February 2021

miRNeasy Tissue/Cells Advanced Mini Kit Handbook

For purification of total RNA, including miRNA from tissue and cells

Sample to Insight

QIAGEN

Contents

Kit Contents

miRNeasy Tissue/Cells Advanced Mini Kit	**(50)**
Catalog no.	**217604**
Number of Reactions	**50**
RNeasy® Mini Spin Columns (each packaged with a 2 ml Collection Tube)	50
gDNA Eliminator Column	50
Collection Tubes (1.5 ml)	50
Collection Tubes (2 ml)	50
Buffer RLT*	45 ml
Buffer AL	12 ml
Buffer RPP	8 ml
Buffer RWT*†	15 ml
Buffer RPE‡	11 ml
RNase-Free water	10 ml
Quick-Start Protocol	1

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

† Buffer RWT is supplied as a concentrate. Before using for the first time, add 2 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.

‡ 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.

Storage

The miRNeasy Tissue/Cells Advanced Mini Kit (cat. no. 217604) is shipped at ambient temperature. All components can be stored dry at room temperature. Under these conditions, all kit components are stable for at least 9 months, if not otherwise stated on the label.

Intended Use

The miRNeasy Tissue/Cells Advanced Mini Kit is intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

QIAcube® Connect is designed to perform fully automated purification of nucleic acids and proteins in molecular biology applications. The system is intended for use by professional users trained in molecular biological techniques and the operation of QIAcube Connect.

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.

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 a 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.

WARNING

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

Buffer RWT contains guanidine thiocyanate, which can form highly reactive compounds when combined with bleach. If liquid containing these solutions is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of miRNeasy Tissue/Cells Advanced Mini Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

Interest in smaller RNA species, such as miRNA, has increased over the past years as researchers understand the regulatory role of small non-coding RNAs. The miRNeasy Tissue/Cells Advanced Mini Kit is designed for purification of total RNA — including miRNA and other small RNA — from cultured cells and various animal and human tissues.

The miRNeasy Tissue/Cells Advanced Mini Kit offers a phenol-free protocol to isolate high yields of total RNA including miRNA from up to 30 mg frozen and 15 mg stabilized tissue or up to 1 x 10^7 cells.

Principles and procedures

The miRNeasy Tissue/Cells Advanced Mini Kit combines guanidine-based lysis of samples, an inhibitor removal step, and silica-membrane-based purification of total RNA, including miRNA and other small RNA. Buffer RLT, included in the kit, contains guanidine thiocyanate, designed to facilitate lysis and to denature protein complexes and RNases. Therefore, RNA in samples lysed in buffer RLT are stable and protected from degradation.

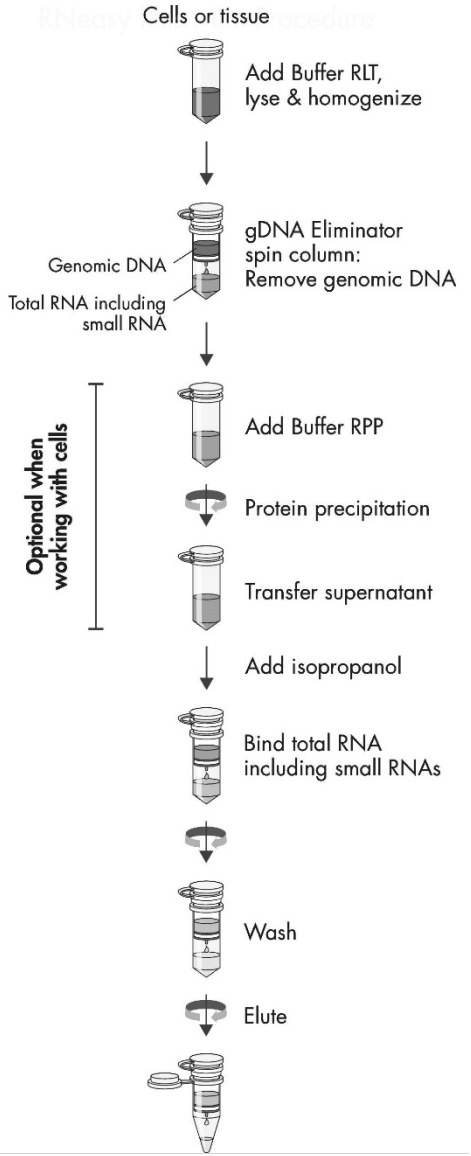
Buffer RLT is added to tissue or cell samples, and a thorough disruption and homogenization is done immediately to ensure a complete lysis and inactivation of RNases. Subsequently, Buffer AL is added to the lysate to optimize the gDNA removal by the gDNA eliminator column. Depending on the type and amount of starting material, an optional on column DNase digestion can be carried out (not included in the kit).

Buffer RPP is added to the flow-through to precipitate inhibitors (mostly proteins, which are highly concentrated in tissue and cell samples and might interfere with the RNA isolation or the downstream analysis) by centrifugation.

The supernatant containing the RNA including miRNA and other small RNA is transferred into a new reaction tube, and isopropanol is added to provide appropriate binding conditions for all RNA molecules from approximately 18 nucleotides (nt) upwards. The sample is then applied to the RNeasy Mini spin column, where the total RNA including miRNA and other small RNA binds to the membrane and all contaminants are efficiently washed away. High-quality RNA including miRNA and other small RNA is eluted in a small volume of RNase-free water.

In this handbook, different protocols are provided for different starting materials. The protocols differ primarily in protein precipitation step. Once the sample is applied to the RNeasy Mini Spin column, the protocols are similar (see flowchart, next page).

miRNeasy Tissue/Cells Advanced Mini Kit procedure



Automated purification of RNA on QIAcube Instruments

Purification of RNA can be fully automated on QIAcube Connect or the classic QIAcube. The innovative QIAcube instruments use advanced technology to process QIAGEN spin columns, enabling seamless integration of automated, low-throughput sample prep into your laboratory workflow. Sample preparation using QIAcube instruments follows the same steps as the manual procedure (i.e., lyse, bind, wash, and elute), enabling you to continue using the miRNeasy Tissue/Cells Advanced Mini Kit for purification of high-quality miRNA.

QIAcube instruments are 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/qiacubeprotocols**.

Figure 1. QIAcube Connect.

Real-time RT-PCR detection of miRNAs using the miRCURY® LNA® miRNA PCR systems

In general, real-time RT-PCR is recommended to accurately quantify yields of miRNA. The miRCURY LNA miRNA PCR system allows sensitive and specific quantification and profiling of miRNA expression using SYBR® Green-based or probe-based real-time PCR. Both the SYBR® Green detection-based miRCURY LNA miRNA PCR system and the probe-based miRCURY LNA miRNA Probe PCR System comprise all the required components to set up and conduct miRNA quantification and expression profiling, from conversion of RNA into cDNA to real-time PCR detection of miRNAs and straightforward data analysis. The systems both use the same miRCURY LNA RT kit for generation of a universal first-strand cDNA synthesis – one cDNA reaction for all miRNAs. Each system then has their own dedicated master mix kit, the miRCURY LNA SYBR® Green PCR kit and the miRCURY LNA miRNA Probe PCR Kit, as well as a broad variety of system-specific LNA-enhanced miRCURY LNA assay and panel products. The RNA Spike-In Kit enables quality control of the RNA isolation, cDNA synthesis, and PCR amplification steps of miRCURY LNA miRNA qPCR experiments.

For both systems, individual assays for mature miRNAs for a variety of different species can be ordered on GeneGlobe® (**www.qiagen.com/GeneGlobe**). Alternatively, for high-throughput and screening experiments, miRCURY LNA PCR Panels and miRCURY LNA Probe PCR Panels enable rapid profiling of the complete miRNome. Other Focus panels like the miRCURY LNA miRNA Serum/Plasma Focus PCR Panel and Serum/Plasma Focus Probe PCR Panel are available for the detection of mature miRNAs. Find out more about the miRCURY LNA miRNA PCR systems at **www.qiagen.com**.

Description of Protocols

This handbook contains two protocols for purification of total RNA, including miRNA, from tissues or cells using the miRNeasy Tissue/Cells Advanced Mini Kit. In addition, protocols are provided for sample disruption, on-column DNase digestion, and for general remarks on handling RNA.

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.

- Ethanol (80%)*
- Isopropanol (100%)
- 14.3 M β-mercaptoethanol (β-ME, commercially available solutions are usually 14.3 M), alternatively dithiothreitol (DTT, 2 M stock solution)
- Sterile, RNase-free pipet tips
- 1.5 ml or 2 ml microcentrifuge tubes
- Microcentrifuge(s) (with rotor for 2 ml tubes) for centrifugation at room temperature (15–25°C)
- Disposable gloves
- Reagent for RNA stabilization (see page-18):
  - For cell samples: RNAprotect® Cell Reagent† or liquid nitrogen
  - For tissue samples: RNAprotect Tissue Reagent† (stabilizes RNA only), Allprotect® Tissue Reagent† (stabilizes DNA, RNA and protein) or liquid nitrogen
- **Optional**: RNase-Free DNase Set (see Ordering Information, page 50)

* Do not use denatured alcohol, which contains other substances, such as methanol and methylethylketone.
† For ordering information, see page 50.

- Equipment and tubes for disruption and homogenization (see page 18). Depending on the method chosen, one or more of the following are required:
  - TissueRuptor® II with TissueRuptor Disposable Probes (see Ordering Information, page 50)
  - TissueLyser II or TissueLyser LT (see Ordering Information, page 50)
  - Mortar and pestle
  - QIAshredder homogenizer (see Ordering Information, page 50)
  - Blunt-ended needle and syringe
  - Trypsin and PBS

Important Notes

Determining the amount of starting material

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

- The volume of Lysis Buffer RLT required for efficient lysis
- The RNA-binding capacity of the RNeasy Mini spin column (100 µg)
- The RNA content of the sample type

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 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 Mini 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 Mini spin column membrane, resulting in lower RNA yield and purity.

Table 1. RNeasy Mini spin column specifications

Maximum binding capacity	**100 µg RNA**
Maximum loading volume	700 µl
RNA size distribution	RNA >18 nucleotides
Minimum elution volume	30 µl
Maximum amount of starting material	
Animal cells	1×10^7
Animal tissues	30 mg flash frozen (15 mg for stabilized tissue)

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

Determining the correct amount of starting material — cells

The minimum amount of starting material is generally 100 cells, while the maximum amount depends on the RNA content of the cell type.

RNA content can vary greatly between cell types. The following examples illustrate how to determine the maximum amount of starting material:

- COS cells have high RNA content (approximately 35 µg RNA per 1×10^6 cells). Do not use more than 3×10^6 cells, otherwise the RNA binding capacity of the RNeasy Mini spin column (100 µg) will be exceeded.
- HeLa cells have average RNA content (approximately 15 µg RNA per 1×10^6 cells). Do not use more than 7×10^6 cells, otherwise the RNA binding capacity of the RNeasy Mini spin column will be exceeded.
- NIH/3T3 cells have low RNA content (approximately 10 µg RNA per 1×10^6 cells). The maximum amount of starting material (1×10^7 cells) can be used.

If processing a cell type where there is no information about its RNA content, we recommend starting with no more than 3–4 $\times 10^6$ cells. Depending on RNA yield and purity, it may be possible to increase the cell number in subsequent preparations.

Counting cells is the most accurate way to quantify 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 2.

Table 2. 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 x 10^4
48-well	1	1 x 10^5
24-well	2	2.5 x 10^5
12-well	4	5 x 10^5
6-well	9.5	1 x 10^6
Dishes		1 x 10^6
35 mm		2.5 x 10^6
60 mm		7 x 10^6
100 mm		2 x 10^7
145-150 mm		

* 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. Cell numbers will vary for different kinds of animal cells, which vary in length from 10 to 30 µm.

Determining the correct amount of starting material — tissue

The maximum amount of tissue that can be processed depends on the RNA content of the tissue. To help to estimate the RNA content of your tissue type, Table 3 shows expected RNA yields from various sources.

In general, a maximum of 30 mg tissue can be processed with the miRNeasy Mini procedure. The binding capacity of the column will not be exceeded by these amounts.

Table 3. Average yields of total RNA with miRNeasy Tissue/Cells Advanced Mini Kit

Sample	Average RNA yield* (µg)
Mouse/rat tissue (10 mg)	
Kidney	5-40
Liver	15–80
Lung	5–15
Heart	5–25
Muscle	5–35
Brain	5–20
Adipose tissue	0.5–2.5
Spleen	15–100
Intestine	10–60
Skin	2–5
Cell culture (1 x 10^6 cells)	
NIH/3T3	10
HeLa	15
COS-7	35
LMH	12
Huh	15
Jurkat	15

* Amounts can vary due to species, developmental stage, etc.

If you have no information about the nature of your starting material, we recommend starting with no more than 30 mg of tissue.

Weighing tissue is the most accurate way to quantify the amount of starting material. However, the following may be used as a guide. A 3 mm cube (volume, 27 mm^3) of most animal tissues weighs 25–35 mg.

Handling and storing starting material

RNA is not protected after harvesting until the sample is treated with RNAprotect Cell Reagent (cultured cells only) or RNAprotect Tissue Reagent (animal tissues only), 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 samples are immediately frozen in liquid nitrogen and stored at –90°C to –65°C (animal tissues only), processed as soon as harvested, or immediately immersed in RNAprotect Cell Reagent or RNAprotect Tissue Reagent. Animal cells can be pelleted and then stored at –90°C to –65°C until required for RNA purification.

An alternative to RNAprotect Tissue Reagent is Allprotect® Tissue Reagent, which provides immediate stabilization of DNA, RNA, and protein in tissues samples at room temperature.

The procedures for harvesting and RNA protection should be carried out as quickly as possible. Frozen samples should not be allowed to thaw during handling or weighing. After disruption and homogenization in Buffer RLT, samples can be stored at –90°C to –65°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 significantly reduces 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. In the miRNeasy procedure, genomic DNA is removed by organic extraction, which makes it possible to homogenize up to 3 x 10^6 cells by vortexing without additional homogenization. Incomplete homogenization results in inefficient binding of RNA to the RNeasy Mini spin column membrane, significantly reducing RNA yields.

Some disruption methods simultaneously homogenize the sample, while others require an additional homogenization step. Table 4 gives an overview of different disruption and homogenization methods and is followed by a detailed description of each method. This information can be used as a guide to choose the appropriate methods for your starting material.

Table 4. Guide to methods of disruption and homogenization of samples

Sample	Disruption method	Homogenization method	Comments
Animal cells	Addition of lysis buffer	TissueRuptor II or QIAshredder homogenizer or syringe and needle or vortexing (≤3 x 10^6 cells)	If processing ≤3 x 10^6 cells, lysate can be homogenized by vortexing.
Animal tissues	TissueLyser II or TissueLyser LT	TissueLyser II or TissueLyser LT	The TissueLyser II and TissueLyser LT give results comparable to using a rotor–stator homogenizer
	TissueRuptor II	TissueRuptor II	Simultaneously disrupts and homogenizes
	Mortar and pestle	QIAshredder homogenizer or syringe and needle	The TissueRuptor II usually gives higher yields than mortar and pestle

Disruption and homogenization using the TissueRuptor II

The TissueRuptor II is a rotor–stator homogenizer that thoroughly disrupts and simultaneously homogenizes single animal tissue samples in the presence of lysis buffer in 15–90 seconds, depending on the toughness and size of the sample. The TissueRuptor II can also be used to homogenize cell lysates. The blade of the TissueRuptor II 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 disruption and homogenization of animal tissues using the TissueRuptor II, refer to the *TissueRuptor II Handbook*. For other rotor–stator homogenizers, please refer to suppliers' guidelines for further details.

Disruption and homogenization using the TissueLyser II or TissueLyser LT

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

- Size and composition of beads
- Ratio of buffer to beads
- Amount of starting material
- Speed and configuration of the TissueLyser II or TissueLyser LT
- Disintegration time

Stainless-steel beads with a 3–7 mm diameter are optimal for use with animal tissues. All other disruption parameters must be determined empirically for each application. For guidelines on disruption and homogenization of tissues using the TissueLyser II and stainless-steel beads, refer to Appendix A (page 39). For other bead mills, please refer to the suppliers' guidelines for further details.

Disruption using a mortar and pestle

For disruption using a mortar and pestle, freeze the animal tissue 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.

Protocol: Purification of Total RNA, Including Small RNAs, from Animal Cells

Important points before starting

- If using the miRNeasy Tissue/Cells Advanced Mini Kit for the first time, read "Important Notes" (page 14).
- It is important not to overload the RNeasy Mini spin column, as overloading will significantly reduce RNA yield and quality. Read "Determining the amount of starting material" (page 11).
- If working with RNA for the first time, read Appendix C (page 43).
- Cell pellets can be stored at –90°C to –65°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 –90°C to –65°C for several months. To process frozen homogenized lysates, incubate at 37°C in a water bath until completely thawed and salts are dissolved. Avoid prolonged incubation, which may compromise RNA integrity.
- 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.

- Generally, DNase digestion is not required since the RNeasy technologies in combination with the gDNA eliminator efficiently removes most of the DNA without DNase treatment. In addition, miRCURY Assays and most other assays for mature miRNA are not affected by the presence of small amounts of genomic DNA. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA. In these cases, small residual amounts of DNA can be removed by on-column DNase digestion (see Appendix B, page 40) or by DNase digestion after RNA purification (please contact QIAGEN Technical Service for a protocol).
- Buffer RLT and Buffer RWT may form a precipitate upon storage. If necessary, redissolve by warming and then place at room temperature (15–25°C).
- Buffer RLT, Buffer AL, and Buffer RWT contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 6 for Safety Information.

All protocol and centrifugation steps should be performed at room temperature. During the procedure, work quickly.

Things to do before starting

- Equilibrate buffers to room temperature.
- All steps should be performed at room temperature. Work quickly.
- Add ethanol (96–100%) to Buffer RWT and Buffer RPE concentrates before use (see bottle label for volume).
- 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.

Procedure

1. Harvest cells according to step 1a or 1b.

   1a. Cells grown in suspension (do not use more than 1 x 10^7 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 Mini spin column membrane. Both effects may reduce RNA yield.

   1b. Cells grown in a monolayer (do not use more than 1 x 10^7 cells):
   Cells grown in a monolayer in cell-culture vessels can be either lysed directly in the vessel (up to 10 cm diameter) or trypsinized and collected as a cell pellet prior to lysis. Cells grown in a monolayer 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 Mini spin column 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. 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 Mini spin column 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 the appropriate volume of Buffer RLT (see Table 5). 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. Ensure that β-ME is added to Buffer RLT before use (see "Important points before starting").

   For direct lysis of cells grown in a monolayer, add the appropriate volume of Buffer RLT (see Table 5) to the cell-culture dish. 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.

Table 5. Volumes of buffer RLT for sample disruption and homogenization sample

	Amount	Dish	Buffer RLT	Buffer AL	Disruption and homogenization
Pelleted cells	<5 x 10^6	<6 cm	260 µl	80 µl	Add Buffer RLT, vortex (≤1 x 10^5 cells); or use QIAshredder, TissueRuptor II, or needle and syringe
	>5 x 10^6	6–10 cm	450 µl	140 µl	

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

   See "Disrupting and homogenizing starting material", page 18, for more details on homogenization. If processing ≤1 x 10^5 cells, they can be homogenized by vortexing for 1 min. After homogenization, proceed to step 4.

   Note: Incomplete homogenization leads to significantly reduced RNA yields and can cause clogging of the RNeasy spin column. Homogenization with a rotor–stator 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 placed in a 2 ml collection tube, and centrifuge for 2 min at maximum speed. Proceed to step 4.

   3b. Homogenize the lysate for 30 s using the TissueRuptor II. Proceed to step 4.

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

4. Add the appropriate volume of Buffer AL (see Table 5) and mix thoroughly. Incubate at room temperature for 3 min.
5. Transfer the homogenized lysate to a gDNA Eliminator spin column placed in a 2 ml collection tube (supplied). Centrifuge for 30 s at ≥8000 x *g* (≥10,000 rpm). Discard the column and save the flow-through.

   Note: Make sure that no liquid remains on the column membrane after centrifugation. If necessary, repeat the centrifugation until all liquid has passed through the membrane.

   Optional: Step 6 and 7 do not need to be carried out when working with cell samples.
6. Transfer the flow-through to a new 2 ml reaction tube (not provided). Add 20 µl Buffer RPP. Close the tube cap and mix vigorously by vortexing for >20 s. Incubate at room temperature for 3 min.
7. Centrifuge at 12,000 x g for 3 min at room temperature to pellet the precipitate.

   Note: Supernatant should be clear and colorless. Transfer supernatant to a new 2 ml reaction tube.
8. Add 1 volume isopropanol and mix well by pipetting. Do not centrifuge.

   Note: When purifying RNA from certain cell lines, precipitates may be visible after addition of isopropanol. This does not affect the procedure.
9. Transfer up to 700µl of the sample to an RNeasy Mini column placed in a 2ml collection tube (provided). Close the lid and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.

   Reuse the collection tube in step 10.

   If the sample volume exceeds 700 µl, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.

10. Pipet 700 µl Buffer RWT to the RNeasy Mini spin column. Close the lid and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.

    Reuse the collection tube in step 11.

    Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.

11. Pipet 500 µl Buffer RPE onto the RNeasy Mini spin column. Close the lid and centrifuge for 15 s at ≥8000 x *g*. 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 "Important points before starting").

12. Add 500 µl of 80% ethanol to the RNeasy Mini spin column. Close the lid and centrifuge for 2 min at ≥8000 x *g*. Discard the flow-through and the collection tube.

13. Place the RNeasy Mini spin column in a new 2 ml collection tube (supplied). Close the lid of the spin column and centrifuge at full speed for 1 min to dry the membrane. Discard the flow-through and the collection tube.

14. Place the RNeasy Mini spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the center of the spin column membrane and incubate for 1 min. Close the lid and centrifuge for 1 min at full speed to elute the RNA.

15. If the expected RNA yield is >30 µg, repeat step 14 using another 30–50 µl of RNase-free water, or using the eluate from step 14 (if high RNA concentration is required). Reuse the collection tube from step 14.

    If using the eluate from step 14, 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, Including Small RNAs, from Animal Tissues

Determining the correct amount of starting material

It is essential to use the correct amount of starting material to obtain optimal RNA yield and purity. A maximum amount of 30 mg fresh or frozen tissue or 15 mg RNAprotect stabilized tissue (which is partially dehydrated) can generally be processed. For most tissues, the DNA removal capacity of the gDNA Eliminator spin column, the RNA binding capacity of the RNeasy spin column, and the lysing capacity of Buffer RLT will not be exceeded by these amounts. However, smaller amounts may allow more efficient DNA removal. Average RNA yields from various tissues are given in table 3 (page14). Some tissues, such as spleen and thymus, contain very high amounts of DNA, which will overload the gDNA Eliminator spin column. For fibrous tissues, such as muscle and skin, that contain contractile proteins, connective tissue and collagen, we recommend the RNeasy Plus Universal Mini Kit, which efficiently removes these components in the phase separation.

Do not overload the gDNA Eliminator spin column, as this will lead to copurification of DNA with RNA. Do not overload the RNeasy spin column, as this will significantly reduce RNA yield and quality.

Important points before starting

- If using the miRNeasy Tissue/Cells Advanced Mini Kit for the first time, read "Important Notes" (page 14).
- It is important not to overload the RNeasy Mini spin column, as overloading will significantly reduce RNA yield and quality. Read "Determining the amount of starting material" (page 14).
- If working with RNA for the first time, read Appendix C (page 43).

- For optimal results, stabilize harvested tissues immediately in RNAprotect Tissue Reagent or Allprotect Stabilization Reagent. Tissues can be stored in the reagent for up to 1 day at 37°C, 7 days at 15–25°C, or 4 weeks at 2–8°C, or archived at –95°C to –15°C.
- Fresh, frozen, or RNAprotect- or AllProtect-stabilized tissues can be used. Tissues can be stored for several months at –90°C to –65°C. Do not allow tissues to thaw during weighing or handling prior to disruption in Buffer RLT. Homogenized tissue lysates (in Buffer RLT, step 3) can also be stored at –90°C to –65°C for several months. To process frozen homogenized lysates, incubate at 37°C in a water bath until completely thawed and salts are dissolved. Avoid prolonged incubation, which may compromise RNA integrity. Continue with step 4.
- Generally, DNase digestion is not required since the combination of the RNeasy technologies in combination with the gDNA eliminator efficiently removes most of the DNA without DNase treatment. In addition, miRCURY Assays and most other assays for mature miRNA are not affected by the presence of small amounts of genomic DNA. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA. In these cases, small residual amounts of DNA can be removed by on-column DNase digestion (see Appendix B, page 40) or by DNase digestion after RNA purification (please contact QIAGEN Technical Service for a protocol).
- Buffer RLT und Buffer RWT may form a precipitate upon storage. If necessary, redissolve by warming and then place at room temperature (15–25°C).
- Buffer RLT, Buffer AL, and Buffer RWT contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 6 for Safety Information.
- All protocol and centrifugation steps should be performed at room temperature. During the procedure, work quickly.

Things to do before starting

- Buffers RWT and RPE are supplied as concentrates. Before using for the first time, add the required amounts of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.
- If performing optional on-column DNase digestion, prepare DNase I stock solution as described in Appendix B (page 40).

Notes before starting

- Equilibrate buffers to room temperature.
- All steps should be performed at room temperature. Work quickly.
- β-mercaptoethanol (β-ME) must be added to Buffer RLT before use. Add 10 µl β-ME per 1 ml Buffer RLT. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT is stable at room temperature (15–25°C) for 1 month after addition of β-ME. Alternatively, add 20 µl 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. Excise the tissue sample from the animal or remove it from storage. Remove RNAprotect-stabilized tissues from the reagent using forceps. Determine the amount of tissue. Do not use more than 30 mg.

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

2. Follow either step 2a or 2b.

   2a. For RNAprotect or Allprotect-stabilized tissues: If tissue is still submerged in stabilizing reagent remove each tissue sample from the liquid. Remove residual reagent (e.g., by dabbing or rolling the tissue over a paper towel) If using the entire tissue, place it directly into a suitably sized vessel for disruption and homogenization, and proceed to step 3.

   If using only a portion of the tissue, cut it on a clean surface. Weigh the piece to be used, and place it into a suitably sized vessel for disruption and homogenization. Proceed to step 3. RNA in RNAprotect-stabilized tissues is protected during cutting and weighing of tissues at ambient temperature (18–25°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 RNAprotect Tissue Reagent. Previously stabilized tissues can be stored at –80°C without the reagent.

   2b. For unstabilized fresh or frozen tissues:

   If using the entire tissue, place it directly into a suitably sized tube for disruption and homogenization, and proceed immediately to step 3.

   If using only a portion of the tissue, weigh the piece to be used, and place it into a suitably sized vessel for disruption and homogenization. Proceed immediately to step 3.

   RNA in harvested tissues is not protected until the tissues are treated with RNAprotect Tissue 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.

   Note: Remaining fresh tissues can be placed into RNAprotect Tissue Reagent to stabilize RNA (see the *RNAprotect Tissue Handbook*). 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 30 mg tissue) according to step 3a, 3b, 3c, or 3d.

   See "Determining the amount of starting material", page 14, for more details on disruption and homogenization.

   Optional: Ensure that β-ME is added to Buffer RLT before use (see "Important points before starting").

   Note: After storage in RNAprotect Tissue Reagent, tissues may become slightly harder than fresh or thawed tissues. Disruption and homogenization using standard methods is usually not a problem. For easier disruption and homogenization, we recommended using 450 µl Buffer RLT.

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

Table 6. Volumes of buffer RLT for sample disruption and homogenization sample

	Amount	Buffer RLT	Buffer AL	Disruption and homogenization
Animal tissues	<20 mg	260 µl	80 µl	TissueLyser LT, TissueLyser II, TissueRuptor II, or mortar and pestle followed by QIAshredder or needle and syringe.
	20–30 mg	450 µl	140 µl	

3a. Disruption and homogenization using the TissueRuptor II: Place the weighed (fresh, frozen, or RNAprotect Tissue-stabilized) tissue in a suitably sized vessel. Add the appropriate volume of Buffer RLT (see Table 6). Immediately disrupt and homogenize the tissue until it is uniformly homogeneous (usually 20–40 s); see the *TissueRuptor II Handbook*. Proceed to step 4.

3b. Disruption using a mortar and pestle followed by homogenization using a QIAshredder homogenizer: Immediately place the weighed (fresh, frozen, or RNAprotect Tissue-stabilized) tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled, 2 ml microcentrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw. Add the appropriate volume of Buffer RLT (see Table 6). Pipet the lysate directly into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 min at maximum speed. Proceed to step 4.

3c. Disruption using a mortar and pestle followed by homogenization using a needle and syringe: Immediately place the weighed (fresh, frozen, or RNAprotect Tissue-stabilized) tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled, 2 ml microcentrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw. Add the appropriate volume of Buffer RLT (see Table 6), and homogenize by passing lysate at least 5 times through a 20-gauge needle fitted to an RNase-free syringe. Proceed to step 4.

3d. Disruption and homogenization using the TissueLyser II or TissueLyser LT: See the *TissueLyser Handbook* or the *TissueLyser LT Handbook*. Then proceed to step 4.

4. Centrifuge the lysate for 3 min at maximum speed. Carefully remove the supernatant by pipetting into a new tube.

   Note: This step is important, as it removes insoluble material that could clog the gDNA Eliminator spin column and interfere with DNA removal. In some preparations, very small amounts of insoluble material will be present after the 3 min centrifugation, making the pellet invisible.

5. Add the appropriate volume of Buffer AL (see Table 5) and mix thoroughly. Incubate at room temperature for 3 min.

6. Transfer the homogenized lysate to a gDNA Eliminator spin column placed in a 2 ml collection tube (supplied). Centrifuge for 30 s at ≥8000 x *g* (≥10,000 rpm). Discard the column and save the flow-through.

   Note: Make sure that no liquid remains on the column membrane after centrifugation. If necessary, repeat the centrifugation until all liquid has passed through the membrane.

7. Transfer the flow-through to a new 2 ml reaction tube (not provided). Add 20 µl Buffer RPP. Close the tube cap and mix vigorously by vortexing for >20 s. Incubate at room temperature for 3 min.

8. Centrifuge at 12,000 x *g* for 3 min at room temperature to pellet the precipitate.

   Note: Supernatant should be clear and colorless. Transfer supernatant to a new 2 ml reaction tube.

9. Add 1 volume isopropanol and mix well by pipetting. Do not centrifuge.

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

10. Transfer up to 700µl of the sample to an RNeasy Mini column placed in a 2ml collection tube (provided). Close the lid and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.

    Reuse the collection tube in step 11.

    If the sample volume exceeds 700 µl, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.

11. Pipet 700 µl Buffer RWT to the RNeasy Mini spin column. Close the lid and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.

    Reuse the collection tube in step 12.

    Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.

12. Pipet 500 µl Buffer RPE onto the RNeasy Mini spin column. Close the lid and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.

    Reuse the collection tube in step 13.

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

13. Add 500 µl of 80% ethanol to the RNeasy Mini spin column. Close the lid and centrifuge for 2 min at ≥8000 x g. Discard the flow-through and the collection tube.

14. Place the RNeasy Mini spin column in a new 2 ml collection tube (supplied). Close the lid of the spin column and centrifuge at full speed for 1 min to dry the membrane. Discard the flow-through and the collection tube.

15. Place the RNeasy Mini spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the center of the spin column membrane and incubate for 1 min. Close the lid and centrifuge for 1 min at full speed to elute the RNA.

16. If the expected RNA yield is >30 µg, repeat step 15 using another 30–50 µl of RNase-free water, or using the eluate from step 15 (if high RNA concentration is required). Reuse the collection tube from step 15.

    If using the eluate from step 15, 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. For more information, see also the Frequently Asked Questions page at our Technical Support Center: **www.qiagen.com/FAQ/FAQList.aspx**. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit **www.qiagen.com**).

	Comments and suggestions
RNA degraded	
a) Inappropriate handling of starting material	Ensure that tissue samples are properly stabilized and stored in RNAprotect 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 39) and "Handling and storing starting material" (page 17).
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 39) for general remarks on handling RNA.
DNA contamination in downstream experiments	
a) Cell number too high	For some cell types, the efficiency of DNA removal may be reduced when processing very high cell numbers (containing more than 20 µg genomic DNA). If the eluted RNA contains substantial DNA contamination, try processing smaller cell numbers or perform DNase digestion of the eluted RNA followed by RNA cleanup.
b) Incomplete removal of cell-culture medium or stabilization reagent	Be sure to remove any excess cell-culture medium or stabilization reagent to prevent significant dilution of the lysis buffer. The gDNA Eliminator spin column will not work effectively if the lysis buffer is diluted.
c) Tissue has high DNA content	For certain tissues with extremely high DNA content (e.g., thymus), DNA may not be completely removed. Try using smaller samples (containing less than 20 µg genomic DNA) or perform DNase I digestion of the eluted RNA followed by RNA cleanup.

	Comments and suggestions
Clogged gDNA Eliminator spin column	
a) Inefficient disruption and/or homogenization	See "Determining the amount of starting material" (page 14) 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 protocols, pages 22 and 28) and/or increase the homogenization time.
b) Too much starting material	Reduce the amount of starting material. It is essential to use the correct amount of starting material (see page 14).
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 gDNA Eliminator spin column. If this happens, set the centrifugation temperature to 25°C. Warm the lysate to 37°C before transferring it to the gDNA Eliminator spin column.
Clogged RNeasy Mini column	
a) Too much starting material	In subsequent preparations, reduce the amounts of starting material. It is essential to use the correct amount of starting material (see page 14).
b) Inefficient disruption and/or	See "Disrupting and homogenizing starting material" (page 18) for a detailed description of homogenization methods. Increase g-force and centrifugation time if necessary. In subsequent preparations, reduce the amount of starting material (see page 14) and/or increase the homogenization time.
c) Centrifugation temperature too low	All centrifugation steps should be performed at room temperature (15–25°C). Some centrifuges may cool to below 20°C even when set at 20°C. This can cause precipitates to form that can clog the RNeasy Mini spin column and reduce RNA yield. If this happens, set the centrifugation temperature to 25°C. Warm the ethanol-containing lysate to 37°C before transferring to the RNeasy Mini spin column.
Low RNA yield or poor performance of RNA in downstream applications	
a) Incorrect ethanol concentration	Be sure to use the ethanol concentrations specified in the protocol steps.
b) Interference from large RNAs	In some assays, the presence of mRNA and rRNA can result in increased background. In this case, follow the protocol in Appendix A: Preparation of miRNA-Enriched Fractions Separate from Larger RNAs (>200 nt) of the *miRNeasy Mini Kit Handbook* to isolate a separate, miRNA-enriched fraction. An additional kit, the RNeasy MinElute® Cleanup Kit, is required for this protocol.
c) Elution volume too high	Elute RNA with less than 2 x 50 µl of water. Do not use less than 1 x 30 µl of water. Although eluting with less than 2 x 50 µl of water results in increased RNA concentrations, RNA yields may be reduced.

	Comments and suggestions
d) Salt carryover during elution	Ensure that Buffer RPE is at 20–30°C. When reusing collection tubes between washing steps, remove residual flow-through from the rim by blotting on clean paper towels.
Low or no recovery of RNA	
a) Too much starting material	In subsequent preparations, reduce the amounts of starting material. It is essential to use the correct amount of starting material (see page 14).
b) Inefficient disruption and/or homogenization	See "Disrupting and homogenizing starting material" (page 18) for a detailed description of homogenization methods. Increase g-force and centrifugation time if necessary. In subsequent preparations, reduce the amount of starting material (see page 14) and/or increase the homogenization time.
c) Elution buffer incorrectly dispensed	Add elution buffer to the center of the RNeasy Mini spin column membrane to ensure that the buffer completely covers the membrane.
d) RNA still bound to the membrane	Repeat the elution step of the protocol, but incubate the RNeasy Mini spin column on the bench for 10 min after adding RNase- free water and before centrifugation.
Low A260/A280 value	
a) Not enough Buffer RLT used for homogenization	Reduce the amount of starting material and/or increase the volume of Lysis Buffer RLT and the homogenization time.
b) Sample not incubated for 3min after homogenization	Place the sample at room temperature (15–25°C) for 3 min after homogenization, as indicated in the protocols (step 3). This step is important to promote dissociation of nucleoprotein complexes.
c) Water used to dilute RNA for A260/A280 measurement	Use 10 mM Tris·Cl, pH 7.5, not RNase-free water, to dilute the sample before measuring purity (see Appendix D, page 43).
RNA degraded	
a) Sample inappropriately handled	For frozen tissue samples, ensure that they were flash-frozen immediately in liquid nitrogen and properly stored at –90°C to –65°C. Perform the protocol quickly, especially the first few steps. See "Appendix C: General Remarks on Handling RNA" (page 43) and "Handling and storing starting material" (page 17).
b) RNase contamination	Although all buffers have been tested and are guaranteed RNase-free, RNases can be introduced during use. Make sure not to introduce any RNases during the procedure or later handling. See "Appendix C: General Remarks on Handling RNA" (page 43). Do not put RNA samples into a vacuum dryer that has been used in DNA preparations where RNases may have been used.

Appendix A: Guidelines for Disruption and Homogenization of Tissues using the TissueLyser II

The TissueLyser II and TissueLyser Adapter Set 2 x 24 allow high-throughput, rapid, and effective disruption of 48 biological samples in 2–4 minutes. Homogenization and disruption with the TissueLyser II give results comparable to using rotor–stator homogenization.

The following guidelines can be used for disruption and homogenization of tissues using the TissueLyser II. Alternatively, for small sample numbers, we recommend the TissueLyser LT. For more information, consult the *TissueLyser LT Handbook*. Be sure to work quickly to prevent RNA degradation.

Procedure

1. Pipet the appropriate volume of Buffer RLT into a 2 ml collection tube (for further information, refer to Table 5 on page 25).
2. Add one stainless steel bead to each tube. For best results, we recommend using a 5 mm (mean diameter) stainless steel bead.
3. Add up to 30 mg tissue per tube.
4. Homogenize on the TissueLyser II for 2 min at 20 Hz.

   Homogenization time depends on the tissue used and can be extended until the tissue is completely homogenized (up to 5 min at 25 Hz).
5. Rotate the TissueLyser rack to allow even homogenization and homogenize for another 2 min at 20 Hz.

   The TissueLyser Adapter Set should be disassembled, and the rack of tubes should be rotated so that the tubes that were nearest to the TissueLyser are now outermost.
6. Carefully transfer the homogenate to a new microcentrifuge tube (not supplied) by pipetting. Do not reuse the stainless-steel bead.
7. Proceed with step 4 of the protocol on page 33.

Appendix B: Optional On-column DNase digestion with the RNase-Free DNase Set

The RNase-Free DNase Set (cat. no. 79254) provides efficient on-column digestion of DNA during RNA purification. The DNase is efficiently removed in subsequent wash steps.

Note: Standard DNase buffers are not compatible with on-column DNase digestion. Use of other buffers may affect the binding of RNA to the RNeasy membrane, reducing RNA yield and integrity.

Lysis and homogenization of the sample and binding of RNA to the RNeasy membrane are performed according to the standard protocol. After washing with a reduced volume of Buffer RWT, the RNA is treated with DNase I while bound to the RNeasy membrane. The DNase I is removed by a second wash with Buffer RWT. Washing with Buffer RPE and elution of RNA are then performed according to the standard protocol.

Important points before starting

- Generally, DNase digestion is not required since DNA levels in plasma/serum samples are very low and RNeasy technologies efficiently remove 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-abundant target).
- 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.

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 –30°C to –15°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

Prepare and load samples onto the RNeasy Mini Spin Column as indicated in steps 1–9 of "Protocol: Purification of Total RNA, Including Small RNAs, from Animal Cells" on pages 24–26 or steps 1–10 of "Protocol: Purification of Total RNA, Including Small RNAs, from Animal Tissues" on pages 30–34. Instead of performing step 10 of "Protocol: Purification of Total RNA, Including Small RNAs, from Animal Cells" on page 22 or step 11 of "Protocol: Purification of Total RNA, Including Small RNAs, from Animal Tissues" on page 28, follow steps 1–3 below.

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

   Reuse the collection tube in step 2.

* Flow-through contains Buffer RWT and is therefore not compatible with bleach. See page 6 for Safety Information.

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

   Buffer RDD is supplied with the RNase-Free DNase Set.

   Note: DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube. Do not vortex. Add 80µl of the DNase I incubation mix 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.

3. Add 350 µl Buffer RWT to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 8000 x *g* (10,000 rpm). Discard the flow-through and place the spin column in a new 2 ml collection tube (supplied).

4. Continue with step 10 of "Protocol: Purification of Total RNA, Including Small RNAs, from Animal Cells" on page 22 or step 11 of "Protocol: Purification of Total RNA, Including Small RNAs, from Animal Tissues" on page 28.

Appendix C: 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. 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. To remove RNase contamination from bench surfaces, nondisposable plasticware and laboratory equipment (e.g., pipets and electrophoresis tanks), use general laboratory reagents. To decontaminate plasticware, rinse with 0.1 M NaOH, 1 mM EDTA,* followed by RNase-free water (see "Solutions", page 44), or rinse with chloroform* if the plasticware is chloroform-resistant. To decontaminate electrophoresis tanks, clean with detergent (e.g., 0.5% SDS),* rinse with RNase-free water, then rinse with ethanol (if the tanks are ethanol resistant) and allow to dry.

* 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.

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.* 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 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 diethyl pyrocarbonate (DEPC)*, as described in "Solutions" below.

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 has been modified. Residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 minutes.

* 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.

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

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

Storage of RNA

Purified RNA may be stored at –70°C to –15°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 quantified using quantitative RT-PCR or fluorometric quantification.

Spectrophotometric quantification of RNA

Using the QIAxpert UV/VIS Spectrophotometer for microvolume analysis

To determine the concentration of your RNA sample purified with RNeasy QIAGEN kit, use the corresponding RNeasy App on the QIAxpert. For more information, see the QIAxpert product page (**www.qiagen.com/qiaxpert-system**).

Using a standard spectrophotometer

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 → 4 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 47), 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 44). 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
Total amount	= concentration x volume in milliliters
	= 440 µg/ml x 0.1 ml
	= 44 µg of RNA

Purity of RNA

The assessment of RNA purity will be performed routinely, when using the QIAxpert with the corresponding RNeasy App. See the QIAxpert user manual for more information (**www.qiagen.com/qiaxpert-system/user manual**)

* 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 standard photometric measurements, 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, such as protein, that absorb in the UV spectrum. 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 when using pure water. 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 46).

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. RNeasy Kits will, however, remove the vast majority of cellular DNA. gDNA Eliminator Solution helps to further reduce genomic DNA contamination; however, trace amounts of genomic DNA 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.

To prevent any interference by DNA in real-time RT-PCR applications, such as with Applied Biosystems® and Rotor-Gene® 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

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

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

DNA 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 50).

Integrity of RNA

The integrity and size distribution of total RNA purified with RNeasy Plus Universal Kits can be checked by denaturing agarose gel electrophoresis and ethidium bromide staining* or by using the QIAxcel® system or Agilent® 2100 Bioanalyzer. Ribosomal RNAs should appear as sharp bands or peaks. The apparent ratio of 28S rRNA to 18S rRNA 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. As a useful measure of RNA integrity, the QIAxcel Advanced system and the Agilent 2100 Bioanalyzer provide an RNA integrity score (RIS) and an RNA integrity number (RIN), respectively. Ideally, the value should be close to 10, but in many cases (particularly with tissue samples), RNA quality is greatly influenced by how well the original sample was preserved.

* 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.

Ordering Information

Product	Contents	Cat. no.
miRNeasy Tissue/Cells Advanced Mini Kit (50)	For 50 preps: RNeasy Mini Spin Columns, gDNA Eliminator Spin Columns, Collection Tubes, RNase-Free Water and Buffers	217604
QIAcube Connect — for fully automated nucleic acid extraction with QIAGEN spin-column kits		
QIAcube Connect*	Instrument, connectivity package, 1-year warranty on parts and labor	Inquire
Starter Pack, QIAcube	200 µl filter-tips (1024); 1000 µl filter-tips (1024); 30 ml reagent bottles (12); rotor adapters (240); 1.5 ml elution tubes (240); rotor adapter holder (1)	990395
Related products		
RNAprotect Cell Reagent (250 ml)	250 ml RNAprotect Cell Reagent	76526
RNAprotect Tissue Reagent (50 ml)	50 ml RNAprotect Tissue Reagent for stabilization of RNA in 25 x 200 mg tissue samples	76104
RNAprotect Tissue Reagent (250 ml)	250 ml RNAprotect Tissue Reagent for stabilization of RNA in 125 x 200 mg tissue samples	76106
Allprotect Tissue Reagent (100 ml)	100 ml Allprotect Tissue Reagent, Allprotect Reagent Pump	76405
QIAshredder (50)	50 disposable cell-lysate homogenizers	79654
QIAshredder (250)	250 disposable cell-lysate homogenizers	79656

Product	Contents	Cat. no.
TissueRuptor II	Handheld rotor–stator homogenizer, 5 TissueRuptor Disposable Probes	Varies†
TissueRuptor Disposable Probes (25)	25 nonsterile plastic disposable probes for use with the TissueRuptor II	990890
TissueLyser II	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 II	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
miRNeasy Micro Kit (50)	For 50 total RNA preps: 50 RNeasy MinElute spin columns, Collection Tubes (1.5 ml and 2 ml), QIAzol Lysis Reagent, RNase-free Reagents and Buffers	217084
Collection Tubes (2 ml)	1000 Collection Tubes (2 ml)	19201
Related products for quantitative, real-time RT-PCR		
miRCURY LNA RT Kit	For 8–64 cDNA synthesis reactions: 5x RT SYBR Green Reaction Buffer, 5x RT Probe Reaction Buffer, 10x RT Enzyme Mix, UniSp6, RNA Spike-in template, RNase-Free Water	339340
RNA Spike-in Kit, for RT	Contains the UniSp2, UniSp4, and UniSp5 RNA Spike-in Template Mix and the cel-miR-39-3p RNA Spike-in Template	339390

Product	Contents	Cat. no.
miRCURY Probe PCR Kit (200)‡	For 200 reactions: 2X QuantiNova Probe Master Mix, 10X miRCURY Probe Univ. Primer, ROX Reference Dye, RNase-Free Water	339371
miRCURY LNA miRNA Probe PCR Assay	Complete premixed assays containing LNA-enhanced target-specific forward primer and probe. For 200 reactions	339350§
miRCURY LNA miRNA Custom Probe PCR Assay	Custom-designed, target-specific forward primer and probe for any user-defined miRNA target. Complete premixed assay for 200 reactions	339351§
miRCURY LNA miRNA miRNome Probe PCR Panels	Pre-made panels of human or mouse and rat LNA PCR Assays for miRNome profiling; 384-well format	339361§
miRCURY LNA miRNA Focus Probe PCR Panels	Pre-made panel of LNA PCR Assays focused on research area; 96-well or 384-well format. (e.g. Serum/Plasma Focus Panel)	339362§
miRCURY LNA miRNA Custom Probe PCR Panels	PCR plates for custom building of stocked or custom-designed LNA-optimized PCR assays for miRNA quantification; 96-well or 384-well format	339360§
Related products for next-generation sequencing		
QIAseq miRNA Library Kit (12)	For 12 sequencing prep reactions: 3′ ligation, 5′ ligation, reverse-transcription, cDNA cleanup, library amplification and library cleanup reagents; quality control primers	331502

Product	Contents	Cat. no.
QIAseq miRNA NGS 12 Index IL (12)	Sequencing adapters, primers and indexes compatible with Illumina platforms; 12 indexes for 12 samples	331592
QIAseq miRNA Library Kit (96)	For 96 sequencing prep reactions: 3′ ligation, 5′ ligation, reverse-transcription, cDNA cleanup, library amplification and library cleanup reagents; quality control primers	331505
QIAseq miRNA NGS 48 Index IL (96)	Sequencing adapters, primers and indexes compatible with Illumina platforms; two 48 indexes for 96 samples	331595

* All QIAcube Connect instruments are provided with a region-specific connectivity package, including tablet and equipment necessary to connect to the local network. Further, QIAGEN offers comprehensive instrument service products, including service agreements, installation, introductory training and preventive subscription. Contact your local sales representative to learn about your options.

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

‡ Other kit sizes available, visit www.qiagen.com.

§ Exact catalog number varies depending on the particular assay or panel configuration.

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.

Document Revision History

Date	Changes
September 2019	Initial release
September 2020	Updated branding of RNA protection products and buffer names. Updated information about Allprotect Tissue Reagent.
February 2021	Added last paragraph in "Introduction" and flowchart. Updated RNA stabilization information in "Equipment and Reagents to be supplied by User", protocols and ordering information. Updated Table 1.

Limited License Agreement for miRNeasy Tissue/Cells Advanced Mini Kit (50)

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