March 2021

miRNeasy Micro Handbook

miRNeasy Micro Kit
For purification of total RNA, including miRNA, from animal and human cells and tissues

Sample to Insight

QIAGEN

Contents

Kit Contents

miRNeasy Micro Kit **Catalog no.** **Number of preps**	**(50)** **217084** **50**
RNeasy® MinElute® Spin Columns (each packaged with a 2 ml Collection Tube)	50
Collection Tubes (1.5 ml)	50
Collection Tubes (2 ml)	50
QIAzol® Lysis Reagent*	50 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.

Shipping and Storage

The miRNeasy Micro Kit (cat. no. 217084) is shipped at ambient temperature. Store the RNeasy MinElute spin columns immediately at 2–8°C. QIAzol Lysis Reagent can be stored at room temperature (15–25°C) or at 2–8°C. Store the remaining components dry at room temperature.

Under these conditions, the components are stable for at least 9 months not if not otherwise stated on the label.

Intended Use

The miRNeasy Micro Kit is intended for molecular biology applications. This product is 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 product. 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 convenient and compact PDF format at **www.qiagen.com/safety** where you can find, view, and print the SDS for each QIAGEN kit and kit component.

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

QIAzol Lysis Reagent and Buffer RWT contain 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 Micro Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

To date, the majority of gene expression studies have focused on mRNA levels. However in recent years, interest in smaller RNA species, such as miRNA, has increased. Most commercial RNA purification kits do not recover RNA molecules smaller than ~200 nucleotides. The miRNeasy Micro Kit is designed for purification of total RNA, including miRNA and other small RNA molecules, from small amounts of cultured cells and various animal and human tissues.

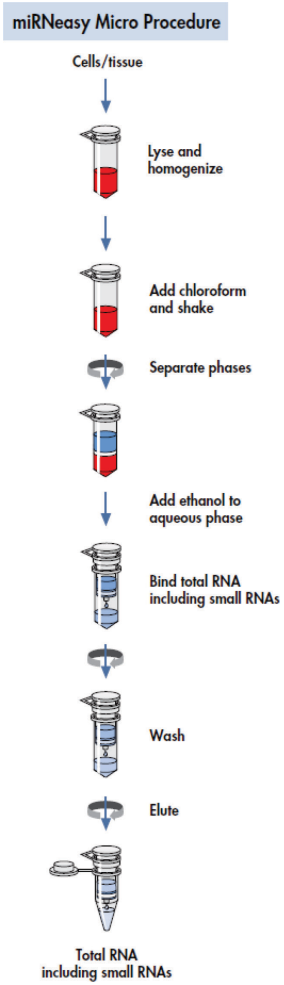
Principle and procedure

The miRNeasy Micro Kit combines phenol/guanidine-based lysis of samples and silica-membrane–based purification of total RNA. QIAzol Lysis Reagent, included in the kit, is a monophasic solution of phenol and guanidine thiocyanate, designed to facilitate lysis of tissues, inhibit ribonucleases (RNases), and remove most of the cellular DNA and proteins from the lysate by organic extraction.

Cells or tissue samples are homogenized in QIAzol Lysis Reagent. After addition of chloroform, the homogenate is separated into aqueous and organic phases by centrifugation. RNA partitions to the upper aqueous phase, while DNA partitions to the interphase and proteins to the lower organic phase or the interphase.

The upper aqueous phase is extracted, and ethanol 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 MinElute spin column, where the total RNA binds to the membrane and phenol and other contaminants are efficiently washed away. High-quality RNA is then eluted in a small volume of RNase-free water.

For enrichment of miRNAs and other small RNAs (less than ~200 nt) from cells and tissues in a separate fraction, a specialized protocol is provided in Appendix A, page 35. Enrichment of small RNAs in a separate fraction may be advantageous for certain applications where mRNA and rRNA could lead to increased background. For this specialized protocol, an additional kit, the RNeasy MinElute Cleanup Kit (cat. no. 74204) is recommended.



Description of protocols

This handbook contains two protocols. The protocol on page 21 is for purification of total RNA, including miRNA, from animal cells using the miRNeasy Micro Kit. The second protocol (page 27) is for purification of total RNA, including miRNA, from animal tissues using the miRNeasy Micro Kit.

miRNA purification in 96 wells, from FFPE tissues and from serum or plasma

The miRNeasy Micro Kit enables low-throughput RNA purification from small amounts of sample using spin columns. For high-throughput purification in a 96-well format, the miRNeasy 96 Kit is recommended. Total RNA and miRNA can also be copurified from formalin-fixed, paraffin-embedded (FFPE) tissue sections using the miRNeasy FFPE Kit. For purification of total RNA, including miRNA, from serum or plasma samples, we recommend the miRNeasy Serum/Plasma Kit (see Ordering Information, page 49).

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**.

Automated purification of RNA on QIAcube instruments

Purification of RNA, including miRNA, 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 Micro Kit for purification of high-quality miRNA

The 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**.

QIAcube Connect.

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.

- Chloroform (without added isoamyl alcohol)
- Ethanol (70%, 80% and 96–100%)*
- Sterile, RNase-free pipet tips
- Microcentrifuge tubes (1.5 or 2 ml)
- Microcentrifuge(s) (with rotor for 2 ml tubes) for centrifugation at 4°C and at room temperature (15–25°C)
- Disposable gloves
- For animal tissues: RNAprotect® Tissue Stabilization Reagent (see Ordering Information, page 49) or liquid nitrogen
- **Optional**: RNase-Free DNase Set (see Ordering Information, page 49)
- Equipment and tubes for disruption and homogenization (see pages 17–20). Depending on the method chosen, one or more of the following are required:
  - TissueRuptor® II with TissueRuptor Disposable Probes (see Ordering Information, page 49)
  - TissueLyser system (see Ordering Information, page 49)
  - Mortar and pestle
  - QIAshredder homogenizer (see Ordering Information, page 49)
  - Blunt-ended needle and syringe
  - Trypsin and PBS

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

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:

- Volume of QIAzol Lysis Reagent required for efficient lysis
- RNA binding capacity of the RNeasy MinElute spin column (45 µg)
- 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 MinElute spin column is not reached, the maximum amount of starting material must not be exceeded. Otherwise, lysis will be incomplete and cellular debris may interfere with the binding of RNA to the RNeasy MinElute spin column membrane, resulting in lower RNA yield and purity.

Table 1. RNeasy MinElute spin column specifications

Maximum binding capacity	45 µg RNA
Maximum loading volume	700 µl
RNA size distribution	RNA >18 nucleotides approximately
Minimum elution volume	10 µl
Maximum amount of starting material	
Animal cells	1×10^6
Animal tissues	5 mg (10 mg for adipose tissue)

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

Determining the correct amount of starting material — cells

The maximum amount of starting material depends on the RNA content of the cell type. RNA content can vary greatly between cell types and growth rates. If processing a cell type where there is no information about its RNA content, we recommend starting with no more than 5×10^5 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	$4–5 \times 10^4$
48-well	1	1×10^5
24-well	2	2.5×10^5
12-well	4	5×10^5
6-well	9.5	1×10^6
Dishes		
35 mm	8	1×10^6
Flasks		
40–50 ml	25	25

* 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 5 mg tissue can be processed with the miRNeasy Micro procedure. For adipose tissues, up to 10 mg can be processed. The binding capacity of the column (45 µg RNA) and the lysing capacity of QIAzol Lysis Reagent will not be exceeded by these amounts.

Table 3. Average yields of total RNA with miRNeasy Micro Kit

Sample	Average RNA yield* (µg)
Mouse/rat tissue (5 mg)	
Kidney	2–20
Liver	8–40
Lung	2–8
Heart	2–12
Muscle	2–15
Brain	2–10
Adipose tissue	0.2–1.5
Spleen	8–50
Intestine	5–30
Skin	1–3
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.

Weighing tissue is the most accurate way to quantify the amount of starting material. However, the following may be used as a guide. A 1.5 mm cube (volume, 3.4 mm^3) of most animal tissues weighs 3.5–4.5 mg.

Handling and storage of cells and tissue

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 –70°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 –70°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 (15–25°C).

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 QIAzol Lysis Reagent, samples can be stored at –70°C for months.

Disrupting and homogenizing starting material

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

- **Disruption**: Complete disruption of plasma membranes of cells and organelles is absolutely required to release all the RNA contained in the sample. Different samples require different methods to achieve complete disruption. Incomplete disruption 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 MinElute 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	Vortexing (or TissueRuptor II or QIAshredder homogenizer or syringe and needle)	For cell numbers recommended with the miRNeasy Micro Kit, lysate can usually be homogenized by thorough vortexing
Animal tissues	TissueLyser II	TissueLyser II	The TissueLyser II gives results comparable to using a rotor–stator homogenizer Simultaneously disrupts and homogenizes
	TissueRuptor II	TissueRuptor II	The TissueRuptor II usually gives higher yields than mortar and pestle
	Mortar and pestle	QIAshredder homogenizer or syringe and needle	

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 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 Handbook*. For other rotor–stator homogenizers, please refer to suppliers' guidelines for further details.

Disruption and homogenization using the TissueLyser system

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
- 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 system and stainless steel beads, refer to the appropriate TissueLyser handbook and user manual. 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 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 miRNA, from Animal Cells

Important points before starting

- If using the miRNeasy Micro Kit for the first time, read "Important Notes" (page 13).
- It is important not to overload the RNeasy MinElute spin column, as overloading will significantly reduce RNA yield and quality. Read "Determining the amount of starting material" (page 13).
- If working with RNA for the first time, read Appendix C (page 43).
- Cell pellets can be stored at –70°C for later use or used directly in the procedure. Determine the number of cells before freezing. Frozen cell pellets should be thawed slightly so that they can be dislodged by flicking the tube in step 2. Homogenized cell lysates from step 3 can be stored at –70°C for several months. 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.
- Generally, DNase digestion is not required since the combined QIAzol and RNeasy technologies efficiently remove most of the DNA without DNase treatment. In addition, miScript® Primer 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 particularly 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 39) or by DNase digestion after RNA purification (please contact QIAGEN Technical Service for a protocol).
- Buffer RWT may form a precipitate upon storage. If necessary, redissolve by warming and then place at room temperature (15–25°C).
- QIAzol Lysis Reagent and Buffer RWT contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 6 for Safety Information.

- Except for phase separation (step 7), 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 volumes 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 39).

Procedure

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

   1a. Cells grown in suspension (do not use more than 1 x 10^6 cells):
   Determine the number of cells. Pellet the appropriate number of cells by centrifuging for 5 min at 300 x *g* in a centrifuge tube (not supplied). Carefully remove all supernatant by aspiration and proceed to step 2.

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

   1b. Cells grown in a monolayer (do not use more than 1 x 10^6 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 MinElute 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 in PBS. After the cells detach from the dish or flask, add medium (containing serum to inactivate the trypsin), transfer the cells to an RNase-free glass or polypropylene centrifuge tube (not supplied), and centrifuge at 300 x *g* for 5 min. Completely aspirate the supernatant and proceed to step 2.

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

2. Disrupt the cells by adding QIAzol Lysis Reagent.

   For pelleted cells, loosen the cell pellet thoroughly by flicking the tube. Add 700 µl QIAzol Lysis Reagent. Vortex or pipet to mix.

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

   For direct lysis of cells grown in a monolayer, add 700 µl QIAzol Lysis Reagent 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.

3. Homogenize the cells by vortexing for 1 min.

   Note: Incomplete homogenization leads to significantly reduced RNA yields and can cause clogging of the RNeasy MinElute spin column. See “Disrupting and homogenizing starting material”, pages 17–20, for more details on homogenization.

   Note: Homogenized cell lysates can be stored at –70°C for several months.

4. Place the tube containing the homogenate on the benchtop at room temperature (15–25°C) for 5 min.

   This step promotes dissociation of nucleoprotein complexes.

5. Add 140 µl chloroform to the tube containing the homogenate and cap it securely. Shake the tube vigorously for 15 s.

   Thorough mixing is important for subsequent phase separation.

6. Place the tube containing the homogenate on the benchtop at room temperature for 2–3 min.

7. Centrifuge for 15 min at 12,000 x *g* at 4°C. After centrifugation, heat the centrifuge up to room temperature if the same centrifuge will be used for the next centrifugation steps.

   After centrifugation, the sample separates into 3 phases: an upper colorless aqueous phase containing RNA, a white interphase, and a lower red organic phase. The volume of the aqueous phase should be approximately 350 µl.

   Note: If you want to purify a separate miRNA-enriched fraction, follow the steps in Appendix A (page 35) after performing this step.

8. Transfer the upper aqueous phase to a new collection tube (not supplied). Add 1.5 volumes (usually 525 µl) of 100% ethanol and mix thoroughly by pipetting up and down several times. Do not centrifuge. Continue without delay with step 9.

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

9. Pipet up to 700 µl of the sample, including any precipitate that may have formed, into an RNeasy MinElute spin column in a 2 ml collection tube (supplied). Close the lid gently and centrifuge at ≥8000 x *g* (≥10,000 rpm) for 15 s at room temperature. Discard the flow-through.*

   Reuse the collection tube in step 10.

10. Repeat step 9 using the remainder of the sample. Discard the flow-through.*

    Reuse the collection tube in step 11.

    Optional: If performing optional on-column DNase digestion (see "Important points before starting"), follow steps 1–4 on page 39 after performing this step.

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

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

    Skip this step if performing the optional on-column DNase digestion (page 39).

    Reuse the collection tube in step 12.

12. Pipet 500 µl Buffer RPE onto the RNeasy MinElute spin column. Close the lid gently and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the column. Discard the flow-through.

    Reuse the collection tube in step 13.

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

    Note: Ethanol, 80%, should be prepared with ethanol (96–100%) and RNase-free water.

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

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

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

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

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

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

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

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

Protocol: Purification of Total RNA, Including miRNA, from Animal Tissues

Important points before starting

- If using the miRNeasy Micro Kit for the first time, read "Important Notes" (page 13).
- It is important not to overload the RNeasy MinElute spin column, as overloading will significantly reduce RNA yield and quality. Read "Determining the amount of starting material" (page 13).
- 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 –20°C or –80°C.
- Fresh, frozen or RNAprotect Tissue Reagent- or AllProtect-stabilized tissues can be used. Tissues can be stored for several months at –70°C. Do not allow tissues to thaw during weighing or handling prior to disruption in QIAzol Lysis Reagent. Homogenized tissue lysates (in QIAzol Lysis Reagent, step 3) can also be stored at –70°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 because the combined QIAzol and RNeasy technologies efficiently remove most of the DNA without DNase treatment. In addition, miScript Primer 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 particularly 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 39) or by DNase digestion after RNA purification (please contact QIAGEN Technical Service for a protocol).

- Buffer RWT may form a precipitate upon storage. If necessary, redissolve by warming and then place at room temperature (15–25°C).
- QIAzol Lysis Reagent and Buffer RWT contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 6 for Safety Information.
- Except for phase separation (step 7), 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 volumes 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 39).

Procedure

1. Excise the tissue sample from the animal or remove it from storage. Determine the amount of tissue. Do not use more than 5 mg tissue or 10 mg adipose tissue.

   Unless you are working with RNAprotect Tissue Reagent- or AllProtect-stabilized tissue, do not allow the tissue to thaw before placing in QIAzol Lysis Reagent.

2. If the entire piece of tissue can be used for RNA purification, place it directly into 700 µl QIAzol Lysis Reagent in a suitably sized vessel for disruption and homogenization.

   If only a portion of the tissue is to be used, determine the weight of the piece to be used and place it into 700 µl QIAzol Lysis Reagent in a suitably sized vessel for disruption and homogenization.

   RNA in tissues is not protected after harvesting until the sample is stabilized in RNAprotect Tissue Reagent or AllProtect Reagent, flash-frozen, or disrupted and homogenized in step 3. Unstabilized, frozen animal tissue should not be allowed to thaw during handling.

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

3. Homogenize immediately using the TissueLyser system, the TissueRuptor II or another method until the sample is uniformly homogeneous (usually 20–40 s).

   See page 17 for a more detailed description of disruption and homogenization methods.

   Note: Homogenization with the TissueRuptor or the TissueLyser system (see page 18) generally results in higher total RNA yields than with other homogenization methods.

   Foaming may occur during homogenization, especially of brain tissue. If this occurs, let the homogenate stand at room temperature (15–25°C) for 2–3 min until the foam subsides before continuing with the protocol.

   Note: Homogenized tissue lysates can be stored at –70°C for several months.

4. Place the tube containing the homogenate on the benchtop at room temperature for 5 min.

   This step promotes dissociation of nucleoprotein complexes.

5. Add 140 µl chloroform to the tube containing the homogenate and cap it securely. Shake the tube vigorously for 15 s.

   Thorough mixing is important for subsequent phase separation.

6. Place the tube containing the homogenate on the benchtop at room temperature for 2–3 min.

7. Centrifuge for 15 min at 12,000 x *g* at 4°C. After centrifugation, heat the centrifuge up to room temperature if the same centrifuge will be used for the next centrifugation steps.

   After centrifugation, the sample separates into 3 phases: an upper colorless aqueous phase containing RNA, a white interphase, and a lower red organic phase. The volume of the aqueous phase should be approximately 350 µl.

   Note: If you want to purify a separate miRNA-enriched fraction, follow the steps in Appendix A (page 35) after performing this step.

8. Transfer the upper aqueous phase to a new collection tube (not supplied). Add 1.5 volumes (usually 525 µl) of 100% ethanol and mix thoroughly by pipetting up and down several times. Do not centrifuge. Continue without delay with step 9.

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

9. Pipet up to 700 µl of the sample, including any precipitate that may have formed, into an RNeasy MinElute spin column in a 2 ml collection tube (supplied). Close the lid gently and centrifuge at ≥8000 x *g* (≥10,000 rpm) for 15 s at room temperature. Discard the flow-through.*

   Reuse the collection tube in step 10.

10. Repeat step 9 using the remainder of the sample. Discard the flow-through.*

    Reuse the collection tube in step 11.

    Optional: If performing optional on-column DNase digestion (see "Important points before starting"), follow steps 1–4 on page 39 after performing this step.

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

    Skip this step if performing the optional on-column DNase digestion (page 39).

    Reuse the collection tube in step 12.

12. Pipet 500 µl Buffer RPE onto the RNeasy MinElute spin column. Close the lid gently and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the column. Discard the flow-through.

    Reuse the collection tube in step 13.

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

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

    Note: Ethanol, 80%, should be prepared with ethanol (96–100%) and RNase-free water.

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

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

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

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

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

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

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

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
Phases do not separate completely		
a)	No chloroform added or chloroform not pure	Make sure to add chloroform that does not contain isoamyl alcohol or other additives.
b)	Homogenate not sufficiently mixed before centrifugation	After addition of chloroform (step 5), the homogenate must be vigorously shaken. If the phases are not well separated, shake the tube vigorously for at least 15 s and repeat the incubation and centrifugation in steps 6 and 7 of the protocol.
c)	Organic solvents present in samples used for RNA purification	Make sure that the starting sample does not contain organic solvents (e.g., ethanol, DMSO), strong buffers or alkaline reagents. These can interfere with the phase separation.
Clogged 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 13).
b)	Inefficient disruption and/or homogenization	See "Disrupting and homogenizing starting material" (starting on page 17) for a detailed description of homogenization methods. Increase *g*-force and centrifugation time if necessary. In subsequent preparations, reduce the amount of starting material (see page 13) and/or increase the homogenization time.
c)	Centrifugation temperature too low	Except for phase separation (step 7), 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 MinElute 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 MinElute spin column.
Low miRNA yield or poor performance of miRNA in downstream applications		
a)	Incorrect ethanol concentration	Be sure to use the ethanol concentrations specified in the protocol steps.

		Comments and suggestions
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 (page 35) to isolate a separate, miRNA-enriched fraction. An additional kit, the RNeasy MinElute Cleanup Kit, is required for this protocol.
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 13).
b)	Inefficient disruption and/or homogenization	See "Disrupting and homogenizing starting material" (starting on page 17) for a detailed description of homogenization methods. Increase *g*-force and centrifugation time if necessary. In subsequent preparations, reduce the amount of starting material (see page 13) and/or increase the homogenization time.
c)	Elution buffer incorrectly dispensed	Add elution buffer to the center of the RNeasy MinElute 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 MinElute spin column on the bench for 10 min after adding RNase-free water and before centrifugation.
Low A_{260}/A_{280} value		
a)	Not enough QIAzol Lysis Reagent used for homogenization	Reduce the amount of starting material and/or increase the volume of QIAzol Lysis Reagent and the homogenization time.
b)	Sample not incubated for 5 min after homogenization	Place the sample at room temperature for 5 min after homogenization, as indicated in the protocols (step 4). This step is important to promote dissociation of nucleoprotein complexes.
c)	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 D, page 45)
RNA degraded		
a)	Sample inappropriately handled	For frozen tissue samples, ensure that they were flash-frozen immediately in liquid nitrogen and properly stored at –70°C. Perform the protocol quickly, especially the first few steps. See "Appendix C: General Remarks on Handling RNA" (page 43) and "Handling and storage of cells and tissue" (page 16).
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.

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

		Comments and suggestions
DNA contamination in downstream experiments		
a)	Phase separation performed at too high a temperature	The phase separation in step 7 should be performed at 4°C. Make sure that the centrifuge does not heat above 10°C during centrifugation.
b)	Interphase contamination of aqueous phase	Contamination of the aqueous phase with the interphase results in increased DNA content in the RNA eluate. Make sure to transfer the aqueous phase without interphase contamination.
c)	No DNase treatment (cell or tissue samples)	Perform optional on-column DNase digestion using the RNase-Free DNase Set (Appendix B, page 39) at the point indicated in the protocol. Alternatively, after the miRNeasy procedure, DNase digest the eluate containing the RNA. After inactivating DNase by heat treatment, the RNA can be used directly in the subsequent application without further treatment. Alternatively, the RNA can be repurified using an RNeasy RNA cleanup protocol (see RNA Cleanup and Concentration Protocol in the *RNeasy Micro Handbook*) with one change to the protocol: the volume of ethanol added to the sample should be increased from 250 µl to 700 µl.
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	After the final membrane wash, be sure to dry the RNeasy MinElute spin column by centrifugation at full speed with open lids for 5 min (protocol step 14).

Appendix A: Preparation of miRNA-Enriched Fractions Separate from Larger RNAs (>200 nt)

This protocol allows purification of a separate fraction, enriched in miRNA and other small RNA species. Removal of larger RNAs, such as mRNA and rRNA, may reduce background in certain downstream applications.

For recovery of the miRNA-enriched fraction, an RNeasy MinElute Cleanup Kit (cat. no. 74204) is required. Alternatively, RNeasy MinElute spin columns contained in the miRNeasy Micro Kit can be used for this protocol. However, this reduces the number of preps that can be performed with the miRNeasy Micro Kit and is less economical than purchase of an RNeasy MinElute Cleanup Kit.

Quantification of miRNA

The miRNA-enriched fraction obtained using this protocol is enriched in various RNAs of <200 nucleotides (e.g., tRNAs). For this reason, the miRNA yield cannot be quantified by OD measurement or fluorogenic assays. To determine yield, we recommend using quantitative, real-time RT-PCR assays specific for the type of small RNA under study. For example, to estimate miRNA yield, an assay directed against any miRNA known to be adequately expressed in the samples being processed may be used.

Procedure

Carry out steps 1–7 as indicated in the protocol (on page 21 or 27). Instead of continuing with step 8, follow steps 1–10 below to isolate the miRNA-enriched fraction only, or steps 1–16 to isolate separate fractions of small RNA and total RNA >200 nt.

1. Transfer the upper aqueous phase to a new reaction tube (not supplied). Add 1 volume of 70% ethanol (usually 350 µl) and mix thoroughly by vortexing. Do not centrifuge. Proceed immediately to step 2.

2. Pipet the sample (approx. 700 µl), including any precipitate that may have formed, into an RNeasy MinElute spin column placed in a 2 ml collection tube. Close the lid gently and centrifuge at ≥8000 x *g* (≥10,000 rpm) for 15 s at room temperature (15–25°C). Pipet the flow-through (which contains miRNA) into a 2 ml reaction tube (not supplied).
3. If purifying the miRNA-enriched fraction only, discard the RNeasy MinElute spin column and follow steps 4–10 only.

   If purifying both the miRNA-enriched fraction and larger RNAs (>200 nt), save the RNeasy MinElute spin column for use in step 11 (the spin column can be stored at 4°C or at room temperature, but not for long periods). Follow steps 4–10 to purify miRNA and then steps 11–16 to purify large RNAs.

Purifying the miRNA-enriched fraction using the RNeasy MinElute Cleanup Kit (steps 4–10)

4. Add 450 µl of 100% ethanol (0.65 volumes) to the flow-through from step 2 and mix thoroughly by vortexing. Do not centrifuge. Proceed immediately to step 5.
5. Pipet 700 µl of the sample into an RNeasy MinElute spin column placed in a 2 ml collection tube. Close the lid gently and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) at room temperature (15–25°C). Discard the flow-through.*

   Repeat this step until the whole sample has been pipetted into the spin column. Discard the flow-through each time.

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

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

   Do not perform this step if you are purifying both the miRNA-enriched fraction and larger RNAs (>200 nt).

   This step is optional for the miRNA-enriched fraction because most impurities removed by this wash have already been removed on the first RNeasy MinElute spin column.

7. Pipet 500 µl Buffer RPE into the RNeasy MinElute spin column. Close the lid gently and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.

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

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

9. Place the RNeasy MinElute spin column into a new 2 ml collection tube. Open the lid and centrifuge for 5 min at ≥8000 x *g* (≥10,000 rpm).

10. Place the RNeasy MinElute spin column into a 1.5 ml collection tube and pipet 14 µl RNase-free water onto the spin column membrane. Close the lid gently and centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm) to elute the miRNA-enriched fraction.

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

Purifying total RNA (>200 nt) using the RNeasy MinElute spin column (steps 11–16)

11. Pipet 700 µl Buffer RWT into the RNeasy MinElute spin column from step 3. Close the lid gently and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.*

    Optional: If on-column DNase digestion using the RNase-Free DNase Set is desired, perform steps 1–4 (Appendix B, page 39) instead of this step. Then proceed to step 12.

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

13. Pipet another 500 µl Buffer RPE into the RNeasy MinElute spin column. Close the lid gently and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through and the collection tube.

14. Place the RNeasy MinElute spin column in a new 2 ml collection tube. Open the lid and centrifuge at full speed for 1 min.

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

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

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: Buffer RDD supplied with the RNase-Free DNase Set is specially optimized for on-column DNase digestion. However, to prevent losses of small RNAs, a modified DNase digestion procedure is recommended for samples containing less than approximately 1 µg total RNA (equivalent to about 1 x 10^5 cells). In the modified procedure, the spin column flow-through after on-column digestion and washing is reapplied to the membrane (page 36). Buffer RWT used in this protocol should be prepared with isopropanol instead of ethanol, as is usually recommended (pages 22 and 28). Therefore, if not all preps will be performed using the procedure for DNase digestion for samples containing <1 µg total RNA approximately, it will be necessary to purchase additional Buffer RWT (cat. no. 1067933), which should be prepared with isopropanol.

For larger sample amounts containing greater than approximately 1 µg total RNA, recovery of tRNA may be reduced by DNase digestion, but miRNA yields are not affected.

Use of DNase buffers other than that supplied with the RNase-Free DNase Set may affect the binding of the RNA to the RNeasy MinElute spin column membrane, reducing the yield and integrity of the RNA.

Important points before starting

- Generally, DNase digestion is not required since the integrated QIAzol and RNeasy technologies efficiently remove most of the DNA without DNase treatment. In addition, miScript Primer 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 (e.g., QuantiFast® RT-PCR analysis with a low-abundance target). Alternatively, DNA can be removed by DNase digestion following RNA purification.
- DNase digestion is not necessary for miRNA-enriched fractions prepared using the protocol in Appendix A. This is because any residual DNA not removed in the organic extraction step will be retained together with larger RNAs by the first RNeasy MinElute spin column (step 2 of Appendix A).
- **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 reconstituted DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.
- For the protocol for samples containing <1 µg total RNA approximately, prepare Buffer RWT by adding 45 ml isopropanol to the concentrate (instead of 30 ml ethanol as usually recommended). Buffer RWT can be ordered separately for this protocol (cat. no.1067933).

Procedure: DNase digestion for samples containing >1 µg total RNA approximately

Carry out steps 1–10 as indicated in the protocol (on page 21 or 27) or steps 1–3 as indicated in Appendix A (page 35). Then follow steps 1–4 below.

1. Pipet 350 µl Buffer RWT into the RNeasy MinElute spin column and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash. Discard the flow-through.*

   Reuse the collection tube in step 4.

2. Add 10 µl DNase I stock solution to 70 µl Buffer RDD. Mix by gently inverting the tube. Do not vortex.

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

3. Pipet the DNase I incubation mix (80 µl) directly onto the RNeasy MinElute spin column membrane and place on the benchtop at 20–30°C for 15 min.

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

4. Pipet 350 µl Buffer RWT into the RNeasy MinElute spin column and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.* Continue with step 12 of the "Protocol: Purification of Total RNA, Including miRNA, from Animal Cells" or "Protocol: Purification of Total RNA, Including miRNA, from Animal Tissues" or step 12 of Appendix A (if performing the protocol in Appendix A).

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

Procedure: DNase digestion for samples containing <1 µg total RNA approximately

Carry out steps 1–10 as indicated in the cells or tissue protocol (on page 21 or 27) or steps 1–3 of Appendix A (page 35). Then follow steps 1–4 below.

1. Pipet 350 µl Buffer RWT (prepared with isopropanol) into the RNeasy MinElute spin column and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash. Discard the flow-through.*

   Reuse the collection tube in step 4.

2. Add 10 µl DNase I stock solution to 70 µl Buffer RDD. Mix by gently inverting the tube. Do not vortex.

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

3. Pipet the DNase I incubation mix (80 µl) directly onto the RNeasy MinElute spin column membrane and place on the benchtop at 20–30°C for 15 min.

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

4. Pipet 500 µl Buffer RWT (prepared with isopropanol) into the RNeasy MinElute spin column and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Keep the flow-through.

5. Reapply the flow-through to the RNeasy MinElute spin column and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.*

6. Continue with step 12 of the cells or tissue protocol or step 12 of Appendix A (if performing the protocol in Appendix A).

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

Appendix C: General Remarks on Handling RNA

Handling RNA

RNases are very stable and active enzymes that generally do not require cofactors to function. Because 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.*

Glassware

Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent,* thoroughly rinsed and oven baked at 240°C for at least 4 hours (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware can be treated with 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.

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

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

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

Storage of RNA

Purified RNA may be stored at temperatures between –70 and –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, which allows analysis at the level of microvolumes. 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 milliliter (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 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 measurement. 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 blank 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

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

Purity of RNA

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

For standard photometric measurements, the ratio of the readings at 260 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 45).

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. miRNeasy kits will, however, remove the vast majority of cellular DNA. The optional on-column DNase digestion 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.

* 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 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 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 49).

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 and an RNA integrity number, 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 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
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	9002864
Starter Pack, QIAcube	Filter-tips, 200 µl (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		
miRNeasy Mini Kit (50)	For 50 total RNA 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 total RNA 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

* 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.

Product	Contents	Cat. no.
miRNeasy Serum/Plasma 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	217184
miRNeasy FFPE Kit (50)	For 50 total RNA preps: 50 RNeasy MinElute Spin Columns, 50 gDNA Eliminator Spin Columns, Collection Tubes, Proteinase K and RNase-Free Reagents and Buffers.	217504
Collection Tubes (2 ml)	1000 Collection Tubes (2 ml)	19201
Accessories		
Buffer RWT	80 ml wash buffer concentrate for use with miRNeasy Kits	1067933
TissueLyser II*	Universal laboratory bead mill disruptor	85300
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
TissueRuptor II	Handheld rotor–stator homogenizer, 5 TissueRuptor Disposable Probes	9002755
TissueRuptor Disposable Probes (25)	25 nonsterile plastic disposable probes for use with the TissueRuptor II	990890
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

* For more information, visit **www.qiagen.com/products/accessories**.

Product	Contents	Cat. no.
Collection Tubes (2 ml)	1000 Collection Tubes (2 ml)	19201
QIAshredder (50)	50 disposable cell-lysate homogenizers	79654
QIAshredder (250)	250 disposable cell-lysate homogenizers	79656
RNase-Free DNase Set (50)	1500 units RNase-free DNase I, RNase-Free Buffer RDD and RNase-Free Water for 50 RNA minipreps	79254
RNeasy MinElute Cleanup Kit (50)	50 RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-Free Reagents and Buffers	74204
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-miR39-3p RNA Spike-in Template	339390
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 LNAenhanced 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

Product	Contents	Cat. no.
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

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
03/2021	Updated branding of RNA protection products, Appendices C and D, and Ordering Information section.

Notes

Limited License Agreement for miRNeasy Micro Kit

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