November 2006

RNeasy® Fibrous Tissue Handbook

RNeasy Fibrous Tissue Mini Kit

RNeasy Fibrous Tissue Midi Kit

For purification of total RNA from heart, skeletal muscle, aorta, and other fiber-rich tissues

QIAGEN

Contents

Kit Contents

RNeasy Fibrous Tissue Kit	Mini (50)	Midi (10)
Catalog no.	**74704**	**75742**
Number of preps	**50**	**10**
RNeasy Mini Spin Columns (each in a 2 ml Collection Tube)	50	–
RNeasy Midi Spin Columns (each in a 15 ml Collection Tube)	–	10
Collection Tubes (1.5 ml)	50	–
Collection Tubes (2 ml)	50	–
Collection Tubes (15 ml)	–	10
Proteinase K	1.4 ml	1.4 ml
Buffer RLT*	45 ml	45 ml
Buffer RW1*	45 ml	45 ml
Buffer RPE† (concentrate)	11 ml	11 ml
RNase-Free Water	4 x 10 ml	5 x 10 ml
RNase-Free DNase Set:		
■ RNase-Free DNase I (lyophilized)	1500 units	1500 units
■ Buffer RDD	2 x 2 ml	2 x 2 ml
■ RNase-Free Water	1.5 ml	1.5 ml
Handbook	1	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

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

The ready-to-use proteinase K included in the kit is dissolved in a specially formulated storage buffer. The proteinase K is stable for up to 1 year after delivery when stored at room temperature. To prolong the lifetime of the proteinase K, we recommend storage at 2–8°C.

Quality Control

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

Product Use Limitations

RNeasy Fibrous Tissue Kits are intended for research use. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

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

Product Warranty and Satisfaction Guarantee

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

Technical Assistance

At QIAGEN we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in molecular biology and the use of QIAGEN products. If you have any questions or experience any difficulties regarding RNeasy Fibrous Tissue Kits or QIAGEN products in general, please do not hesitate to contact us.

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

For technical assistance and more information please call one of the QIAGEN Technical Service Departments or local distributors (see back cover).

Safety Information

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

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

Buffer RLT contains guanidine thiocyanate and Buffer RW1 contains a small amount of guanidine thiocyanate. Guandine 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.

The following risk and safety phrases apply to the components of RNeasy Fibrous Tissue Kits.

Buffer RLT

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

Buffer RW1

Contains ethanol: flammable. Risk phrase:* R10

RNase-Free DNase I

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

Proteinase K

Contains proteinase K: sensitizer, irritant. Risk and safety phrases:* R36/37/38-42/43, S23-24-26-36/37

24-hour emergency information

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

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

* R10: Flammable; R20/21/22: Harmful by inhalation, in contact with skin and if swallowed; R32: Contact with acids liberates very toxic gas; R36/37/38: Irritating to eyes, respiratory system and skin; R42/43: May cause sensitization by inhalation and skin contact; S13: Keep away from food, drink and animal feedingstuffs; S22: Do not breathe dust; S23: Do not breathe vapor; S24: Avoid contact with skin; S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice; S36: Wear suitable protective clothing; S36/37: Wear suitable protective clothing and gloves; S46: If swallowed, seek medical advice immediately and show the container or label.

Introduction

RNeasy Fibrous Tissue Kits are designed for optimal lysis of fiber-rich tissues and purification of high-quality total RNA. QIAGEN provides a wide range of other kits for purification of total RNA from different sample sources (visit www.qiagen.com/RNA).

Principle and procedure

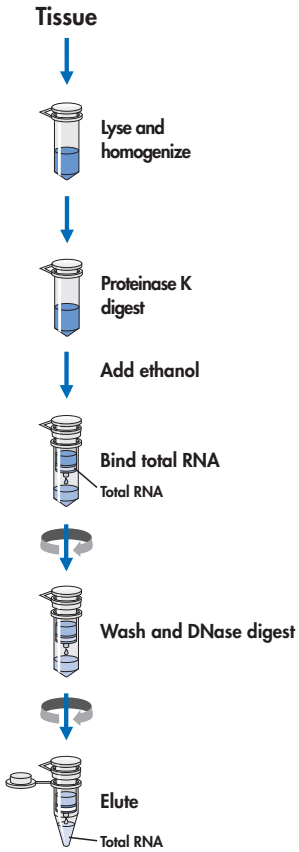
Total RNA purification from fibrous tissues, such as skeletal muscle, heart, and aorta tissue, can be difficult due to the abundance of contractile proteins, connective tissue, and collagen. RNeasy Fibrous Tissue Kits are supplied with proteinase K, which removes these proteins. Tissue samples are first lysed in Buffer RLT and then diluted before being treated with proteinase K. Debris is pelleted by centrifugation, and the supernatant is removed. The supernatant is mixed with ethanol and then centrifuged through an RNeasy spin column, where RNA binds to the silica membrane. Traces of DNA that may copurify with the RNA are removed by DNase treatment on the silica membrane. DNase and any contaminants are efficiently washed away, and high-quality total RNA is eluted in RNase-free water (see flowchart, next page).

RNeasy Fibrous Tissue Kits are available in two formats:

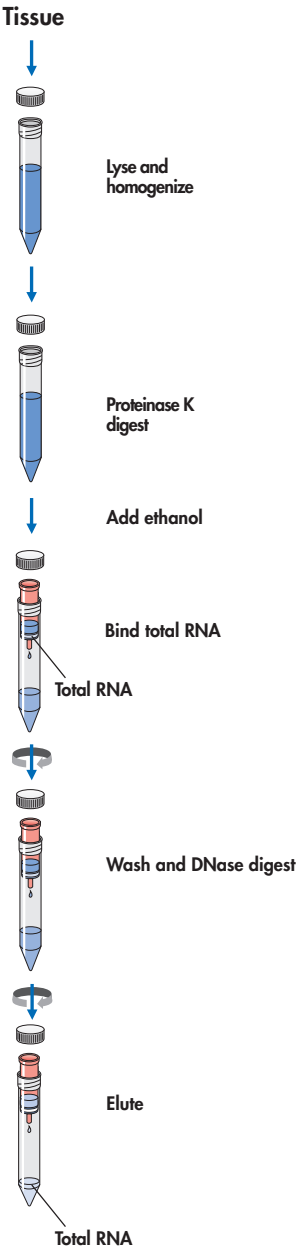
- **RNeasy Fibrous Tissue Mini Kit** — for RNA purification from up to 30 mg tissue using RNeasy Mini spin columns
- **RNeasy Fibrous Tissue Midi Kit** — for RNA purification from up to 250 mg tissue using RNeasy Midi spin columns

With RNeasy Fibrous Tissue Kits, all RNA molecules longer than 200 nucleotides are purified. The procedure provides an enrichment for mRNA since most RNAs <200 nucleotides (such as 5.8S rRNA, 5S rRNA, and tRNAs, which together comprise 15–20% of total RNA) are selectively excluded. The size distribution of the purified RNA is comparable to that obtained by centrifugation through a CsCl cushion, where small RNAs do not sediment efficiently. For purification of small RNA, including microRNA, from tissues and cells, we recommend using miRNeasy Kits (see ordering information, page 39).

RNeasy Fibrous Tissue Mini Procedure



RNeasy Fibrous Tissue Midi Procedure



Equipment and Reagents to Be Supplied by User

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

- 14.3 M β-mercaptoethanol (β-ME) (commercially available solutions are usually 14.3 M) or, as alternative, dithiothreitol (DTT)
- Ethanol (96–100%)*
- Sterile, RNase-free pipet tips
- Disposable gloves
- Water bath or heating block capable of reaching 55°C
- Equipment for tissue disruption and homogenization (see page 12): we recommend either the TissueRuptor with TissueRuptor Disposable Probes or the TissueLyser system (see ordering information, page 36)
- For stabilization of RNA in tissues (see page 12): RNA*later*® RNA Stabilization Reagent (see ordering information, page 36) or liquid nitrogen and dry ice

For users of RNeasy Fibrous Tissue Mini Kit

- 1.5 ml or 2 ml microcentrifuge tubes
- Microcentrifuge (with rotor for 2 ml tubes)

For users of RNeasy Fibrous Tissue Midi Kit

- 10–15 ml centrifuge tubes
- Laboratory centrifuge capable of 3000–5000 x *g* (e.g., Centrifuge 4-15C or Centrifuge 4K15C from QIAGEN; please inquire) with a swinging bucket rotor for 15 ml centrifuge tubes†

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

† A maximum speed of 3500–5000 rpm corresponds to 3000–5000 x *g* for most rotors. RNeasy Midi spin columns fit into 15 ml collection tubes supplied with the kit. These fit into the rotor of almost every standard laboratory centrifuge available. In the unlikely event that the tubes do not fit, RNeasy Midi spin columns can also be inserted into different 12–15 ml RNase-free glass or polypropylene tubes.

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 tissue and its RNA content
- The volume of Buffer RLT required for efficient lysis
- The RNA binding capacity of the RNeasy 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 spin column is not exceeded.

When processing samples containing 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 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 the protocols. Table 2 shows expected RNA yields from various sources.

Table 1. RNeasy Spin Column Specifications

Specification	RNeasy Mini spin column	RNeasy Midi spin column
Maximum binding capacity	100 µg RNA	1 mg RNA
Maximum loading volume	700 µl	4 ml
RNA size distribution	RNA >200 nucleotides	RNA >200 nucleotides
Minimum elution volume	30 µl	150 µl
Maximum amount of starting tissue	≤30 mg	20–250 mg

Note: If the binding capacity of the RNeasy 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 spin column is not exceeded.

Table 2. Typical Yields of Total RNA with RNeasy Fibrous Tissue Kits

Mouse/rat tissue (10 mg)	Yield of total RNA (µg)*
Heart	8–12
Aorta	8–12
Trachea	8–12
Esophagus	8–12
Muscle	5–10
Skin	4–8
Eye	8–12
Ear	10–15

* Amounts can vary due to factors such as species and developmental stage. Since the RNeasy procedures enrich 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.

Handling and storing starting material

RNA in tissues is not protected after harvesting until the sample is treated with RNA*later* RNA Stabilization Reagent, flash-frozen, or disrupted and homogenized in the presence of RNase-inhibiting or denaturing reagents. It is therefore important that tissue samples are immediately immersed in RNA*later* RNA Stabilization Reagent (see *RNA*later *Handbook*), or immediately frozen in liquid nitrogen and stored at –70°C. Otherwise, unwanted changes in the gene expression profile will occur.

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. Incomplete disruption results in significantly reduced RNA yields.

- **Homogenization:** Homogenization is necessary to reduce the viscosity of the lysates produced by disruption. Homogenization shears high-molecular-weight genomic DNA and other high-molecular-weight cellular components to create a homogeneous lysate. Incomplete homogenization results in inefficient binding of RNA to the RNeasy spin column membrane and therefore significantly reduced RNA yields.

Disruption and homogenization of tissue samples can be carried out rapidly and efficiently using either the TissueRuptor (for processing samples individually) or the TissueLyser (for processing multiple samples simultaneously). Disruption and homogenization with the TissueRuptor or TissueLyser generally results in higher RNA yields than with other methods, such as disruption in liquid nitrogen using a mortar and pestle followed by homogenization using a syringe and needle.*

Disruption and homogenization using the TissueRuptor

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

Disruption and homogenization using the TissueLyser

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. 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.

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. In this case, we recommend using the RNeasy 96 Universal Tissue Kit, which provides high-throughput RNA purification from all types of tissue, including fiber-rich tissues, in 96-well format. For ordering information, see page 39.

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

Protocol: Purification of Total RNA Using the RNeasy Fibrous Tissue Mini Kit

Determining the correct amount of starting material

It is essential to use the correct amount of tissue in order to obtain optimal RNA yield and purity. With the RNeasy Fibrous Tissue Mini Kit, a maximum of 30 mg tissue can generally be processed. For most tissues, the RNA binding capacity of the RNeasy spin column and the lysing capacity of Buffer RLT and proteinase K will not be exceeded by this amount. Average RNA yields from various tissues are given in Table 2 (page 12).

If there is no information about the nature of your starting material, we recommend starting with no more than 10 mg tissue. Depending on the RNA yield from the tissue sample, it may be possible to use up to 30 mg tissue in subsequent preparations.

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

Weighing tissue is the most accurate way to quantify the amount of starting material. As a guide, a 3 mm cube (27 mm^3) of most animal tissues weighs 25–35 mg.

Important points before starting

- If using RNeasy Fibrous Tissue Kits for the first time, read "Important Notes" (page 11).
- If working with RNA for the first time, read Appendix A (page 30).
- 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*.
- 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 pancreas or intestine.
- Fresh, frozen, or RNA*later* stabilized tissues can be used. If freezing tissues, flash-freeze in liquid nitrogen and immediately transfer to –70°C, where they can be stored for several months. Do not allow tissues to thaw during weighing or handling prior to disruption in Buffer RLT. Homogenized tissue lysates from step 4 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 5. Avoid prolonged incubation, which may compromise RNA integrity.
- **Do not vortex reconstituted DNase I.** DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube.
- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature (15–25°C).

- 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. During the procedure, work quickly.
- Perform all centrifugation steps at 20–25°C. 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 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.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- Prepare DNase I stock solution before using the RNase-Free DNase Set for the first time. Dissolve the lyophilized DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided in the RNase-Free DNase Set box. 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 DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.

Procedure

1. **Heat a water bath or heating block to 55°C for proteinase K digestion in step 6.**
2. **If using the TissueLyser, add one stainless steel bead (5 mm mean diameter) per 2 ml microcentrifuge tube (not supplied). If working with tissues that are not stabilized in RNA*later* RNA Stabilization Reagent, place the tubes on dry ice.**
3. **Excise the tissue sample from the animal or remove it from storage. Determine the amount of tissue. Do not use more than 30 mg. Proceed immediately to step 4.**

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

If the tissue sample was stored in RNA*later* RNA Stabilization Reagent, remove it from the reagent using forceps and be sure to remove any crystals that may have formed.

RNA in harvested tissues is not protected until the tissues are treated with RNA*later* RNA Stabilization Reagent, flash-frozen, or disrupted and homogenized in step 4. Frozen tissues should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible.

4. Disrupt the tissue and homogenize the lysate using either the TissueRupter (follow step 4a) or TissueLyser (follow step 4b).

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

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

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

4a. Disruption and homogenization using the TissueRuptor:

- **Place the tissue in a suitably sized vessel. Add 300 µl Buffer RLT.**

  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 uniformly homogeneous (usually 20–40 s). Proceed to step 5.**

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

4b. Disruption and homogenization using the TissueLyser:

- **Place the tissues in the tubes prepared in step 2.**
- **If the tubes were stored on dry ice, place them at room temperature. Then immediately add 300 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 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 5.**

  Do not reuse the stainless steel beads.

5. **Add 590 µl RNase-free water to the lysate. Then add 10 µl proteinase K solution, and mix thoroughly by pipetting.**
6. **Incubate at 55°C for 10 min.**
7. **Centrifuge at 20–25°C for 3 min at 10,000 x *g*.**

   A small pellet of tissue debris will form, sometimes accompanied by a thin layer or film on top of the supernatant.
8. **Pipet the supernatant (approximately 900 µl) into a new 1.5 ml or 2 ml microcentrifuge 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.
9. **Add 0.5 volumes (usually 450 µl) of ethanol (96–100%) to the cleared lysate. Mix well by pipetting. Do not centrifuge.**

   Precipitates may be visible after addition of ethanol. This does not affect the procedure.
10. **Transfer 700 µl of the sample, including any precipitate that may have formed, to an RNeasy Mini spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge at 20–25°C for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.***

    Reuse the collection tube in step 11.
11. **Repeat step 10 using the remainder of the sample. Discard the flow-through.***

    Reuse the collection tube in step 12.
12. **Add 350 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge at 20–25°C for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the membrane. Discard the flow-through.***

    Reuse the collection tube in step 15.

    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* (but not the collection tube). Proceed to step 16.

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

13. **Add 10 µl DNase I stock solution to 70 µl Buffer RDD. Mix by gently inverting the tube, and centrifuge briefly to collect residual liquid from the sides of 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.

14. **Add the DNase I incubation mix (80 µl) directly to the RNeasy 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 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.

15. **Add 350 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) at 20–25°C. Discard the flow-through.***

    Reuse the collection tube in step 16.

16. **Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge at 20–25°C for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the membrane. Discard the flow-through.**

    Reuse the collection tube in step 17.

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

17. **Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge at 20–25°C for 2 min at ≥8000 x *g* (≥10,000 rpm) to wash the membrane.**

    The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

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

18. **Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min.**

    Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 17.

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

19. **Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the RNeasy spin column membrane. Close the lid gently. To elute the RNA, centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm) at 20–25°C.**

20. **Repeat step 19 using another 30–50 µl RNase-free water, or using the eluate from step 19 (if high RNA concentration is required). Reuse the collection tube from step 19. If the expected RNA yield is >30 µg, there is no need to repeat step 19.**

    If using the eluate from step 19, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.

Protocol: Purification of Total RNA Using the RNeasy Fibrous Tissue Midi Kit

Determining the correct amount of starting material

It is essential to use the correct amount of tissue in order to obtain optimal RNA yield and purity. With the RNeasy Fibrous Tissue Midi Kit, a maximum of 250 mg tissue can generally be processed. For most tissues, the RNA binding capacity of the RNeasy spin column and the lysing capacity of Buffer RLT and proteinase K will not be exceeded by this amount. Average RNA yields from various tissues are given in Table 2 (page 12).

If there is no information about the nature of your starting material, we recommend starting with no more than 100 mg tissue. Depending on the RNA yield from the tissue sample, it may be possible to use up to 250 mg tissue in subsequent preparations.

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

Weighing tissue is the most accurate way to quantify the amount of starting material. As a guide, a 5 mm cube (125 mm^3) of most animal tissues weighs 150–175 mg.

Important points before starting

- If using RNeasy Fibrous Tissue Kits for the first time, read "Important Notes" (page 11).
- If working with RNA for the first time, read Appendix A (page 30).
- 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*.
- 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 pancreas or intestine.
- Fresh, frozen, or RNA*later* stabilized tissues can be used. If freezing tissues, flash-freeze in liquid nitrogen and immediately transfer to –70°C, where they can be stored for several months. Do not allow tissues to thaw during weighing or handling prior to disruption in Buffer RLT. Homogenized tissue lysates from step 4 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 5. Avoid prolonged incubation, which may compromise RNA integrity.
- **Do not vortex reconstituted DNase I**. DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube.
- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature (15–25°C).

- 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. During the procedure, work quickly.
- Perform all centrifugation steps at 20–25°C. 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 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.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- Prepare DNase I stock solution before using the RNase-Free DNase Set for the first time. Dissolve the lyophilized DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided in the RNase-Free DNase Set box. 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 DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.

Procedure

1. **Heat a water bath or heating block to 55°C for proteinase K digestion in step 6.**
2. **If using the TissueLyser, add one stainless steel bead (5 mm mean diameter) per 2 ml microcentrifuge tube (not supplied). If working with tissues that are not stabilized in RNA*later* RNA Stabilization Reagent, place the tubes on dry ice.**
3. **Excise the tissue sample from the animal or remove it from storage. Determine the amount of tissue. Do not use more than 250 mg. Proceed immediately to step 4.**

If using the TissueLyser, we recommend no more than 150 mg tissue for optimal lysis. Depending on the organism and the type of tissue, up to 250 mg tissue may be possible.

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

If the tissue sample was stored in RNA*later* RNA Stabilization Reagent, remove it from the reagent using forceps and be sure to remove any crystals that may have formed.

RNA in harvested tissues is not protected until the tissues are treated with RNA*later* RNA Stabilization Reagent, flash-frozen, or disrupted and homogenized in step 4. Frozen tissues should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible.

4. Disrupt the tissue and homogenize the lysate using either the TissueRupter (follow step 4a) or TissueLyser (follow step 4b).

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

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

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

4a. Disruption and homogenization using the TissueRuptor:

- **Place the tissue in a suitably sized vessel. Add 2 ml Buffer RLT.**

  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 uniformly homogeneous (usually 45–60 s). Proceed to step 5.**

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

4b. Disruption and homogenization using the TissueLyser:

- **Place the tissues in the tubes prepared in step 2.**
- **If the tubes were stored on dry ice, place them at room temperature. Then immediately add 1 ml Buffer RLT per tube.**
- **Place the tubes in the TissueLyser Adapter Set 2 x 24.**
- **Operate the TissueLyser for 3 min at 20 Hz.**

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

- **Rearrange the tubes so that the outermost tubes are innermost and the innermost tubes are outermost. Operate the TissueLyser for another 3 min at 20 Hz.**

  Rearranging the tubes allows even homogenization.

- **Carefully pipet the lysates into new microcentrifuge tubes (not supplied). Adjust the volume to 2 ml with Buffer RLT. Proceed to step 5.**

  Do not reuse the stainless steel beads.

5. **Add 4 ml RNase-free water to the lysate. Then add 65 µl proteinase K solution, and mix thoroughly by pipetting.**
6. **Incubate at 55°C for 20 min.**
7. **Centrifuge at 20–25°C for 5 min at 3000–5000 x *g*.**

   A small pellet of tissue debris will form, sometimes accompanied by a thin layer or film on top of the supernatant.
8. **Pipet the supernatant (approximately 6 ml) into a new 10–15 ml centrifuge 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.
9. **Add 0.5 volumes (usually 3 ml) of ethanol (96–100%) to the cleared lysate. Mix well by pipetting. Do not centrifuge.**

   Precipitates may be visible after addition of ethanol. This does not affect the procedure.
10. **Transfer 3 ml of the sample, including any precipitate that may have formed, to an RNeasy Midi spin column placed in a 15 ml collection tube. Close the lid gently, and centrifuge at 20–25°C for 5 min at 3000–5000 x *g*. Discard the flow-through.***

    Reuse the collection tube in step 11.
11. **Repeat step 10 twice, the first time using 3 ml of the sample, and the second time using the remainder of the sample (about 3 ml). Discard the flow-through.***

    Reuse the collection tube in step 12.
12. **Add 2 ml Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge at 20–25°C for 5 min at 3000–5000 x *g* to wash the membrane. Discard the flow-through.***

    Reuse the collection tube in step 15.

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

Optional: If on-column DNase digestion is not desired, add 4 ml Buffer RW1 instead, centrifuge for 5 min at 3000–5000 x *g*, and discard the flow-through* (but not the collection tube). Proceed to step 16.

13. **Add 20 µl DNase I stock solution to 140 µl Buffer RDD. Mix by gently inverting the tube, and centrifuge briefly to collect residual liquid from the sides of 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.

14. **Add the DNase I incubation mix (160 µl) directly to the RNeasy 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 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.

15. **Add 2 ml Buffer RW1 to the RNeasy spin column. Close the lid gently, and wait for 5 min and then centrifuge for 5 min at 3000–5000 x *g* at 20–25°C. Discard the flow-through.***

    Reuse the collection tube in step 16.

16. **Add 2.5 ml Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge at 20–25°C for 2 min at 3000–5000 x *g* to wash the membrane. Discard the flow-through.**

    Reuse the collection tube in step 17.

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

17. **Add 2.5 ml Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge at 20–25°C for 5 min at 3000–5000 x *g* to wash the membrane.**

    The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

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

18. **Place the RNeasy spin column in a new 15 ml collection tube (supplied). Add 150 µl RNase-free water directly to the RNeasy spin column membrane. Close the lid gently. To elute the RNA, wait for 1 min and then centrifuge for 3 min at 3000–5000 x *g* at 20–25°C.**

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

19. **Repeat step 18 using another 150 µl RNase-free water, or using the eluate from step 18 (if high RNA concentration is required). Reuse the collection tube from step 18.**

    If using the eluate from step 18, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or molecular biology applications (see back cover for contact information).

		Comments and suggestions
Clogged RNeasy spin column		
a)	Inefficient disruption and/or homogenization	See "Disrupting and homogenizing starting material" (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 page 11 and protocol, page 14 or 20) and/or increase the homogenization time.
b)	Too much starting material	In subsequent preparations, reduce the amount of starting material. It is essential to use the correct amount of starting material (see page 11 and protocol, page 14 or 20).
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 RNeasy spin column. If this happens, set the centrifugation temperature to 25°C. Warm the ethanol-containing lysate to 37°C before transferring it to the RNeasy spin column.
Low RNA yield		
a)	Insufficient disruption and homogenization	See "Disrupting and homogenizing starting material" (page 12) for details on disruption and homogenization methods. Increase *g*-force and centrifugation time if necessary.

		Comments and suggestions
		In subsequent preparations, reduce the amount of starting material (see page 11 and protocol, page 14 or 20) and/or increase the volume of lysis buffer and the homogenization time.
b)	Too much starting material	In subsequent preparations, reduce the amount of starting material. It is essential to use the correct amount of starting material (see page 11 and protocol, page 14 or 20).
c)	RNA still bound to RNeasy spin column membrane	Repeat RNA elution, but incubate the RNeasy spin column on the benchtop for 10 min with RNase-free water before centrifuging.
d)	Ethanol carryover	During the second wash with Buffer RPE (step 17, page 18 or 24), be sure to dry the RNeasy spin column membrane by centrifuging at ≥8000 x *g* (≥10,000 rpm) for 2 min at 20–25°C (RNeasy Fibrous Tissue Mini Kit) or at 3000–5000 x *g* for 5 min at 20–25°C (RNeasy Fibrous Tissue Midi Kit). After centrifugation, carefully remove the column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur. To eliminate any chance of possible ethanol carryover with the RNeasy Fibrous Tissue Mini Kit, place the RNeasy Mini spin column in a new 2 ml collection tube and perform the optional 1-min centrifugation step as described in step 18 of the protocol (page 18).
Low or no recovery of RNA		
RNase-free water incorrectly dispensed		Add RNase-free water to the center of the RNeasy spin column membrane to ensure that the membrane is completely covered.

	Comments and suggestions
Low A_{260}/A_{280} value	
Water used 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 32)
RNA degraded	
a) Inappropriate handling of starting material	For optimal results, ensure that tissue samples are properly stabilized and stored in RNA*later* RNA Stabilization Reagent (for details, see the *RNA*later *Handbook*). For 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 30) and "Handling and storing starting material" (page 12).
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 30) for general remarks on handling RNA. Do not put RNA samples into a vacuum dryer that has been used in DNA preparations where RNases may have been used.
DNA contamination in downstream experiments	
No DNase treatment	Perform on-column DNase digestion using the RNase-Free DNase Set as described in the protocol. Alternatively, perform DNase digestion after RNA purification (see Appendix C, page 34). For real-time, two-step RT-PCR experiments, carry out the RT step using the QuantiTect Reverse Transcription Kit, which provides cDNA synthesis with integrated removal of genomic DNA contamination. For ordering information, see page 38.

RNA does not perform well in downstream experiments

a)	Salt carryover during elution	Ensure that Buffer RPE is at 20–30°C.
b)	Ethanol carryover	During the second wash with Buffer RPE (step 17, page 18 or 24), be sure to dry the RNeasy spin column membrane by centrifuging at ≥8000 x *g* (≥10,000 rpm) for 2 min at 20–25°C (RNeasy Fibrous Tissue Mini Kit) or at 3000–5000 x *g* for 5 min at 20–25°C (RNeasy Fibrous Tissue Midi Kit). After centrifugation, carefully remove the column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur. To eliminate any chance of possible ethanol carryover with the RNeasy Fibrous Tissue Mini Kit, place the RNeasy Mini spin column in a new 2 ml collection tube and perform the optional 1-min centrifugation step as described in step 18 of the protocol (page 18).

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 31). Alternatively, chloroform-resistant plasticware can be rinsed with chloroform* to inactivate RNases.

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

Glassware

Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent,* thoroughly rinsed, and oven baked at 240ºC 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 material safety data sheets (MSDSs), available from the product supplier.

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

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

Storage of RNA

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

Quantification of RNA

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

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 33), 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 31). 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 = 100 µl

Dilution = 10 µl of RNA sample + 490 µl of 10 mM Tris·Cl,* pH 7.0 (1/50 dilution)

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

A_{260} = 0.2

Concentration of RNA sample = 44 µg/ml x A_{260} x dilution factor
= 44 µg/ml x 0.2 x 50
= 440 µg/ml

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

Total amount	= concentration x volume in milliliters
	= 440 µg/ml x 0.1 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 32).

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 RNeasy Kits will remove the vast majority of cellular DNA, trace amounts may still remain, 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 ABI PRISM® 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 are designed for SYBR® Green based real-time RT-PCR analysis of RNA sequences (without detection of genomic DNA) where possible (see www.qiagen.com/GeneGlobe). For real-time RT-PCR assays where amplification of genomic DNA cannot be avoided, we recommend using the QuantiTect Reverse Transcription Kit for reverse transcription. The kit integrates fast cDNA synthesis with rapid removal of genomic DNA contamination (see ordering information, page 38).

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 sample suffered major degradation either before or during RNA purification.

Appendix C: DNase Digestion of RNA Eluates

As an alternative to on-column DNase digestion (steps 12–15, page 17 or 23), DNase digestion of RNA eluates can be performed instead. For samples with high DNA content, we recommend DNase digestion of RNA eluates, as it is more efficient than on-column DNase digestion.

Important points before starting

- **Do not vortex the reconstituted DNase I.** DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube.
- In the procedure below, ▲ refers to use of the RNeasy Fibrous Tissue Mini Kit, and ● refers to use of the RNeasy Fibrous Tissue Midi Kit.

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

Things to do before starting

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

Procedure

C1. Purify RNA according to the standard protocol (page 14 or 20), but without performing the on-column DNase digestion described in steps 12–15 (instead, perform the optional wash with Buffer RW1 described in step 12).

C2. Mix the following in a microcentrifuge tube:

- **▲ ≤87.5 µl or ● ≤175 µl RNA eluate**
- **▲ 10 µl or ● 20 µl Buffer RDD**
- **▲ 2.5 µl or ● 5 µl DNase I stock solution**

Make the volume up to ▲ 100 µl or ● 200 µl with RNase-free water.

The reaction volumes can be doubled if necessary (to ▲ 200 µl or ● 400 µl final volume).

C3. Incubate on the benchtop (20–25°C) for 10 min.

C4. Clean up the RNA using the ▲ RNeasy Mini Kit or ● RNeasy Midi Kit (see ordering information, page 38).

Ordering Information

Product	Contents	Cat. no.
RNeasy Fibrous Tissue Mini Kit (50)	50 RNeasy Mini Spin Columns, Collection Tubes, Proteinase K, RNase-Free DNase I, RNase-Free Reagents and Buffers	74704
RNeasy Fibrous Tissue Midi Kit (10)	10 RNeasy Midi Spin Columns, Collection Tubes, Proteinase K, RNase-Free DNase I, RNase-Free Reagents and Buffers	75742
Accessories		
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
TissueRuptor	Handheld rotor–stator homogenizer, 5 TissueRuptor Disposable Probes	9001271* 9001272† 9001273‡ 9001274§
TissueRuptor Disposable Probes (25)	25 nonsterile plastic disposable probes for use with the TissueRuptor	990890

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

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

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

§ 235 V, 50/60 Hz (for Australia)

Ordering Information

Product	Contents	Cat. no.
TissueLyser	Universal laboratory mixer-mill disruptor	85200* 85210† 85220‡
TissueLyser Adapter Set 2 x 24	2 sets of Adapter Plates and 2 racks for use with 2 ml microcentrifuge tubes on the TissueLyser	69982
TissueLyser Single-Bead Dispenser, 5 mm	For dispensing individual beads (5 mm diameter)	69965
Stainless Steel Beads, 5 mm (200)	Stainless Steel Beads, suitable for use with the TissueLyser system	69989
Buffer RLT (220 ml)	220 ml lysis buffer for RNeasy Kits	79216
QIAGEN Proteinase K (2 ml)	2 ml (>600 mAU/ml, solution)	19131
QIAGEN Proteinase K (10 ml)	10 ml (>600 mAU/ml, solution)	19133
RNase-Free DNase Set (50)	1500 units RNase-Free DNase I, RNase-Free Buffer RDD, and RNase-Free Water	79254
Collection Tubes (2 ml)	1000 x 2 ml Collection Tubes	19201
TissueLyser Adapter Set 2 x 96	2 sets of Adapter Plates for use with Collection Microtubes (racked) on the TissueLyser	69984
Collection Microtubes (racked)	Nonsterile polypropylene tubes (1.2 ml), 960 in racks of 96	19560
Collection Microtube Caps (120 x 8)	Nonsterile polypropylene caps for collection microtubes (1.2 ml), 960 in strips of 8	19566

* 100 V, 50/60 Hz (for Japan)

† 120 V, 50/60 Hz (for North America)

‡ 220–240 V, 50/60 Hz (for Europe and Australia)

Ordering Information

Product	Contents	Cat. no.
Related products		
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, and RNase-Free Water	205311
RNeasy Kits — for purification of total RNA from animal cells or tissues, or yeast†		
RNeasy Mini Kit (50)	50 RNeasy Mini Spin Columns, Collection Tubes, RNase-Free Reagents and Buffers	74104
RNeasy Midi Kit (10)	10 RNeasy Midi Spin Columns, Collection Tubes, RNase-Free Reagents and Buffers	75142
RNeasy Plus Mini Kit — for purification of total RNA from cultured cells and tissues using gDNA Eliminator columns		
RNeasy Plus Mini Kit (50)	50 RNeasy Mini Spin Columns, 50 gDNA Eliminator Mini Spin Columns, Collection Tubes, RNase-Free Reagents and Buffers	74134
RNeasy Lipid Tissue Kits — for purification of total RNA from all types of animal tissue, including fatty tissues		
RNeasy Lipid Tissue Mini Kit (50)	50 RNeasy Mini Spin Columns, Collection Tubes, QIAzol Lysis Reagent, RNase-Free Reagents and Buffers	74804
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* Larger kit size available; see www.qiagen.com/products/PCR .

† Larger kit sizes and format available; see www.qiagen.com/RNA .

Ordering Information

Product	Contents	Cat. no.
RNeasy 96 Universal Tissue Kit — for high-throughput RNA purification from any type of animal tissue		
RNeasy 96 Universal Tissue Kit (4)*†	For 4 x 96 total RNA preps: 4 RNeasy 96 Plates, Collection Microtubes, Elution Microtubes CL, Caps, S-Blocks, AirPore Tape Sheets, QIAzol Lysis Reagent, RNase-Free Reagents and Buffers	74881
miRNeasy Kits — for purification of microRNA and total RNA from a wide range of animal tissues and cells		
miRNeasy Mini Kit (50)	For 50 preps: 50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), QIAzol Lysis Reagent, RNase-Free Reagents and Buffers	217004
miRNeasy 96 Kit (4)†	For 4 x 96 preps: 4 RNeasy 96 plates, Collection Microtubes (racked), Elution Microtubes CL, Caps, S-Blocks, AirPore Tape Sheets, QIAzol Lysis Reagent, RNase-Free Reagents and Buffers	217061

RNA*later* RNA Stabilization Reagent, RNA*later* TissueProtect Tubes, the TissueRuptor, the TissueLyser, the RNeasy Midi Kit, the RNeasy Plus Mini Kit, RNeasy Lipid Tissue Kits, and QuantiTect Kits and Assays are intended for research use. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease. The RNeasy Mini Kit is for general laboratory use. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

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

† Requires use of the Plate Rotor 2 x 96 and Centrifuge 4K15C (TissueLyser system recommended for disruption and homogenization; QIAvac 96 optional).

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Bench Protocol: RNA Purification Using the RNeasy Fibrous Tissue Mini Kit

Note: Before using this bench protocol, you should be completely familiar with the safety information and protocols in the *RNeasy Fibrous Tissue Handbook*.

Please tear off here

Important points before starting

- Except for step 4 and 9, perform the procedure at 15–25°C. Work quickly.
- Perform centrifugation at 20–25°C.
- Redissolve any precipitate in Buffer RLT by warming. Add β-ME before use.
- Before using Buffer RPE for the first time, ensure ethanol is added.
- Prepare DNase I stock solution.

Procedure

1. **Heat water bath or heating block to 55°C.**
2. **Disrupt and homogenize ≤30 mg tissue in 300 µl Buffer RLT using the TissueRuptor or TissueLyser.**
3. **Add 590 µl RNase-free water, then 10 µl Proteinase K, and mix.**
4. **Incubate at 55°C for 10 min.**
5. **Centrifuge at 10,000 x *g* for 3 min.**
6. **Transfer supernatant to new tube, add 0.5 volumes of 96–100% ethanol, and mix. Do not centrifuge.**
7. **Transfer sample to RNeasy column in 2 ml tube. Close lid, centrifuge for 15 s at ≥8000 x *g*, and discard flow-through.**
8. **Add 350 µl Buffer RW1 to RNeasy column. Close lid, centrifuge for 15 s at ≥8000 x *g*, and discard flow-through.**
9. **Mix 10 µl DNase stock solution with 70 µl Buffer RDD, add to RNeasy membrane, and incubate for 15 min at 20–30°C.**
10. **Add 350 µl Buffer RW1 to RNeasy column. Close lid, centrifuge for 15 s at ≥8000 x *g*, and discard flow-through.**
11. **Add 500 µl Buffer RPE to RNeasy column. Close lid, centrifuge for 15 s at ≥8000 x *g*, and discard flow-through.**
12. **Add 500 µl Buffer RPE to RNeasy column. Close lid and centrifuge for 2 min at ≥8000 x *g*.**
13. **Optional: Place RNeasy column in new 2 ml tube, close lid, and centrifuge at full speed for 1 min.**
14. **Place RNeasy column in new 1.5 ml tube. Add 30–50 µl RNase-free water, close lid, and centrifuge for 1 min at ≥8000 x *g*.**

    Optional: Repeat elution with another volume of water or with RNA eluate.

Bench Protocol: RNA Purification Using the RNeasy Fibrous Tissue Midi Kit

Note: Before using this bench protocol, you should be completely familiar with the safety information and protocols in the *RNeasy Fibrous Tissue Handbook*.

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Important points before starting

- Except for steps 4 and 9, perform the procedure at 15–25°C. Work quickly.
- Perform centrifugation at 20–25°C.
- Redissolve any precipitate in Buffer RLT by warming. Add β-ME before use.
- Before using Buffer RPE for the first time, ensure ethanol is added.
- Prepare DNase I stock solution.

Procedure

1. **Heat water bath or heating block to 55°C.**
2. **For the TissueRuptor, disrupt and homogenize ≤250 mg tissue in 2 ml Buffer RLT. For the TissueLyser, disrupt and homogenize ≤150 mg tissue in 1 ml Buffer RLT, and adjust volume to 2 ml with Buffer RLT.**
3. **Add 4 ml RNase-free water, then 65 µl Proteinase K, and mix.**
4. **Incubate at 55°C for 20 min.**
5. **Centrifuge at 3000–5000 x *g* for 5 min.**
6. **Transfer supernatant to new tube, add 0.5 volumes of 96–100% ethanol, and mix. Do not centrifuge.**
7. **Transfer sample to RNeasy column in 15 ml tube. Close lid, centrifuge for 5 min at 3000–5000 x *g*, and discard flow-through.**
8. **Add 2 ml Buffer RW1 to RNeasy column. Close lid, centrifuge for 5 min at 3000–5000 x *g*, and discard flow-through.**
9. **Mix 20 µl DNase stock solution with 140 µl Buffer RDD, add to RNeasy membrane, and incubate for 15 min at 20–30°C.**
10. **Add 2 ml Buffer RW1 to RNeasy column. Close lid, wait 5 min, centrifuge for 5 min at 3000–5000 x *g*, and discard flow-through.**
11. **Add 2.5 ml Buffer RPE to RNeasy column. Close lid, centrifuge for 2 min at 3000–5000 x *g*, and discard flow-through.**
12. **Add 2.5 ml Buffer RPE to RNeasy column. Close lid and centrifuge for 5 min at 3000–5000 x *g*.**
13. **Place RNeasy column in new 15 ml tube. Add 150 µl RNase-free water, close lid, wait 1 min, and centrifuge for 3 min at 3000–5000 x *g*.**

    Optional: Repeat elution with another volume of water or with RNA eluate.