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miRNeasy Mini Handbook

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

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Kit Contents

miRNeasy Mini Kit	(50)
Catalog no.	217004
Number of preps	50
RNeasy® Mini 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 5 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 Mini Kit should be stored dry at room temperature (15–25°C). All components are stable for at least 9 months under these conditions, if not otherwise stated on the label.

QIAzol Lysis Reagent can be stored at room temperature or at 2–8°C.

Intended Use


The miRNeasy Mini 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 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 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.

<p>CAUTION</p> 	<p>CAUTION: DO NOT add bleach or acidic solutions directly to the sample-preparation waste.</p>
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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 Mini 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 Mini Kit is designed for purification of total RNA, including miRNA and other small RNA molecules, from cultured cells and various animal and human tissues.

Principle and procedure

The miRNeasy Mini 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, to inhibit RNases, and also to 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.

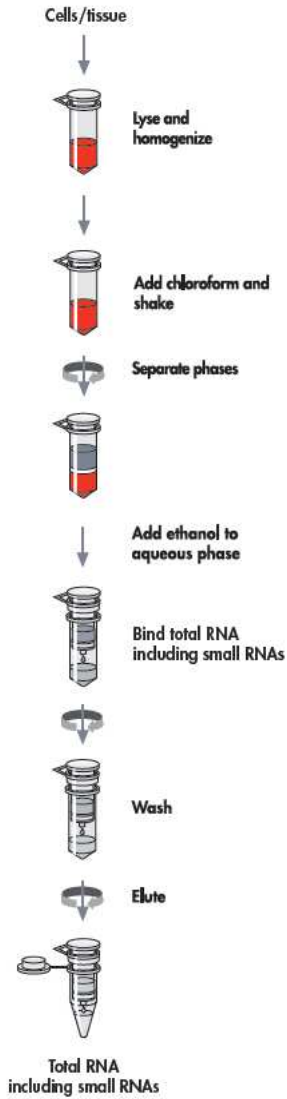
The upper, aqueous phase is extracted, and ethanol is added to provide appropriate binding conditions for all RNA molecules from 18 nucleotides (nt) upwards. The sample is then applied to the RNeasy Mini spin column, where the total RNA binds to the membrane and phenol and other contaminants are efficiently washed away. High-quality RNA is then eluted in RNase-free water.

For enrichment of miRNAs and other small RNAs (less than ~200 nt) in a separate fraction, a specialized protocol is provided in Appendix A, page 39. 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 required.

For purification of total RNA from leukocytes, a protocol is provided in Appendix D, page 49. Purchase of Buffer EL (cat. no. 79217) is required for this protocol.

miRNeasy Mini Procedure



The miRNeasy Mini Kit enables low-throughput miRNA and total RNA purification using spin columns. The miRNeasy Micro Kit is designed for purification from small amounts of cultured cells, sorted cells, laser capture microdissection samples from cryosections, and various animal and human tissues. For high-throughput purification in a 96-well format, the miRNeasy 96 Kit is available. For purification of total RNA, including miRNA, from serum or plasma samples, we recommend the miRNeasy Serum/Plasma Kit. Total RNA and miRNA can also be copurified from formalin-fixed, paraffin-embedded (FFPE) tissue sections using the miRNeasy FFPE Kit (see ordering information, starting on page 59). Total RNA and miRNA can be purified from animal cells without the use of QIAzol Lysis Reagent by using the RNeasy Plus Mini Kit along with a protocol which is available at www.qiagen.com/miRNA. For more information about miRNeasy Kits and other miRNA solutions from QIAGEN, visit www.qiagen.com/miRNA.

miRNA quantification using the miScript® PCR System

The miScript PCR System allows sensitive and specific quantification and profiling of miRNA expression using SYBR® Green-based real-time PCR. The robust miScript PCR System comprises the miScript II RT Kit, the miScript PreAMP PCR Kit, the miScript SYBR Green PCR Kit, miScript Assays and miScript miRNA PCR Arrays. It covers all the steps of miRNA quantification, from conversion of RNA into cDNA to real-time PCR detection of miRNAs and straightforward data analysis.

Individual assays for mature miRNAs, precursor miRNAs and other small noncoding RNAs can be ordered at the GeneGlobe® Web portal (www.qiagen.com/GeneGlobe). Alternatively, for high-throughput experiments, miScript miRNA PCR Arrays enable rapid profiling of the complete miRNome or pathway-focused panels of mature miRNAs for a variety of species. Find out more about the miScript PCR System at www.qiagen.com/miRNA.

Automated purification of RNA on QIAcube Instruments

Purification of RNA, including mi 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 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.



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% and 96–100%)*
- Sterile, RNase-free pipet tips
- 1.5 ml or 2 ml microcentrifuge tubes
- 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: RNAlater® RNA Stabilization Reagent (see ordering information, starting on page 59) or liquid nitrogen
- **Optional:** RNase-Free DNase Set (see ordering information, starting on page 59)
- Equipment and tubes for disruption and homogenization (see “Disrupting and homogenizing starting material”, page 18). Depending on the method chosen, one or more of the following are required:
 - TissueRuptor® II with TissueRuptor Disposable Probes (see ordering information, starting on page 59)
 - TissueLyser II or TissueLyser LT (see ordering information, starting on page 59)
 - Mortar and pestle
 - QIAshredder homogenizer (see ordering information, starting on page 59)
 - Blunt-ended needle and syringe
 - Trypsin and phosphate-buffered saline (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 Mini spin column (100 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 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 x 10 ⁷
Animal tissues	50 mg (100 mg for adipose 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 10⁶ cells). Do not use more than 3 × 10⁶ 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 10⁶ cells). Do not use more than 7 × 10⁶ 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 10⁶ cells). The maximum amount of starting material (1 × 10⁷ 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 × 10⁶ 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 × 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
60 mm	21	2.5 × 10 ⁶
100 mm	56	7 × 10 ⁶
145–150 mm	145	2 × 10 ⁷
Flasks		
40–50 ml	25	3 × 10 ⁶
250–300 ml	75	1 × 10 ⁷
650–750 ml	162–175	2 × 10 ⁷

* 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 50 mg tissue can be processed with the miRNeasy Mini procedure. For adipose tissues, up to 100 mg can be processed. The binding capacity of the column (100 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 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 10⁶ 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. Depending on the yield and purity of RNA obtained, it may be possible to increase the amount of tissue to 100 mg.

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 mm³) of most animal tissues weighs 25–35 mg.

Handling and storage of starting material

RNA is not protected after harvesting until the sample is treated with RNAprotect® Cell Reagent (cultured cells only) or RNA/later RNA Stabilization 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 RNA/later RNA Stabilization Reagent. Animal cells can be pelleted and then stored at -90°C to -65°C until required for RNA purification.

An alternative to RNA/later RNA Stabilization Reagent is Allprotect® Tissue Reagent, which provides immediate stabilization of DNA, RNA and protein in tissues samples at room temperature ($15-25^{\circ}\text{C}$).

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 -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 3×10^6 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 ($\leq 3 \times 10^6$ cells)	If processing $\leq 3 \times 10^6$ 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 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 C (page 47). 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 Mini Kit for the first time, read “Important Notes” (page 13).
- 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 13).
- If working with RNA for the first time, read Appendix E (page 51).
- 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.
- 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 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 43) 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 ($15\text{--}25^{\circ}\text{C}$).

- QIAzol Lysis Reagent and Buffer RWT contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 5 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 43).

Procedure

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

1a. Cells grown in suspension (do not use more than 1×10^7 cells):

Determine the number of cells. Pellet the appropriate number of cells by centrifuging for 5 min at $300 \times g$ 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 1×10^7 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 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 Mini 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 μ l 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 μ l 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. If processing $\leq 3 \times 10^6$ cells, homogenize the cells by vortexing for 1 min. If processing $> 3 \times 10^6$ cells, they can be homogenized using a QIAshredder homogenizer, the TissueRuptor II or a syringe and needle. See “Disrupting and homogenizing starting material”, page 18, for more details on homogenization.

Note: Incomplete homogenization leads to significantly reduced RNA yields and can cause clogging of the RNeasy Mini spin column.

Note: Homogenized cell lysates can be stored at -90°C to -65°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 39) after performing this step.

8. Transfer the upper aqueous phase to a new collection tube (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 Mini spin column in a 2 ml collection tube (supplied). Close the lid gently and centrifuge at $\geq 8000 \times g$ ($\geq 10,000$ rpm) for 15 s at room temperature. Discard the flow-through.*

Reuse the collection tube in step 10.

* Flow-through contains QIAzol Lysis Reagent and is therefore not compatible with bleach. See page 5 for safety information.

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 the steps in Appendix B (page 43) after performing this step.

11. **Optional:** Add 700 μ l Buffer RWT to the RNeasy Mini spin column. Close the lid gently and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the column.

Discard the flow-through.*

Skip this step if performing the optional on-column DNase digestion (page 43).

Reuse the collection tube in step 12.

12. Pipet 500 μ l Buffer RPE onto the RNeasy Mini spin column. Close the lid gently and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the column. Discard the flow-through.

Reuse the collection tube in step 13.

13. Add another 500 μ l Buffer RPE to the RNeasy Mini spin column. Close the lid gently and centrifuge for 2 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to dry the RNeasy Mini spin column membrane.

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

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

* Flow-through contains QIAzol Lysis Reagent or Buffer RWT and is therefore not compatible with bleach. See page 5 for safety information.

14. **Optional:** Place the RNeasy Mini spin column into a new 2 ml collection tube (not supplied), and discard the old collection tube with the flow-through. Centrifuge in a microcentrifuge at full speed for 1 min.

Perform this step to eliminate any possible carryover of Buffer RPE or if residual flow-through remains on the outside of the RNeasy Mini spin column after step 13.

15. Transfer the RNeasy Mini spin column to a new 1.5 ml collection tube (supplied). Pipet 30–50 μ l RNase-free water directly onto the RNeasy Mini spin column membrane. Close the lid gently and centrifuge for 1 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to elute the RNA.

16. If the expected RNA yield is $>30 \mu\text{g}$, repeat step 15 with a second volume of 30–50 μ l RNase-free water. Elute into the same collection tube.

To obtain a higher total RNA concentration, this second elution step may be performed by using the first eluate (from step 15). The yield will be 15–30% less than the yield obtained using a second volume of RNase-free water, but the final concentration will be higher.

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

Important points before starting

- If using the miRNeasy Mini Kit for the first time, read “Important Notes” (page 13).
- 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 13).
- If working with RNA for the first time, read Appendix E (page 51).
- For optimal results, stabilize harvested tissues immediately in RNA^{later} RNA Stabilization 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 –30°C to –15°C or –90°C to –65°C.
- Fresh, frozen or RNA^{later} 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 QIAzol Lysis Reagent. Homogenized tissue lysates (in QIAzol Lysis Reagent, 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 QIAzol and RNeasy technologies efficiently removes 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. In these cases, small residual amounts of DNA can be removed by on-column DNase digestion (see Appendix B, page 43) 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 5 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 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 43).

Procedure

1. Excise the tissue sample from the animal or remove it from storage. Determine the amount of tissue. Do not use more than 50 mg flash-frozen tissue, 25 mg liver, thymus, spleen or RNA*later* stabilized tissue, or 100 mg adipose tissue.

Unless you are working with RNA*later* 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 RNA*later* or AllProtect Reagent, flash-frozen or disrupted and homogenized in step 3. 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 II, TissueLyser LT, TissueRuptor II or another method until the sample is uniformly homogeneous (usually 20–40 s).

See “Disrupting and homogenizing starting material”, page 18, for a more detailed description of disruption and homogenization methods.

Note: Homogenization with the TissueRuptor II or the TissueLyser II/TissueLyser LT (see Appendix C, page 47, or the appropriate *TissueLyser Handbook*) 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 –90°C to –65°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. For tissues with an especially high fat content, an additional, clear phase may be visible below the 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 39) after performing this step.
8. Transfer the upper aqueous phase to a new collection tube (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 RNeasy procedure.
9. Pipet up to 700 µl of the sample, including any precipitate that may have formed, into an RNeasy Mini spin column in a 2 ml collection tube (supplied). Close the lid gently and centrifuge at $\geq 8000 \times g$ ($\geq 10,000$ 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 the steps in Appendix B (page 43) after performing this step. 11.

* Flow-through contains QIAzol Lysis Reagent and is therefore not compatible with bleach. See page 5 for safety information.

11. Add 700 μ l Buffer RWT to the RNeasy Mini spin column. Close the lid gently and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the column. Discard the flow-through.*

Skip this step if performing the optional on-column DNase digestion (page 43).

Reuse the collection tube in step 12.

12. Pipet 500 μ l Buffer RPE into the RNeasy Mini spin column. Close the lid gently and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the column. Discard the flow-through.

Reuse the collection tube in step 13.

13. Add another 500 μ l Buffer RPE to the RNeasy Mini spin column. Close the lid gently and centrifuge for 2 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to dry the RNeasy Mini spin column membrane.

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

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

14. **Optional:** Place the RNeasy Mini spin column into a new 2 ml collection tube (not supplied), and discard the old collection tube with the flow-through. Centrifuge in a microcentrifuge at full speed for 1 min.

Perform this step to eliminate any possible carryover of Buffer RPE or if residual

flow-through remains on the outside of the RNeasy Mini spin column after step 13.

15. Transfer the RNeasy Mini spin column to a new 1.5 ml collection tube (supplied). Pipet 30–50 μ l RNase-free water directly onto the RNeasy Mini spin column membrane. Close the lid gently and centrifuge for 1 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm)

to elute the RNA.

16. If the expected RNA yield is >30 µg, repeat step 15 with a second volume of 30–50 µl RNase-free water. Elute into the same collection tube.

To obtain a higher total RNA concentration, this second elution step may be performed by using the first eluate (from step 15). The yield will be 15–30% less than the yield obtained using a second volume of RNase-free water, but the final concentration will be higher.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or 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 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” (page 18) for a detailed description of homogenization methods.

Increase <i>g</i> -force and centrifugation time if necessary. In subsequent preparations, reduce the amount of starting material (see page 13) and/or increase the homogenization time. |

Comments and suggestions

- 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 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 F, page 54).

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 E: General Remarks on Handling RNA” (page 51) and “Handling and storage of starting material” (page 17).

* 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

- 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 E: General Remarks on Handling RNA” (page 51).
- Do not put RNA samples into a vacuum dryer that has been used in DNA preparations where RNases may have been used.

DNA contamination in downstream experiments

- 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
- Perform optional on-column DNase digestion using the RNase-Free DNase Set (Appendix B, page 43) 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 *RNeasy Mini Handbook*) with one change to the protocol: the volume of ethanol added to the sample should be increased from 250 µl to 700 µl.

Comments and suggestions

RNA does not perform well in downstream experiments

- a) Salt carryover during elution Ensure that Buffer RPE is at 20–30°C.
- b) Ethanol carryover During the second Buffer RPE wash (step 13), be sure to dry the RNeasy Mini spin column membrane by centrifugation at $\geq 8000 \times g$ ($\geq 10,000$ rpm) for 2 min at 20–25°C. Following the centrifugation, remove the RNeasy Mini spin column from the centrifuge tube carefully so the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.
- To eliminate any chance of possible ethanol carryover, transfer the RNeasy Mini spin column to a new 2 ml collection tube and perform the recommended 1 min centrifugation step as described in step 14 of the protocols.

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 the recovery of the miRNA-enriched fraction, an RNeasy MinElute Cleanup Kit (cat. no. 74204) is required.

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 22 or 28). Instead of continuing with step 8, follow steps A1–A10 below to isolate the miRNA-enriched fraction only, or steps A1–A16 to isolate separate fractions of small RNA and total RNA >200 nt.

- A1. Transfer the upper aqueous phase to a new reaction tube (not supplied). Add 1 volume of 70% ethanol (usually 350 μ l) and mix thoroughly by vortexing. Do not centrifuge. Proceed immediately to step A2.
- A2. Pipet the sample (approx. 700 μ l), including any precipitate that may have formed, into an RNeasy Mini spin column placed in a 2 ml collection tube. Close the lid gently and centrifuge at $\geq 8000 \times g$ ($\geq 10,000$ 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).

A3. If purifying the miRNA-enriched fraction only, discard the RNeasy Mini spin column and follow steps A4–A10 only.

If purifying both the miRNA-enriched fraction and larger RNAs (>200 nt), save the RNeasy Mini spin column for use in step A11 (the spin column can be stored at 4°C or at room temperature, but not for long periods). Follow steps A4–A10 to purify miRNA and then steps A11–A16 to purify large RNAs.

Purifying the miRNA-enriched fraction using the RNeasy MinElute Cleanup Kit (steps A4–A10)

A4. Add 450 µl of 100% ethanol (0.65 volumes) to the flow-through from step A2 and mix thoroughly by vortexing. Do not centrifuge. Proceed immediately to step A5.

A5. 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 $\geq 8000 \times g$ ($\geq 10,000$ rpm) at room temperature. Discard the flow-through.*

Repeat this step until the whole sample has been pipetted into the spin column.

Discard the flow-through each time.

A6. **Optional:** Add 700 µl Buffer RWT to the RNeasy MinElute spin column. Close the lid gently and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ 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 have already been removed on the first RNeasy Mini spin column.

* Flow-through contains QIAzol Lysis Reagent or Buffer RWT and is therefore not compatible with bleach. See page 5 for safety information.

A7. Pipet 500 μ l Buffer RPE into the RNeasy MinElute spin column. Close the lid gently and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.

A8. Add 500 μ l of 80% ethanol to the RNeasy MinElute spin column. Close the lid gently and centrifuge for 2 min at $\geq 8000 \times g$ ($\geq 10,000$ 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.

A9. Place the RNeasy MinElute spin column into a new 2 ml collection tube. Open the lid and centrifuge for 5 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm).

A10. Place the RNeasy MinElute spin column into a 1.5 ml collection tube and pipet 14 μ l RNase-free water onto the spin column membrane. Close the lid gently and centrifuge for 1 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to elute the miRNA-enriched fraction.

Purifying total RNA (>200 nt) using the RNeasy Mini spin column (steps A11–A16)

A11. Pipet 700 μ l Buffer RWT into the RNeasy Mini spin column from step A3. Close the lid gently and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ 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 the steps in Appendix B (page 43) instead of this step. Then proceed to step A12.

A12. Add 500 μ l Buffer RPE to the RNeasy Mini spin column. Close the lid gently and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the spin column membrane. Discard the flow-through.

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

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- A13. Pipet another 500 μ l Buffer RPE into the RNeasy Mini spin column. Close the lid gently and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the spin column membrane. Discard the flow-through and the collection tube.
- A14. Place the RNeasy Mini spin column in a new 2 ml collection tube. Open the lid and centrifuge at full speed for 1 min.
- A15. Place the RNeasy Mini spin column into a new 1.5 ml collection tube. Pipet 30–50 μ l RNase-free water directly onto the spin column membrane. Close the lid gently and centrifuge for 1 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to elute the total RNA.
- A16. If the expected RNA yield is $>30 \mu\text{g}$, repeat step A15 with a second volume of 30–50 μ l RNase-free water. Elute into the same collection tube.

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 1×10^5 cells). In the modified procedure, the spin column flow-through after on-column digestion and washing is reapplied to the membrane (page 46).

IMPORTANT: Buffer RWT used in the procedure “DNase digestion for samples containing <1 µg total RNA approximately” should be prepared with isopropanol instead of ethanol, as is usually recommended (pages 22 and 28). Buffer RWT is prepared by adding 45 ml isopropanol to the concentrate instead of 30 ml ethanol. 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 A2).
- 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 -30°C to -15°C for up to 9 months. Thawed aliquots can be stored at $2-8^{\circ}\text{C}$ for up to 6 weeks. Do not refreeze the aliquots after thawing.
- For the protocol for samples containing $<1\ \mu\text{g}$ 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 22 or 28) or steps A1–A3 as indicated in Appendix A (page 39). Then follow steps B1–B4 below.

B1. Pipet 350 µl Buffer RWT into the RNeasy Mini Spin Column and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash. Discard the flow-through.*

Reuse the collection tube in step B4.

B2. 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.

B3. Pipet the DNase I incubation mix (80 µl) directly onto the RNeasy Mini 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 Mini Spin Column.

B4. Pipet 350 µl Buffer RWT into the RNeasy Mini Spin Column and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.* Continue with step 12 of the protocol or step A12 (if performing the protocol in Appendix A).

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

Procedure

DNase digestion for samples containing <1 µg total RNA approximately Carry out steps 1–10 as indicated in the protocol (on page 22 or 28) or steps A1–A3 as indicated in Appendix A (page 39). Then follow steps B1–B6 below.

B1. Pipet 350 µl Buffer RWT (prepared with isopropanol) into the RNeasy Mini Spin Column and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash. Discard the flow-through.*

Reuse the collection tube in step B4.

B2. 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.

B3. Pipet the DNase I incubation mix (80 µl) directly onto the RNeasy Mini 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 Mini Spin Column.

B4. Pipet 500 µl Buffer RWT (prepared with isopropanol) into the RNeasy Mini Spin Column and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Keep the flow-through.

B5. Reapply the flow-through to the RNeasy Mini Spin Column and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.*

B6. Continue with step 12 of the protocol or step A12 (if performing the protocol in Appendix A).

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

Appendix C: 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

- C1. Pipet 700 µl QIAzol Lysis Reagent into a 2 ml collection tube.
- C2. Add one stainless steel bead to each tube. For best results, we recommend using a 5 mm (mean diameter) stainless steel bead.
- C3. Add up to 50 mg tissue per tube.
- C4. 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).
- C5. 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.

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- C6. Carefully transfer the homogenate to a new microcentrifuge tube (not supplied) by pipetting. Do not reuse the stainless steel bead.
- C7. Proceed with step 4 of the protocol.

Appendix D: Purification of Total RNA, Including Small RNAs, from Human Leukocytes

This protocol is for purification of total RNA including small RNAs of approximately 18 nucleotides upwards using the miRNeasy Mini Kit. In addition to the kit, this protocol requires purchase of Buffer EL (cat. no. 79217).

Procedure

D1. Mix 1 volume of human whole blood with 5 volumes of Buffer EL in an appropriately sized tube (not provided).

For optimal results, the volume of the mixture (blood + Buffer EL) should not exceed $\frac{3}{4}$ of the volume of the tube to allow efficient mixing.

Note: Use an appropriate volume of whole blood. Up to 1.5 ml of healthy blood (typically 4000–7000 leukocytes per microliter) can be processed. Reduce the amount appropriately if blood with elevated numbers of leukocytes is used.

D2. Incubate for 10–15 min on ice. Mix by vortexing briefly two times during incubation.

The cloudy suspension becomes translucent during incubation, indicating lysis of erythrocytes. If necessary, incubation time can be extended to 20 min.

D3. Centrifuge for 400 x g for 10 min at 4°C and completely remove and discard the supernatant.

Leukocytes will form a pellet after centrifugation. Ensure the supernatant is completely removed. Trace amounts of erythrocytes, which give the pellet a red tint, will be removed in the following wash step.

D4. Add Buffer EL to the cell pellet (use 2 volumes of Buffer EL per volume of whole blood used in step D1). Resuspend cells by vortexing briefly.

For example, add 2 ml of Buffer EL for every 1 ml of whole blood used in step D1.

D5. Centrifuge at 400 x g for 10 min at 4°C and completely remove and discard the supernatant.

Note: Incomplete removal of the supernatant will interfere with lysis and subsequent binding of RNA to the spin column, resulting in lower yield.

D6. Continue with step 2 of the protocol for purification of total RNA, including small RNAs, from cells (page 22).

Appendix E: General Remarks on Handling RNA

Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to destroy RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Great care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the isolation procedure. 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 isolated 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 of RNaseKiller (cat. no. 2500080) from 5 PRIME (www.5prime.com) is recommended. RNase contamination can alternatively be removed using general laboratory reagents. To decontaminate plasticware, rinse with 0.1 M NaOH, 1 mM EDTA* followed by RNase-free water (see "Solutions", page 52), or rinse with chloroform* if the plasticware is chloroform-resistant.

* 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.

To decontaminate electrophoresis tanks, clean with detergent (e.g., 0.5% SDS),* rinse with RNase-free water, rinse with ethanol (if the tanks are ethanol-resistant), and allow to dry.

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

Solutions

Solutions should be purchased RNase-free or treated with 0.1% DEPC. We recommend purchasing RNase-free water rather than DEPC treating water. This is because if trace amounts of DEPC remain after autoclaving, purine residues in RNA may be modified by carbethoxylation, and performance of enzymatic reactions, such as cDNA synthesis or PCR may be negatively affected. Therefore residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 minutes.

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. 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

* 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.

in the presence of Tris buffers and decomposes rapidly into ethanol and CO₂. When preparing Tris buffers, use RNase-free water to dissolve Tris to make the appropriate buffer.

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

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

Storage of RNA

Purified RNA may be stored at -30°C to -15°C or -90°C to -65°C in RNase-free water. Under these conditions, no degradation of RNA is detectable after 1 year.

Quantification of RNA

The concentration of RNA should be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer (see “Spectrophotometric quantification of RNA” below). For small amounts of RNA, however, it may be difficult to determine amounts photometrically. Small amounts of RNA can be accurately quantified using an Agilent® 2100 Bioanalyzer, quantitative RT-PCR or fluorometric quantification. When purifying RNA from particularly small samples (e.g., laser-microdissected samples or from plasma or serum), quantitative, real-time RT-PCR should be used for quantification.

Spectrophotometric quantification of RNA

To ensure significance, A_{260} readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 44 μg of RNA per ml ($A_{260}=1 \rightarrow 44 \mu\text{g/ml}$). 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 56), 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

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with 0.1 M NaOH, 1 mM EDTA,* followed by washing with RNase-free water (see "Solutions", page 52). 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 μ l

Dilution = 10 μ l of RNA sample + 490 μ l 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 μ g/ml $\times A_{260} \times$ dilution factor
= 44 μ g/ml $\times 0.2 \times 50$
= 440 μ g/ml

Total amount = concentration \times volume in milliliters
= 440 μ g/ml $\times 0.1$ ml
= 44 μ g of RNA

Spectrophotometric quantification of miRNA-enriched fractions miRNA-enriched fractions prepared using the protocol in Appendix A contain many types of small RNA, including miRNA, snoRNA and piRNA, as well as tRNA and a significant proportion of the 5S and 5.8S rRNA. Larger species, including tRNA, will dominate the OD measurement of these fractions and also gel staining or assays which use RNA-binding dyes. For this reason, it is not possible to quantify miRNA using OD measurements. To quantify miRNA, we recommend real-time RT-PCR assays (e.g., using the miScript PCR System).

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Purity of RNA

The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV spectrum, such as protein. However, the A_{260}/A_{280} ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting A_{260}/A_{280} ratio can vary greatly. Lower pH results in a lower A_{260}/A_{280} ratio and reduced sensitivity to protein contamination.* For accurate values, we recommend measuring absorbance in 10 mM Tris-Cl, pH 7.5. Pure RNA has an A_{260}/A_{280} ratio of 1.9–2.1† in 10 mM Tris-Cl, pH 7.5. Always be sure to calibrate the spectrophotometer with the same solution used for dilution.

For determination of RNA concentration, however, we recommend dilution of the sample in a buffer with neutral pH since the relationship between absorbance and concentration (A_{260} reading of 1 = 44 µg/ml RNA) is based on an extinction coefficient calculated for RNA at neutral pH (see “Quantification of RNA”, page 54).

DNA contamination

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

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

To prevent any interference by DNA in real-time RT-PCR applications, such as with ABI PRISM® and LightCycler® instruments, we recommend designing primers that anneal at intron splice

* 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.

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 (the assays can be ordered online at 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, starting on page 59).

Alternatively, gene expression analysis can be performed using QuantiFast Probe Assays and the QuantiFast Probe RT-PCR Plus Kit, which includes an integrated genomic DNA removal step.

miScript Primer Assays, used with the miScript PCR System for mature miRNA quantification, do not detect genomic DNA.

For other sensitive applications, DNase digestion of the purified RNA with RNase-free DNase is recommended. A protocol for optional on-column DNase digestion using the RNase-Free DNase Set is provided in Appendix B (page 43). The DNase is efficiently washed away in subsequent wash steps.

Integrity of RNA

The integrity and size distribution of total RNA purified with miRNeasy Kits can be checked by denaturing agarose gel electrophoresis and ethidium bromide* staining or by using the QIAxcel® system or Agilent 2100 Bioanalyzer. The respective 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. The Agilent 2100 Bioanalyzer also

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provides an RNA Integrity Number (RIN) as a useful measure of RNA integrity. Ideally, the RIN 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.

Ordering Information

Product	Contents	Cat. no.
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
Related products		
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
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 Serum/Plasma Spike-In Control	10 pmol lyophilized <i>C. elegans</i> miR-39 miRNA mimic	219610
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
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
Accessories		
Buffer RWT	80 ml wash buffer concentrate for use with miRNeasy Kits	1067933
TissueLyser II*	Universal laboratory mixer mill disruptor	Inquire

Product	Contents	Cat. no.
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
TissueLyser Adapter Set 2 x 96	2 sets of Adapter Plates for use with Collection Microtubes (racked) on the TissueLyser II	69984
TissueLyser LT*	Compact bead mill	Inquire
TissueLyser LT Adapter, 12-Tube	Adapter for disruption of up to 12 samples in 2 ml microcentrifuge tubes on the TissueLyser LT	69980
Stainless Steel Beads, 5 mm (200)	Stainless Steel Beads, suitable for use with TissueLyser systems	69989
TissueRuptor II	Handheld rotor–stator homogenizer, 5 TissueRuptor Disposable Probes	Inquire
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
RNA ^{later} RNA Stabilization Reagent (50 ml)	50 ml RNA ^{later} RNA Stabilization Reagent for stabilization of RNA in animal tissues	76104
RNA ^{later} RNA Stabilization Reagent (250 ml)	250 ml RNA ^{later} RNA Stabilization Reagent for stabilization of RNA in animal tissues	76106
Collection Tubes (2 ml)	1000 Collection Tubes (2 ml)	19201
QIAshredder (50)	50 disposable cell-lysate homogenizers	79654

Product	Contents	Cat. no.
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
Buffer EL (1000 ml)	1000 ml buffer for RNA purification from leukocytes	79217
Related products for quantitative, real-time RT-PCR		
miScript II RT Kit (12)	For 12 cDNA synthesis reactions: miScript Reverse Transcriptase Mix, 10x miScript Nucleics Mix, 5x miScript HiSpec Buffer, 5x miScript HiFlex Buffer, RNase-Free Water	218160
miScript II RT Kit (50)	For 50 cDNA synthesis reactions: miScript Reverse Transcriptase Mix, 10x miScript Nucleics Mix, 5x miScript HiSpec Buffer, 5x miScript HiFlex Buffer, RNase-Free Water	218161
miScript PreAMP PCR Kit (12)	For 12 preamplification reactions: 5x miScript PreAMP Buffer, HotStarTaq DNA Polymerase (2 U/ μ l), miScript PreAMP Universal Primer, miScript Primer Assays (miR-16, SNORD95, miRTC, <i>C. elegans</i> miR-39), RNase-Free Water	331451
miScript PreAMP PCR Kit (60)	For 60 preamplification reactions: 5x miScript PreAMP Buffer, HotStarTaq DNA Polymerase (2 U/ μ l), miScript PreAMP Universal Primer, miScript Primer Assays (miR-16, SNORD95, miRTC, <i>C. elegans</i> miR-39), RNase-Free Water	331452
miScript PreAMP Primer Mix	For preamplification reactions using the miScript PreAMP PCR Kit	Varies

Product	Contents	Cat. no.
miScript SYBR Green PCR Kit (200)	For 200 reactions: QuantiTect SYBR Green PCR Master Mix, miScript Universal Primer	218073
miScript SYBR Green PCR Kit (1000)	For 1000 reactions: QuantiTect SYBR Green PCR Master Mix, miScript Universal Primer	218075
miScript Primer Assay (100)	miRNA-specific primer; available via GeneGlobe	Varies†
Pathway-Focused miScript miRNA PCR Array	Array of assays for a pathway, disease or gene family for human, mouse, rat, dog or rhesus macaque miRNAs; available in 96-well, 384-well or Rotor-Disc® 100 format	Varies
miRNome miScript miRNA PCR Array	Array of assays for the complete human, mouse, rat, dog or rhesus macaque miRNome; available in 96-well, 384-well or Rotor-Disc 100 format	Varies
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	Filter-tips, 200 µl (1024), 1000 µl filter-tips (1024), 30 ml reagent bottles (12), rotor adapters (240), elution tubes (240), rotor adapter holder	990395

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

† Visit www.qiagen.com/GeneGlobe to search for and order these products.

‡ 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

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Document Revision History

Date	Changes
January 2020	Updated text, ordering information and intended use for QIAcube Connect.

Notes

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Notes

Limited License Agreement for miRNeasy Mini Kit

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