during homogenization. If only 75 µl of Buffer RLT was used in step 2, then add only 75 µl of 70% ethanol in this step.

   Note: When purifying RNA from certain cell lines, precipitates may be visible after addition of ethanol. This does not affect the procedure.

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

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

Optional: If recovery of protein is desired, keep the flow-through on ice and follow steps E1–E5 in Appendix E on page 60.

Reuse the collection tube in step 6.

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

   Reuse the collection tube in step 9.

   Optional: If on-column DNase digestion is not desired, add 700 µl Buffer RW1 instead, centrifuge for 15 s at ≥8000 x *g*, and discard the flow-through and collection tube.* Proceed to 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. **Add the DNase I incubation mix (80 µl) directly to the RNeasy MinElute spin column membrane, and place on the benchtop (20–30°C) for 15 min.**

   Note: Be sure to add the DNase I incubation mix directly to the RNeasy MinElute spin column membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.

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

10. **Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Add 500 µl Buffer RPE to the spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. 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 lid gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to wash the spin column 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.

* Flow-through contains Buffer RW1 and is therefore not compatible with bleach. See page 6 for safety information.

Note: After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

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

    To avoid damage to their lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counterclockwise).

    It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.

13. **Place the RNeasy MinElute spin column in a new 1.5 ml collection tube (supplied). Add 14 µl RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.**

    As little as 10 µl RNase-free water can be used for elution if a higher RNA concentration is required, but the yield will be reduced by approximately 20%. Do not elute with less than 10 µl RNase-free water, as the spin column membrane will not be sufficiently hydrated.

    The dead volume of the RNeasy MinElute spin column is 2 µl: elution with 14 µl RNase-free water results in a 12 µl eluate.

    For RT-PCR and real-time RT-PCR with the purified RNA, QIAGEN offers a range of optimized, ready-to-use kits that provide highly specific and sensitive results. For details, visit www.qiagen.com/PCR . For whole transcriptome amplification (WTA) of limited amounts of RNA, we recommend the QuantiTect Whole Transcriptome Kit. For details, visit www.qiagen.com/goto/WTA .

Protocol: Purification of Total RNA from Animal and Human Tissues

This protocol is for the purification of RNA from most animal and human tissues. For fibrous tissues, follow the protocol on page 30. For total RNA purification from frozen, microdissected tissue samples, see page 37.

Determining the correct amount of starting material

It is essential to use the correct amount of starting material in order to obtain optimal RNA yield and purity. A maximum amount of 5 mg fresh or frozen tissue or 2–3 mg RNA*later* or Allprotect stabilized tissue (which is partially dehydrated) can generally be processed. For most tissues, the RNA binding capacity of the RNeasy MinElute spin column and the lysing capacity of Buffer RLT will not be exceeded by these amounts. Typical RNA yields from various tissues are given in Table 2 (page 11).

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

Do not overload the RNeasy MinElute spin column, as this will significantly reduce RNA yield and quality.

Weighing tissue is the most accurate way to quantitate the amount of starting material. As a guide, a 1.5 mm cube (3.4 mm^3) of most animal tissues weighs 3.5–4.5 mg.

Important points before starting

- If using the RNeasy Micro Kit for the first time, read "Important Notes" (page 10).
- If preparing RNA for the first time, read Appendix A (page 50).
- If using the TissueRuptor, ensure that you are familiar with operating it by referring to the *TissueRuptor User Manual* and *TissueRuptor Handbook*.
- If using the TissueLyser, ensure that you are familiar with operating it by referring to the operating instructions and *TissueLyser Handbook*.
- For optimal results, stabilize harvested tissues immediately in RNA*later* RNA Stabilization Reagent (see the *RNA*later *Handbook*) or Allprotect Tissue Reagent (see the *Allprotect Tissue Reagent Handbook*). Tissues can be stored in the reagent at 37°C for up to 1 day, at 15–25°C for up to 7 days, or at 2–8°C for up to 4 weeks (RNA*later*) or 6 months (Allprotect). Alternatively, tissues can be archived at –30 to –15° or –80°C.

- Fresh, frozen, or RNA*later*/Allprotect stabilized tissue can be used. Tissues can be stored at –70°C for several months. Flash-freeze tissues in liquid nitrogen, and immediately transfer to –70°C. Do not allow tissues to thaw during weighing or handling prior to disruption in Buffer RLT. Homogenized tissue lysates from step 3 can also be stored at –70°C for several months. Incubate frozen lysates at 37°C in a water bath until completely thawed and salts are dissolved before continuing with step 4. Avoid prolonged incubation, which may compromise RNA integrity.
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 6 for safety information.
- Perform all steps of the procedure at room temperature (15–25°C). During the procedure, work quickly.
- Perform all centrifugation steps at 20–25°C in a standard microcentrifuge. Ensure that the centrifuge does not cool below 20°C.

Things to do before starting

- 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 containing β-ME can be stored at room temperature (15–25°C) for up to 1 month. Alternatively, add 20 µl of 2 M dithiothreitol (DTT) per 1 ml Buffer RLT. The stock solution of 2 M DTT in water should be prepared fresh, or frozen in single-use aliquots. Buffer RLT containing DTT can be stored at room temperature for up to 1 month.
- When processing less than about 2 µg tissue, carrier RNA may be added to the lysate before homogenization (see "Carrier RNA", page 14). Before using for the first time, dissolve the carrier RNA (310 µg) in 1 ml RNase-free water. Store this stock solution at –30 to –15°, and use it to make fresh dilutions for each set of RNA preps. The concentration of this stock solution is 310 µg/ml (i.e., 310 ng/µl). To make a working solution (4 ng/µl) for 10 preps, add 5 µl stock solution to 34 µl Buffer RLT and mix by pipetting. Add 6 µl of this diluted solution to 54 µl Buffer RLT to give a working solution of 4 ng/µl. Add 5 µl of this solution to the lysate in step 2. **Do not add the carrier RNA to the lysate if purifying RNA for use in oligo-dT–based amplification.**
- 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.
- 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). The procedure also requires 70% ethanol, which can be prepared by diluting ethanol (96–100%) with distilled water (not supplied).

- Buffer RLT may form a precipitate during storage. If necessary, redissolve by warming, and then place at room temperature.
- Prepare DNase I stock solution before using the RNase-Free DNase Set for the first time. Dissolve the lyophilized DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided. To avoid loss of DNase I, do not open the vial. Inject RNase-free water into the vial using an RNase-free needle and syringe. Mix gently by inverting the vial. **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 –30 to –15° 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. **Excise the tissue sample from the animal or remove it from storage. Determine the amount of tissue. Do not use more than 5 mg. Proceed immediately to step 2.**

   Weighing tissue is the most accurate way to determine the amount. If necessary, cut the tissue on a clean surface and weigh the piece to be used.

   For RNA*later* or Allprotect stabilized tissues: Remove the tissue from the stabilization reagent using forceps and be sure to remove any crystals that may have formed. RNA in RNA*later* or Allprotect stabilized tissues is protected during cutting and weighing of tissues at room temperature (15–25°C). It is not necessary to cut the tissues on ice or dry ice or in a refrigerated room. Remaining tissues can be stored in RNA*later* or Allprotect Reagent. Previously stabilized tissues can be stored at –80°C without the reagent.

   For unstabilized fresh or frozen tissues: RNA in harvested tissues is not protected until the tissues are treated with RNA*later* or Allprotect Reagent, flash-frozen, or disrupted and homogenized in step 2. Frozen tissues should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible. Remaining fresh tissues can be placed into RNA*later* Reagent to stabilize RNA or in Allprotect Tissue Reagent to stabilize DNA, RNA, and protein. However, previously frozen tissues thaw too slowly in the reagent, preventing the reagent from diffusing into the tissues quickly enough to prevent RNA degradation.

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

   See “Disrupting and homogenizing starting material”, page 12, for more details on disruption and homogenization.

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

Note: If processing <2 µg tissue, 20 ng carrier RNA (5 µl of a 4 ng/µl solution) may be added to the lysate before homogenization. Prepare the carrier RNA as described in "Things to do before starting".

After storage in RNA*later* or Allprotect Reagent, tissues may become slightly harder than fresh or thawed tissues. Disruption and homogenization using standard methods is usually not a problem.

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

2a. Disruption and homogenization using the TissueRuptor:

- **Place the tissue in a suitably sized vessel. Add 350 µl Buffer RLT.**

  Note: Use a suitably sized vessel with sufficient extra headspace to accommodate foaming, which may occur during homogenization.

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

- **Place the tip of the disposable probe into the vessel and operate the TissueRuptor at full speed until the lysate is homogeneous (usually 30 s). Proceed to step 3.**

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

  Foaming may occur during homogenization. If this happens, let the homogenate stand at room temperature for 2–3 min until the foam subsides before continuing with the procedure.

2b. Disruption and homogenization using the TissueLyser:

- **Place the tissues in 2 ml microcentrifuge tubes containing one stainless steel bead (5 mm mean diameter).**

  If handling fresh or frozen tissue samples, keep the tubes on dry ice.

- **Place the tubes at room temperature. Immediately add 350 µl Buffer RLT per tube.**
- **Place the tubes in the TissueLyser Adapter Set 2 x 24.**
- **Operate the TissueLyser for 2 min at 20 Hz.**

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

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

  Rearranging the tubes allows even homogenization.

- **Proceed to step 3.**

  Do not reuse the stainless steel beads.

2c. Disruption using a mortar and pestle followed by homogenization using a QIAshredder homogenizer or a needle and syringe:

- **Immediately place the weighed 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 into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 min at full speed. Alternatively, pass the lysate at least 5 times through a blunt 20-gauge needle fitted to an RNase-free syringe. Proceed to step 3.**

3. Centrifuge the lysate for 3 min at full speed. 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 after the 3-min centrifugation, making the pellet invisible.

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

Note: The volume of 70% ethanol to add may be less than 350 µl if some lysate was lost during homogenization.

Note: Precipitates may be visible after addition of ethanol, but this does not affect the procedure.

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

Optional: If recovery of protein is desired, keep the flow-through on ice and follow steps E1–E5 in Appendix E on page 60.

Reuse the collection tube in step 6.

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

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

   Reuse the collection tube in step 9.

   Optional: If on-column DNase digestion is not desired, add 700 µl Buffer RW1 instead, centrifuge for 15 s at ≥8000 x *g*, and discard the flow-through and collection tube.* Proceed to 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. **Add the DNase I incubation mix (80 µl) directly to the RNeasy MinElute spin column membrane, and place on the benchtop (20–30°C) for 15 min.**

   Note: Be sure to add the DNase I incubation mix directly to the RNeasy MinElute spin column membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.

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

10. **Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Add 500 µl Buffer RPE to the spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. 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 lid gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through and collection tube.**

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

    Note: After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

* Flow-through contains Buffer RW1 and is therefore not compatible with bleach. See page 6 for safety information.

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

    To avoid damage to their lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counterclockwise).

    It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.

13. **Place the RNeasy MinElute spin column in a new 1.5 ml collection tube (supplied). Add 14 µl RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.**

    As little as 10 µl RNase-free water can be used for elution if a higher RNA concentration is required, but the yield will be reduced by approximately 20%. Do not elute with less than 10 µl RNase-free water, as the spin column membrane will not be sufficiently hydrated.

    The dead volume of the RNeasy MinElute spin column is 2 µl: elution with 14 µl RNase-free water results in a 12 µl eluate.

    For RT-PCR and real-time RT-PCR with the purified RNA, QIAGEN offers a range of optimized, ready-to-use kits that provide highly specific and sensitive results. For details, visit www.qiagen.com/PCR . For whole transcriptome amplification (WTA) of limited amounts of RNA, we recommend the QuantiTect Whole Transcriptome Kit. For details, visit www.qiagen.com/goto/WTA .

Protocol: Purification of Total RNA from Fibrous Tissues

This protocol can be used to purify RNA from skeletal muscle, heart, and skin tissue. RNA purification from these fibrous tissues can be difficult due to the abundance of contractile proteins, connective tissue, and collagen. This protocol is a modification of the protocol for RNA purification from animal and human tissues (page 23): it include a proteinase K digest to remove proteins, which can interfere with RNA purification. 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 membrane. Traces of DNA that may copurify are removed by DNase digestion on the RNeasy MinElute spin column. DNase and any contaminants are washed away, and total RNA is eluted in RNase-free water.

If purifying RNA from other tissues rich in proteins, we recommend comparing this protocol with the protocol on page 23. 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. In general, the protocol on page 23 is the protocol of choice for other tissues.

Determining the correct amount of starting material

It is essential to use the correct amount of starting material in order to obtain optimal RNA yield and purity. A maximum amount of 5 mg fresh or frozen tissue or 2–3 mg RNA*later* or Allprotect stabilized tissue (which is partially dehydrated) can generally be processed. For most tissues, the RNA binding capacity of the RNeasy MinElute spin column and the lysing capacity of Buffer RLT will not be exceeded by these amounts. Typical RNA yields from various tissues are given in Table 2 (page 11).

Do not overload the RNeasy MinElute spin column, as this will significantly reduce RNA yield and quality.

Weighing tissue is the most accurate way to quantitate the amount of starting material. As a guide, a 1.5 mm cube (3.4 mm^3) of most animal tissues weighs 3.5–4.5 mg.

Important points before starting

- If using the RNeasy Micro Kit for the first time, read "Important Notes" (page 10).
- If preparing RNA for the first time, read Appendix A (page 50).
- If using the TissueRuptor, ensure that you are familiar with operating it by referring to the *TissueRuptor User Manual* and *TissueRuptor Handbook*.
- If using the TissueLyser, ensure that you are familiar with operating it by referring to the operating instructions and *TissueLyser Handbook*.

- For optimal results, stabilize harvested tissues immediately in RNA*later* RNA Stabilization Reagent (see the *RNA*later *Handbook*) or Allprotect Tissue Reagent (see the *Allprotect Tissue Reagent Handbook*). Tissues can be stored in the reagent at 37°C for up to 1 day, at 15–25°C for up to 7 days, or at 2–8°C for up to 4 weeks (RNA*later*) or 6 months (Allprotect). Alternatively, tissues can be archived at –30 to –15° or –80°C.
- Fresh, frozen, or RNA*later*/Allprotect stabilized tissue can be used. Tissues can be stored at –70°C for several months. Flash-freeze tissues in liquid nitrogen, and immediately transfer to –70°C. Do not allow tissues to thaw during weighing or handling prior to disruption in Buffer RLT. Homogenized tissue lysates from step 7 can also be stored at –70°C for several months. Incubate frozen lysates at 37°C in a water bath until completely thawed and salts are dissolved before continuing with step 8. Avoid prolonged incubation, which may compromise RNA integrity.
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 6 for safety information.
- Unless otherwise indicated, perform all steps of the procedure at room temperature (15–25°C). During the procedure, work quickly.
- Perform all centrifugation steps at 20–25°C in a standard microcentrifuge. Ensure that the centrifuge does not cool below 20°C.

Things to do before starting

- 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 containing β-ME can be stored at room temperature (15–25°C) for up to 1 month. Alternatively, add 20 µl of 2 M dithiothreitol (DTT) per 1 ml Buffer RLT. The stock solution of 2 M DTT in water should be prepared fresh, or frozen in single-use aliquots. Buffer RLT containing DTT can be stored at room temperature for up to 1 month.
- When processing less than about 2 µg tissue, carrier RNA may be added to the lysate before homogenization (see "Carrier RNA", page 14). Before using for the first time, dissolve the carrier RNA (310 µg) in 1 ml RNase-free water. Store this stock solution at –30 to –15°, and use it to make fresh dilutions for each set of RNA preps. The concentration of this stock solution is 310 µg/ml (i.e., 310 ng/µl). To make a working solution (4 ng/µl) for 10 preps, add 5 µl stock solution to 34 µl Buffer RLT and mix by pipetting. Add 6 µl of this diluted solution to 54 µl Buffer RLT to give a working solution of 4 ng/µl. Add 5 µl of this solution to the lysate in step 3. **Do not add the carrier RNA to the lysate if purifying RNA for use in oligo-dT–based amplification.**

- 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.
- 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).
- Buffer RLT may form a precipitate during storage. If necessary, redissolve by warming, and then place at room temperature.
- Prepare DNase I stock solution before using the RNase-Free DNase Set for the first time. Dissolve the lyophilized DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided. To avoid loss of DNase I, do not open the vial. Inject RNase-free water into the vial using an RNase-free needle and syringe. Mix gently by inverting the vial. **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 –30 to –15° 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 water bath or heating block to 55°C for proteinase K digestion in step 5.**
2. **Excise the tissue sample from the animal or remove it from storage. Determine the amount of tissue. Do not use more than 5 mg. Proceed immediately to step 3.**

   Weighing tissue is the most accurate way to determine the amount. If necessary, cut the tissue on a clean surface and weigh the piece to be used.

   For RNA*later* or Allprotect stabilized tissues: Remove the tissue from the stabilization reagent using forceps and be sure to remove any crystals that may have formed. RNA in RNA*later* or Allprotect stabilized tissues is protected during cutting and weighing of tissues at room temperature (15–25°C). It is not necessary to cut the tissues on ice or dry ice or in a refrigerated room. Remaining tissues can be stored in RNA*later* or Allprotect Reagent. Previously stabilized tissues can be stored at –80°C without the reagent.

   For unstabilized fresh or frozen tissues: RNA in harvested tissues is not protected until the tissues are treated with RNA*later* or Allprotect Reagent, flash-frozen, or disrupted and homogenized in step 3. Frozen tissues should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible. Remaining fresh tissues can be placed into RNA*later* Reagent to stabilize RNA or in Allprotect Tissue Reagent to stabilize DNA, RNA, and protein. However, previously frozen tissues thaw too slowly in the reagent, preventing the reagent from diffusing into the tissues quickly enough to prevent RNA degradation.

3. Disrupt the tissue and homogenize the lysate in Buffer RLT (do not use more than 5 mg tissue) according to step 3a, 3b, or 3c.

See "Disrupting and homogenizing starting material", page 12, for more details on disruption and homogenization.

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

Note: If processing <2 µg tissue, 20 ng carrier RNA (5 µl of a 4 ng/µl solution) may be added to the lysate before homogenization. Prepare the carrier RNA as described in "Things to do before starting".

After storage in RNA*later* or Allprotect Reagent, tissues may become slightly harder than fresh or thawed tissues. Disruption and homogenization using standard methods is usually not a problem.

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

3a. Disruption and homogenization using the TissueRuptor:

- **Place the tissue in a suitably sized vessel. Add 150 µl Buffer RLT.**

  Note: Use a suitably sized vessel with sufficient extra headspace to accommodate foaming, which may occur during homogenization.

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

- **Place the tip of the disposable probe into the vessel and operate the TissueRuptor at full speed until the lysate is homogeneous (usually 30 s). Proceed to step 4.**

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

  Foaming may occur during homogenization. If this happens, let the homogenate stand at room temperature for 2–3 min until the foam subsides before continuing with the procedure.

3b. Disruption and homogenization using the TissueLyser:

- **Place the tissues in 2 ml microcentrifuge tubes containing one stainless steel bead (5 mm mean diameter).**

  If handling fresh or frozen tissue samples, keep the tubes on dry ice.

- **Place the tubes at room temperature. Immediately add 150 µl Buffer RLT per tube.**
- **Place the tubes in the TissueLyser Adapter Set 2 x 24.**
- **Operate the TissueLyser for 2 min at 20 Hz.**

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

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

  Rearranging the tubes allows even homogenization.

- **Carefully pipet the lysates into new microcentrifuge tubes (not supplied). Proceed to step 4.**

  Do not reuse the stainless steel beads.

3c. Disruption using a mortar and pestle followed by homogenization using a QIAshredder homogenizer or a needle and syringe:

- **Immediately place the weighed 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 150 µl Buffer RLT.**
- **Pipet the lysate directly into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 min at full speed. Alternatively, pass the lysate at least 5 times through a blunt 20-gauge needle fitted to an RNase-free syringe. Proceed to 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 (15–25°C).

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 supplied).

Avoid transferring any of the pellet. If this is unavoidable, a small amount of pelleted debris may be carried over without affecting the RNeasy 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 96–100% ethanol to the cleared lysate, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 9.

Note: Precipitates may be visible after addition of ethanol, but this does not affect the procedure.

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

   Optional: If recovery of protein is desired, keep the flow-through on ice and follow steps E1–E5 in Appendix E on page 60.

   Reuse the collection tube in step 10.

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

    Reuse the collection tube in step 13.

    Optional: If on-column DNase digestion is not desired, add 700 µl Buffer RW1 instead, centrifuge for 15 s at ≥8000 x *g*, and discard the flow-through and collection tube.* Proceed to 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. **Add the DNase I incubation mix (80 µl) directly to the RNeasy MinElute spin column membrane, and place on the benchtop (20–30°C) for 15 min.**

    Note: Be sure to add the DNase I incubation mix directly to the RNeasy MinElute spin column membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.

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

14. **Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Add 500 µl Buffer RPE to the spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. 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 lid gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through and collection tube.**

* Flow-through contains Buffer RLT or Buffer RW1 and is therefore not compatible with bleach. See page 6 for safety information.

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

Note: After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

16. **Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the flow-through and collection tube.**

    To avoid damage to their lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counterclockwise).

    It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.

17. **Place the RNeasy MinElute spin column in a new 1.5 ml collection tube (supplied). Add 14 µl RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.**

    As little as 10 µl RNase-free water can be used for elution if a higher RNA concentration is required, but the yield will be reduced by approximately 20%. Do not elute with less than 10 µl RNase-free water, as the spin column membrane will not be sufficiently hydrated.

    The dead volume of the RNeasy MinElute spin column is 2 µl: elution with 14 µl RNase-free water results in a 12 µl eluate.

    For RT-PCR and real-time RT-PCR with the purified RNA, QIAGEN offers a range of optimized, ready-to-use kits that provide highly specific and sensitive results. For details, visit www.qiagen.com/PCR . For whole transcriptome amplification (WTA) of limited amounts of RNA, we recommend the QuantiTect Whole Transcriptome Kit. For details, visit www.qiagen.com/goto/WTA .

Protocol: Purification of Total RNA from Microdissected Cryosections

This protocol is for the purification of total RNA from frozen, microdissected samples of animal and human tissues. For total RNA purification from microdissected, formalin-fixed samples, we recommend the RNeasy FFPE Kit (cat. no. 74404).

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) and P.A.L.M. Microlaser Technologies (www.palm-mikrolaser.com).

Important points before starting

- If using the RNeasy Micro Kit for the first time, read "Important Notes" (page 10).
- If preparing RNA for the first time, read Appendix A (page 50).
- To minimize RNA degradation, avoid prolonged storage of unstabilized samples at room temperature. RNA in tissues is not protected before flash-freezing in liquid nitrogen.
- Tissue lysates from step 4 can be stored at –70°C for several months. Incubate frozen lysates at 37°C in a water bath until completely thawed and salts are dissolved before continuing with step 5. Avoid prolonged incubation, which may compromise RNA integrity.
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 6 for safety information.
- Perform all steps of the procedure at room temperature (15–25°C). During the procedure, work quickly.
- Perform all centrifugation steps at 20–25°C in a standard microcentrifuge. Ensure that the centrifuge does not cool below 20°C.
- In the procedure below, ▲ refers to use of the Leica® AS LMD System (which requires reduced buffer volumes), and ● refers to use of other laser microdissection systems.

Things to do before starting

- 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 containing β-ME can be stored at room temperature (15–25°C) for up to 1 month. Alternatively, add 20 µl of 2 M dithiothreitol (DTT) per 1 ml Buffer RLT. The stock solution of 2 M DTT in water should be prepared fresh, or frozen in single-use aliquots. Buffer RLT containing DTT can be stored at room temperature for up to 1 month.
- When processing <500 cells, carrier RNA may be added to the lysate before homogenization (see "Carrier RNA", page 14). Before using for the first time, dissolve the carrier RNA (310 µg) in 1 ml RNase-free water. Store this stock solution at –30 to –15°, and use it to make fresh dilutions for each set of RNA preps. The concentration of this stock solution is 310 µg/ml (i.e., 310 ng/µl). To make a working solution (4 ng/µl) for 10 preps, add 5 µl stock solution to 34 µl Buffer RLT and mix by pipetting. Add 6 µl of this diluted solution to 54 µl Buffer RLT to give a working solution of 4 ng/µl. Add 5 µl of this solution to the lysate in step 3. **Do not add the carrier RNA to the lysate if purifying RNA for use in oligo-dT–based amplification.**
- 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.
- 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). The procedure also requires 70% ethanol, which can be prepared by diluting ethanol (96–100%) with distilled water (not supplied).
- Buffer RLT may form a precipitate during storage. If necessary, redissolve by warming, and then place at room temperature.
- Prepare DNase I stock solution before using the RNase-Free DNase Set for the first time. Dissolve the lyophilized DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided. To avoid loss of DNase I, do not open the vial. Inject RNase-free water into the vial using an RNase-free needle and syringe. Mix gently by inverting the vial. **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 –30 to –15° 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. **Collect the sample directly into an appropriate volume of Buffer RLT (the volume depends on the collection vessel used for microdissection, but should not be greater than ▲ 65 µl or ● 300 µl).**

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

2. **If necessary, transfer the sample and buffer to a larger vessel (e.g., 1.5 ml or 2 ml 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: If processing <500 cells, 20 ng carrier RNA (5 µl of a 4 ng/µl solution) may be added to the lysate before homogenization. Prepare the carrier RNA as described in "Things to do before starting".

4. **Vortex the sample for 30 s.**

   No further homogenization is necessary.

5. **Add 1 volume of 70% ethanol to the homogenized lysate, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 6.**

   Note: The volume of lysate may be less than ▲ 75 µl or ● 350 µl due to loss during homogenization.

   Note: Precipitates may be visible after addition of ethanol. This does not affect the procedure.

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

   Reuse the collection tube in step 10.

   Optional: If on-column DNase digestion is not desired, add 700 µl Buffer RW1 instead, centrifuge for 15 s at ≥8000 x *g*, and discard the flow-through and collection tube.* Proceed to step 11.

* Flow-through contains Buffer RLT or Buffer RW1 and is therefore not compatible with bleach. See page 6 for safety information.

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. **Add the DNase I incubation mix (80 µl) directly to the RNeasy MinElute spin column membrane, and place on the benchtop (20–30°C) for 15 min.**

   Note: Be sure to add the DNase I incubation mix directly to the RNeasy MinElute spin column membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.

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

11. **Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Add 500 µl Buffer RPE to the spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. 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").

12. **Add 500 µl of 80% ethanol to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to wash the spin column 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: After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

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

    To avoid damage to their lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counterclockwise).

    It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.

* Flow-through contains Buffer RW1 and is therefore not compatible with bleach. See page 6 for safety information.

14. **Place the RNeasy MinElute spin column in a new 1.5 ml collection tube (supplied). Add 14 µl RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.**

    As little as 10 µl RNase-free water can be used for elution if a higher RNA concentration is required, but the yield will be reduced by approximately 20%. Do not elute with less than 10 µl RNase-free water, as the spin column membrane will not be sufficiently hydrated.

    The dead volume of the RNeasy MinElute spin column is 2 µl: elution with 14 µl RNase-free water results in a 12 µl eluate.

    For RT-PCR and real-time RT-PCR with the purified RNA, QIAGEN offers a range of optimized, ready-to-use kits that provide highly specific and sensitive results. For details, visit www.qiagen.com/PCR . For whole transcriptome amplification (WTA) of limited amounts of RNA, we recommend the QuantiTect Whole Transcriptome Kit. For details, visit www.qiagen.com/goto/WTA .

Protocol: RNA Cleanup and Concentration

The RNeasy Micro Kit 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 concentration of total cellular RNA purified using the PAXgene™ Blood RNA Kit, we recommend using the RNeasy MinElute Cleanup Kit (cat. no. 74204).

Determining the correct amount of starting material

A maximum of 45 µg RNA in a maximum volume of 200 µl can be cleaned up in this protocol. This amount corresponds to the binding capacity of the RNeasy MinElute spin column.

Important points before starting

- If preparing RNA for the first time, read Appendix A (page 50).
- Generally, DNase digestion is not required since 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® RT-PCR analysis with a low-abundance target). In these cases, DNA can be removed by a DNase digestion before starting RNA cleanup (see Appendix D, page 59).
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 6 for safety information.
- Perform all steps of the procedure at room temperature (15–25°C). During the procedure, work quickly.
- Perform all centrifugation steps at 20–25°C in a standard microcentrifuge. Ensure that the centrifuge does not cool below 20°C.
- In the procedure below, ▲ refers to use of starting volumes ≤100 µl, and ● refers to use of starting volumes of 100–200 µl.

Things to do before starting

- 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.
- 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).
- Buffer RLT may form a precipitate during storage. If necessary, redissolve by warming, and then place at room temperature (15–25°C).

- Optional: If cleaning up crude RNA preps (e.g., after salting-out methods) or samples rich in RNases, we recommend adding β-mercaptoethanol (β-ME) 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 containing β-ME can be stored at room temperature for up to 1 month. Alternatively, add 20 µl of 2 M dithiothreitol (DTT) per 1 ml Buffer RLT. The stock solution of 2 M DTT in water should be prepared fresh, or frozen in single-use aliquots. Buffer RLT containing DTT can be stored at room temperature for up to 1 month.

Procedure

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

   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 (or DTT) 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 well by pipetting. Do not centrifuge. Proceed immediately to step 3.**

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

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

4. **Optional: Add 700 µl Buffer RW1 to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through and collection tube.***

5. **Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Add 500 µl Buffer RPE to the spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.**

   Reuse the collection tube in step 6.

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

6. **Add 500 µl of 80% ethanol to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through and collection tube.**

* Flow-through contains Buffer RLT or Buffer RW1 and is therefore not compatible with bleach. See page 6 for safety information.

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

Note: After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

7. **Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the flow-through and collection tube.**

   To avoid damage to their lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counterclockwise).

   It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.

8. **Place the RNeasy MinElute spin column in a new 1.5 ml collection tube (supplied). Add 14 µl RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.**

   As little as 10 µl RNase-free water can be used for elution if a higher RNA concentration is required, but the yield will be reduced by approximately 20%. Do not elute with less than 10 µl RNase-free water, as the spin column membrane will not be sufficiently hydrated.

   The dead volume of the RNeasy MinElute spin column is 2 µl: elution with 14 µl RNase-free water results in a 12 µl eluate.

   For RT-PCR and real-time RT-PCR with the purified RNA, QIAGEN offers a range of optimized, ready-to-use kits that provide highly specific and sensitive results. For details, visit www.qiagen.com/PCR . For whole transcriptome amplification (WTA) of limited amounts of RNA, we recommend the QuantiTect Whole Transcriptome Kit. For details, visit www.qiagen.com/goto/WTA .

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 sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

		Comments and suggestions
Clogged RNeasy MinElute spin column		
a)	Inefficient disruption and/or homogenization	See "Disrupting and homogenizing starting materials" (page 12) for details on disruption and homogenization methods. Increase *g*-force and centrifugation time if necessary. In subsequent preparations, reduce the amount of starting material (see the individual protocols) and/or increase the homogenization time. If working with tissues rich in proteins, the protocol for purification of total RNA from fibrous tissues (page 30) may provide better results than the protocol on page 23.
b)	Too much starting material	Reduce the amount of starting material (see the individual protocols). It is essential to use the correct amount of starting material.
c)	Centrifugation temperature too low	The centrifugation temperature should be 20–25°C. Some centrifuges may cool to below 20°C even when set at 20°C. This can cause formation of precipitates that can clog the spin column. If this happens, set the centrifugation temperature to 25°C. Warm the lysate to 37°C before transferring it to the RNeasy MinElute spin column.

		Comments and suggestions
Low RNA yield		
a)	Insufficient disruption and homogenization	See "Disrupting and homogenizing starting materials" (page 12) for details on disruption and homogenization methods. Increase *g*-force and centrifugation time if necessary. In subsequent preparations, reduce the amount of starting material (see the individual protocols) and/or increase the volume of lysis buffer and the homogenization time. If working with tissues rich in proteins, the protocol for purification of total RNA from fibrous tissues (page 30) may provide better results than the protocol on page 23.
b)	Too much starting material	In subsequent preparations, reduce the amount of starting material (see the individual protocols). It is essential to use the correct amount of starting material.
c)	RNA still bound to spin column membrane	Repeat RNA elution, but incubate the RNeasy MinElute spin column on the benchtop for 10 min with RNase-free water before centrifuging.
d)	Ethanol carryover	After the wash with 80% ethanol, be sure to centrifuge at full speed for 5 min to dry the RNeasy MinElute spin column membrane. After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.
e)	80% ethanol not made with RNase-free water	The 80% ethanol used to wash the RNeasy MinElute spin column membrane must be free of RNases. Be sure to prepare the 80% ethanol using ethanol (96–100%) and the RNase-free water supplied with the kit, as described in "Things to do before starting" in each protocol.

	Comments and suggestions
Low or no recovery of RNA	
a) RNase-free water incorrectly dispensed	Pipet RNase-free water to the center of the RNeasy MinElute spin column membrane to ensure that the membrane is completely covered.
b) Ethanol carryover	After the wash with 80% ethanol, be sure to centrifuge at full speed for 5 min to dry the RNeasy MinElute spin column membrane. After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.
c) 80% ethanol not made with RNase-free water	The 80% ethanol used to wash the RNeasy MinElute spin column membrane must be free of RNases. Be sure to prepare the 80% ethanol using ethanol (96–100%) and the RNase-free water supplied with the kit, as described in "Things to do before starting" in each protocol.
Low A_{260}/A_{280} value	
Water use to dilute RNA for A_{260}/A_{280} measurement	Use 10 mM Tris·Cl, pH 7.5, not RNase-free water, to dilute the sample before measuring purity (see Appendix B, page 52)
RNA degraded	
a) Inappropriate handling of starting material	Ensure that tissue samples are properly stabilized and stored in RNA*later* RNA Stabilization Reagent or Allprotect Tissue Reagent. For frozen cell pellets or frozen tissue samples, ensure that they were flash-frozen immediately in liquid nitrogen and properly stored at –70°C. Perform the RNeasy procedure quickly, especially the first few steps. See Appendix A (page 50) and "Handling and storing starting material" (page 11).

		Comments and suggestions
b)	RNase contamination	Although all RNeasy buffers have been tested and are guaranteed RNase-free, RNases can be introduced during use. Be certain not to introduce any RNases during the RNeasy procedure or later handling. See Appendix A (page 50) for general remarks on handling RNA. Do not put RNA samples into a vacuum dryer or microcentrifuge that has been used in DNA preparations where RNases may have been used.
c)	80% ethanol not made with RNase-free water	The 80% ethanol used to wash the RNeasy MinElute spin column membrane must be free of RNases. Be sure to prepare the 80% ethanol using ethanol (96–100%) and the RNase-free water supplied with the kit, as described in "Things to do before starting" in each protocol.
DNA contamination in downstream experiments		
	No DNase treatment	Be sure to perform the on-column DNase digestion as described in the protocols.
RNA does not perform well in downstream experiments		
a)	Ethanol carryover	After the wash with 80% ethanol, be sure to centrifuge at full speed for 5 min to dry the RNeasy MinElute spin column membrane. After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.
b)	Salt carryover during elution	Ensure that buffers are at 20–30°C. Ensure that the correct buffer is used for each step of the procedure. When reusing collection tubes between washing steps, remove residual flow-through from the rim by blotting on clean paper towels.

		Comments and suggestions
c)	Reverse transcription with too small an amount of RNA	When performing reverse transcription with very small amounts of RNA, we recommend using the Sensiscript® RT Kit, which is specially designed for cDNA synthesis from <50 ng RNA. If synthesizing cDNA for use in real-time PCR, we recommend the QuantiTect Reverse Transcription Kit, which is compatible with a wide range of RNA amounts (10 pg to 1 µg), or the QuantiTect Whole Transcriptome Kit, which provides whole transcriptome amplification from as little as 1 ng RNA. For ordering information, see page 61.

Appendix A: General Remarks on Handling RNA

Handling RNA

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

General handling

Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles 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 purified RNA on ice when aliquots are pipetted for downstream applications.

Disposable plasticware

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

Nondisposable plasticware

Nondisposable plasticware should be treated before use to ensure that it is RNase-free. Plasticware should be thoroughly rinsed with 0.1 M NaOH, 1 mM EDTA* followed by RNase-free water (see "Solutions", page 51). Alternatively, chloroform-resistant plasticware can be rinsed with chloroform* to inactivate RNases.

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

Glassware

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

Electrophoresis tanks

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

Solutions

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

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

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

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

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

Storage of RNA

Purified RNA may be stored at –30 to –15° 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 can be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer (see "Spectrophotometric quantification of RNA" below). For small amounts of RNA, however, it may not be possible to accurately determine amounts photometrically. Small amounts of RNA can be quantified using an Agilent® 2100 bioanalyzer, fluorometric quantification, or quantitative, real-time RT-PCR. When purifying RNA from particularly small samples (e.g., laser-microdissected samples), quantitative, real-time RT-PCR should be used for quantification.

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 53), 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 0.1 M NaOH, 1 mM EDTA* followed by washing with RNase-free water (see "Solutions", page 51). Use the buffer in which the RNA is diluted to zero the spectrophotometer. 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} = 0.2

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

Concentration of RNA sample	= 44 µg/ml x A_{260} x dilution factor
	= 44 µg/ml x 0.2 x 500
	= 4400 µg/ml
Total amount	= concentration x volume in milliliters
	= 4400 µg/ml x 0.01 ml
	= 44 µg of RNA

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 spectrum, 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 used for dilution.

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 = 44 µg/ml RNA) is based on an extinction coefficient calculated for RNA at neutral pH (see "Spectrophotometric quantification of RNA", page 52).

DNA contamination

No currently available purification method can guarantee that RNA is completely free of DNA, even when it is not visible on an agarose gel. While the RNeasy Micro Kit will remove the vast majority of cellular DNA, trace amounts may still remain in the purified RNA, depending on the amount and nature of the sample.

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

* 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.

To prevent any interference by DNA in real-time RT-PCR applications, such as with Applied Biosystems® and LightCycler® instruments, we recommend designing primers that anneal at intron splice junctions so that genomic DNA will not be amplified. QuantiTect Primer Assays from QIAGEN (www.qiagen.com/GeneGlobe) are designed for SYBR® Green based real-time RT-PCR analysis of RNA sequences (without detection of genomic DNA) where possible. For real-time RT-PCR assays where amplification of genomic DNA cannot be avoided, the QuantiTect Reverse Transcription Kit provides fast cDNA synthesis with integrated removal of genomic DNA contamination (see ordering information, page 61).

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 by 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 either before or during RNA purification.

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

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

QIAzol Lysis Reagent is a monophasic solution of phenol and guanidine thiocyanate that can be used for sample lysis and partial purification of total RNA (see page 61 for ordering information). The RNA can be cleaned up using the RNeasy Micro Kit, which removes any contaminating phenol. The aqueous phase from QIAzol lysis is used as starting material in this protocol. It is therefore not necessary to precipitate the RNA and redissolve it prior to RNA cleanup.*

Determining the correct amount of starting material

This protocol is designed for QIAzol preps with a maximum starting volume of 1 ml QIAzol Lysis Reagent. This corresponds to a final volume of approximately 600 µl (aqueous phase) for RNA cleanup.

A maximum of 45 µg RNA can be cleaned up in this protocol. This amount corresponds to the binding capacity of the RNeasy MinElute spin column. If the expected RNA yield is >45 µg, use an appropriate proportion of the QIAzol lysate per RNeasy MinElute spin column.

Important points before starting

- If preparing RNA for the first time, read Appendix A (page 50).
- 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 RT-PCR analysis with a low-abundance target). In these cases, residual DNA can be removed by the recommended on-column DNase digestion steps in this 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.
- Unless otherwise indicated, perform all steps of the procedure at room temperature (15–25°C). During the procedure, work quickly.
- Perform all centrifugation steps at 20–25°C in a standard microcentrifuge. Ensure that the centrifuge does not cool below 20°C.

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

Things to do before starting

- 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.
- 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). The procedure also requires 70% ethanol, which can be prepared by diluting ethanol (96–100%) with distilled water (not supplied).
- Recommended: For on-column DNase digestion, prepare DNase I stock solution before using the RNase-Free DNase Set for the first time. Dissolve the lyophilized DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided. To avoid loss of DNase I, do not open the vial. Inject RNase-free water into the vial using an RNase-free needle and syringe. Mix gently by inverting the vial. **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 –30 to –15° 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 less than about 2 µg tissue, carrier RNA may be added to the lysate before homogenization (see "Carrier RNA", page 14). Before using for the first time, dissolve the carrier RNA (310 µg) in 1 ml RNase-free water. Store this stock solution at –30 to –15°, and use it to make fresh dilutions for each set of RNA preps. The concentration of this stock solution is 310 µg/ml (i.e., 310 ng/µl). To make a working solution (4 ng/µl) for 10 preps, add 5 µl stock solution to 34 µl Buffer RLT and mix by pipetting. Add 6 µl of this diluted solution to 54 µl Buffer RLT to give a working solution of 4 ng/µl. Add 5 µl of this solution to the lysate in step C2. **Do not add the carrier RNA to the lysate if purifying RNA for use in oligo-dT–based amplification.**

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 for 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. Proceed immediately to step C3.

Note: If processing <2 µg tissue, 20 ng carrier RNA (5 µl of a 4 ng/µl solution) may be added to the aqueous phase before adding ethanol. Prepare the carrier RNA as described in "Things to do before starting".

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

If the sample is >700 µl, transfer the remaining sample (up to 700 µl) and repeat the centrifugation. Discard the flow-through.*

For on-column DNase digestion (recommended), proceed immediately to steps C4–C7. Otherwise, proceed directly to step C8.

C4. Recommended: Add 350 µl Buffer RW1 to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.*

Reuse the collection tube in step C7.

C5. Recommended: 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.

C6. Recommended: Add the DNase I incubation mix (80 µl) directly to the RNeasy MinElute spin column membrane, and place on the benchtop (20–30°C) for 15 min.

Note: Be sure to add the DNase I incubation mix directly to the RNeasy MinElute spin column membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.

C7. Recommended: Add 350 µl Buffer RW1 to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through and collection tube.*

C8. Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Add 500 µl Buffer RPE to the spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.

Reuse the collection tube in step C9.

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

C9. Add 500 µl of 80% ethanol to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to wash the spin column 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.

* Flow-through contains QIAzol Lysis Reagent or Buffer RW1 and is therefore not compatible with bleach. See the QIAzol Handbook and page 6 for safety information.

Note: After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

C10. Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the flow-through and collection tube.

To avoid damage to their lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counterclockwise).

It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.

C11. Place the RNeasy MinElute spin column in a new 1.5 ml collection tube (supplied). Add 14 µl RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.

As little as 10 µl RNase-free water can be used for elution if a higher RNA concentration is required, but the yield will be reduced by approximately 20%. Do not elute with less than 10 µl RNase-free water, as the spin column membrane will not be sufficiently hydrated.

The dead volume of the RNeasy MinElute spin column is 2 µl: elution with 14 µl RNase-free water results in a 12 µl eluate.

For RT-PCR and real-time RT-PCR with the purified RNA, QIAGEN offers a range of optimized, ready-to-use kits that provide highly specific and sensitive results. For details, visit www.qiagen.com/PCR . For whole transcriptome amplification (WTA) of limited amounts of RNA, we recommend the QuantiTect Whole Transcriptome Kit. For details, visit www.qiagen.com/goto/WTA .

Appendix D: DNase Digestion of RNA before RNA Cleanup

This protocol describes how to digest contaminating DNA in RNA solutions prior to RNA cleanup and concentration. This protocol requires use of the RNase-Free DNase Set supplied with the RNeasy Micro Kit.

Important points before starting

- **Do not vortex reconstituted DNase I.** DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the vial.

Things to do before starting

- Prepare DNase I stock solution before using the RNase-Free DNase Set for the first time. Dissolve the lyophilized DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided. To avoid loss of DNase I, do not open the vial. Inject RNase-free water into the vial using an RNase-free needle and syringe. Mix gently by inverting the vial. **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 –30 to –15° 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

D1. Mix the following in a microcentrifuge tube:

- **≤87.5 µl RNA solution (contaminated with genomic DNA)**
- **10 µl Buffer RDD**
- **2.5 µl DNase I stock solution**

Make the volume up to 100 µl with RNase-free water.

The reaction volumes can be doubled if necessary (to 200 µl final volume).

D2. Incubate on the benchtop (20–25°C) for 10 min.

D3. Clean up the RNA according to "Protocol: RNA Cleanup and Concentration" on page 42.

Appendix E: Acetone Precipitation of Protein from Lysates

The following procedure describes how to recover denatured protein by acetone precipitation from lysates of cells and tissues.

Reagents to be supplied by user

- Ice
- Acetone*
- Optional: Ethanol*
- Buffer* for downstream application (e.g., loading buffer for SDS-PAGE gel)

Important points before starting

- **Do not use trichloroacetic acid (TCA) to precipitate protein from Buffer RLT lysates.** This buffer contains guanidine thiocyanate, which can form highly reactive compounds when combined with acidic solutions.

Procedure

Bind total RNA to the RNeasy MinElute spin column as described in the cell protocol (from page 16, steps 1–5), the tissue protocol (from page 23, steps 1–5), or the fibrous tissue protocol (from page 30, steps 1–9). Then follow steps E1–E5 below to precipitate protein from the flow-through.

E1. Add 4 volumes of ice-cold acetone to the flow-through from the RNeasy MinElute spin column.

E2. Incubate for 30 min on ice or at –30 to –15°.

E3. Centrifuge for 10 min at full speed in a benchtop centrifuge. Discard the supernatant and air-dry the pellet.†

E4. Optional: Wash the pellet with 100 µl ice-cold ethanol and air-dry.

Do not overdry the pellet as this may make resuspension more difficult.

E5. Resuspend the pellet in the buffer for your downstream application.

Sodium dodecyl sulfate (SDS) causes guanidine salts to precipitate. In case the pellet contains traces of guanidine thiocyanate, load the sample onto an SDS-PAGE gel immediately after heating for 7 minutes at 95°C.

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

† Supernatant contains guanidine thiocyanate and is therefore not compatible with bleach. See page 6 for safety information.

Ordering Information

Product	Contents	Cat. no.
RNeasy Micro Kit (50)	50 RNeasy MinElute Spin Columns, Collection Tubes, RNase-Free DNase I, Carrier RNA, RNase-Free Reagents and Buffers	74004
Accessories		
RNAprotect Cell Reagent (250 ml)	250 ml RNAprotect Cell Reagent	76526
RNA*later* RNA Stabilization Reagent (50 ml)	For stabilization of RNA in 25 x 200 mg tissue samples: 50 ml RNA*later* RNA Stabilization Reagent	76104
RNA*later* RNA Stabilization Reagent (250 ml)	For stabilization of RNA in 125 x 200 mg tissue samples: 250 ml RNA*later* RNA Stabilization Reagent	76106
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
RNA*later* TissueProtect Tubes (20 x 5 ml)	For stabilization of RNA in 20 x 500 mg tissue samples: 20 screw-top tubes containing 5 ml RNA*later* RNA Stabilization Reagent each	76163
Allprotect Tissue Reagent (100 ml)	For stabilization of DNA/RNA/protein in 50 x 200 mg tissue samples: 100 ml Allprotect Tissue Reagent, Allprotect Reagent Pump	76405
RNase-Free DNase Set (50)	For 50 RNA minipreps: 1500 units RNase-Free DNase I, RNase-Free Buffer RDD, and RNase-Free Water	79254
QIAGEN Proteinase K (2 ml)	2 ml (>600 mAU/ml, solution)	19131
QIAGEN Proteinase K (10 ml)	10 ml (>600 mAU/ml, solution)	19133
Collection Tubes (2 ml)	1000 x 2 ml Collection Tubes	19201

Ordering Information

Product	Contents	Cat. no.
QIAshredder (50)	50 disposable cell-lysate homogenizers	79654
QIAshredder (250)	250 disposable cell-lysate homogenizers	79656
TissueRuptor	Handheld rotor–stator homogenizer, 5 TissueRuptor Disposable Probes	Varies*
TissueRuptor Disposable Probes (25)	25 nonsterile plastic disposable probes for use with the TissueRuptor	990890
TissueLyser	Universal laboratory mixer-mill disruptor	Varies*
TissueLyser Adapter Set 2 x 24	2 sets of Adapter Plates and 2 racks for use with 2 ml microcentrifuge tubes on the TissueLyser	69982
Stainless Steel Beads, 5 mm (200)	Stainless Steel Beads, suitable for use with the TissueLyser system	69989
TissueLyser Single-Bead Dispenser, 5 mm	For dispensing individual beads (5 mm diameter)	69965
Related products		
RNeasy FFPE Kit — for purification of high yields of usable RNA from FFPE tissue sections		
RNeasy FFPE Kit (50)	50 RNeasy MinElute Spin Columns, 50 gDNA Eliminator Mini Spin Columns, Collection Tubes, RNase-Free Reagents and Buffers	74404
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, RNase-Free Reagents and Buffers	74204

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

Ordering Information

Product	Contents	Cat. no.
QuantiTect Whole Transcriptome Kit — for unlimited real-time PCR analysis from precious RNA samples		
QuantiTect Whole Transcriptome Kit (25)*	For 25 x 50 µl whole-transcriptome-amplification reactions: T-Script Enzyme and Buffer, Ligation Enzymes, Reagent, and Buffer, and REPLI-g® DNA Polymerase and Buffer	207043
Sensiscript RT Kit — for reverse transcription using less than 50 ng RNA per reaction		
Sensiscript RT Kit (50)*	For 50 x 20 µl reactions: Sensiscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix, RNase-Free Water	205211
QuantiTect Reverse Transcription Kit — for fast cDNA synthesis for sensitive real-time two-step RT-PCR		
QuantiTect Reverse Transcription Kit (50)*	For 50 x 20 µl reactions: gDNA Wipeout Buffer, Quantiscript® Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, RNase-Free Water	205311

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

* Larger kit size available; see www.qiagen.com.

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