February 2023

miRNeasy 96 Tissue/Cell Advanced Handbook

Sample to Insight

Contents

Kit contents

Kit **Catalog no.** **Number of preps**	**(4)** **217661** **4 x 96**
RNeasy® 96 Plates	4
Register Cards (96-well)	4
gDNA Eliminator 96 plates	4
Collection Microtube Caps	100 x 8
S-Blocks	8
Elution Microtubes CL	4 x 96
Elution Microtube Caps (strips)	50 x 8
AirPore Tape Sheets	1x 25
Buffer RWT*†	2 x 80 mL
Buffer RPE‡	2 x 65 mL
RNase-Free Water	3 x 30 mL
Buffer RLT	1 x 220 mL
Proteinase K	1 x 10mL
Buffer AL	1 x 33 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.

Additional Buffers, S-Blocks, Elution Microtubes CL, Collection Microtubes (racked) and AirPore Tape Sheets are available separately. See ordering information (page 62).

Storage

The miRNeasy 96 Tissue/Cell Advanced Kit is shipped at room temperature (15–25°C). It should be stored at room temperature. Under these conditions, the components are stable for at least 9 months without showing any reduction in performance and quality, unless otherwise indicated on the label.

Intended Use

The miRNeasy 96 Tissue/Cell Advanced Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

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

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.

Quality Control

In accordance with QIAGEN's ISO-certified Total Quality Management System, each lot of the miRNeasy 96 Tissue/Cell Advanced Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

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

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

Principle and procedure

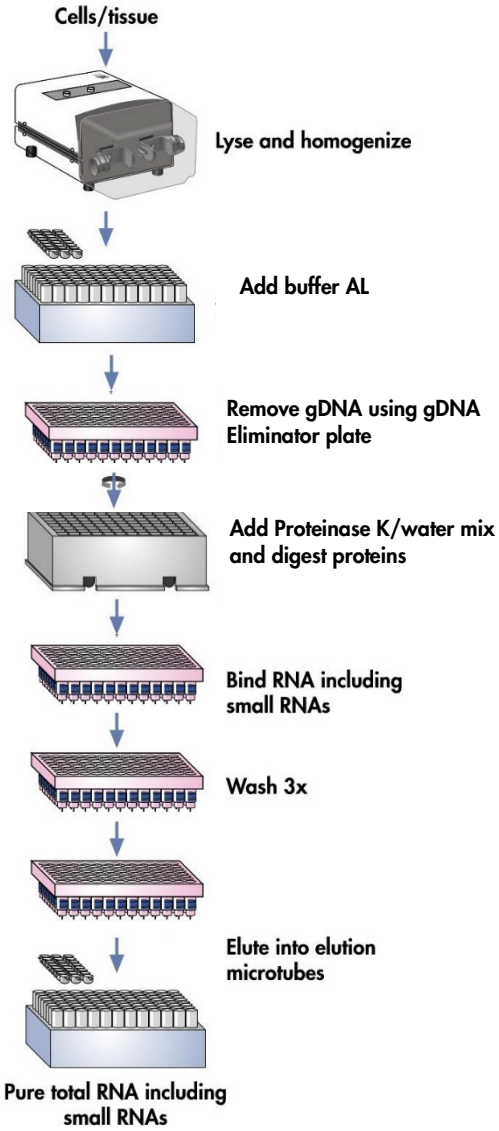
The miRNeasy 96 Tissue/Cell Advanced Kit combines guanidine-based sample lysis and silica-membrane–based RNA purification with the speed of vacuum and/or spin processing (see flowchart, page 9).The procedure starts with a lysis in buffer RLT, which is designed to facilitate lysis and to denature protein complexes and RNases. Therefore, RNA in samples lysed in buffer RLT are stable and protected from degradation.

Buffer RLT is added to tissue or cell samples, immediately followed by a thorough disruption and homogenization to ensure a complete lysis and inactivation of RNases. Buffer AL is added to the lysate to optimize the gDNA removal by the gDNA eliminator column. Subsequently, a Proteinase K digestion is carried out under optimized conditions to ensure complete lysis of even difficult-to-lyse tissue and thereby release of RNA.

Isopropanol is added to provide appropriate binding conditions for recovery of all RNA, including miRNA and other small RNA molecules ≥18 nucleotides. Samples are then applied to the wells of the RNeasy 96 plate, where total RNA binds and contaminants are efficiently washed away. High-quality RNA is then eluted in a small volume of water, ready for use in any downstream application.

RNA is eluted in 50 or 100 µL RNase-free water. Isolated RNA is compatible with RT-PCR, digital PCR, and NGS workflows. If necessary, RNA can be stored long term at –30 to –15°C.

Flowchart of miRNeasy 96 Tissue/Cell Advanced Kit process



Description of protocols

The protocols in this handbook provide two different handling options, using either a combination of vacuum and spin technology, or spin technology alone. Both handling options provide high yields of high-quality RNA.

I. Vacuum/spin technology

Using vacuum/spin technology, all protocol steps from the binding step until the final wash step are performed on the QIAvac 96 vacuum manifold (see page 15). RNA binding, membrane drying and the elution steps are performed in the Centrifuge 4–16K (see page 14). The Plate Rotor 2 x 96 holds two RNeasy 96 plates, allowing up to 192 RNA samples to be prepared in parallel. Residual traces of salt are removed by centrifugation in the final wash step. RNA purified using vacuum/spin technology can be used for any nonenzymatic or enzymatic downstream application, including quantitative RT-PCR analysis with QuantiNova® technology and NGS with QIAseq® miRNA technologies.

II. Spin technology

Using spin technology, all protocol steps are performed in the Centrifuge 4–16K (see page 14). The Plate Rotor 2 x 96 holds 2 RNeasy 96 plates, allowing up to 192 RNA samples to be prepared in parallel. RNA purified using spin technology can be used for any nonenzymatic or enzymatic downstream application, including quantitative RT-PCR analysis with QuantiNova technology and NGS with QIAseq miRNA technologies.

The miRNeasy 96 Tissue/Cell Advanced Kit can also be used in combination with the QIAcube® HT . Using spin technology, gDNA Elimantion is done manually followed by RNA isolation realized on the QCHT instrument (please refer to product page for more information, **https://www.qiagen.com/us/products/instruments-and-automation/nucleic-acid-purification/qiacube-ht** .

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

In general, 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. Both systems use the same miRCURY LNA RT Kit for generating universal first-strand cDNA synthesis – one cDNA reaction for all miRNAs. Each system 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 Panesls enable rapid profiling of the complete miRNome. Other Focus panels such as the miRCURY LNA miRNA Serum/Plasma Focus PCR Panel and the 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**

Equipment and Reagents to Be Supplied by User

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

For all protocols

- Multichannel pipette with tips. For the most efficient sample processing in the miRNeasy 96 Tissue/Cell Advanced protocol, we recommend the use of an electric multichannel pipet with a minimum capacity of 650 µL per pipet tip. Matrix® Impact® cordless electronic multichannel pipettes have a unique expandable tip-spacing system allowing the user to transfer liquid directly from racks of tubes to 96-well microplates. Matrix Impact pipets can be purchased from Matrix Technologies Corporation (**www.matrixtechcorp.com**)*
- Reagent reservoirs for multichannel pipettes
  Note: Make sure that reagent reservoirs used for chloroform are chloroform-resistant.
- Disposable gloves
- Centrifuge 4–16K (see page 14)
- Plate Rotor 2 x 96 (see page 14)
- QIAGEN's TissueLyser II (see page 13) or a rotor–stator homogenizer
- Chloroform (without added isoamyl alcohol)
- Isopropanol (96–100%)
- Dry ice
- For animal tissues: RNAprotect® Tissue Reagent, RNAprotect Tissue Tubes (see Ordering Information, page 62) or liquid nitrogen

* This is not a complete list of suppliers and does not include many important vendors of biological supplies.

- **Optional**: additional S-Blocks (24) (cat. no. 19585). Some S-Blocks are supplied with the kit for use as waste-trays. If several RNeasy 96 plates are processed per day, it may be convenient to keep extra S-Blocks on hand.
- Collection Microtubes rack (10 x 96) (cat. no. 19560) and Collection Microtube Caps (120 x 8) (cat. no. 19566) or similar format for sample disruption

For protocol using vacuum/spin technology

- QIAvac 96 vacuum manifold
- Vacuum source capable of generating a vacuum pressure of –800 to –900 mbar (see pages 15–18). Vacuum Pumps (capacity 34 liter/min) can be purchased from QIAGEN (see Ordering Information, page 62)

Optional reagents

- RNase inhibitors (available from Promega, Applied Biosystems, Amersham Pharmacia and Stratagene)*

TissueLyser II

The TissueLyser II provides high-throughput processing for simultaneous, rapid, and effective disruption of up to 192 biological samples, including all types of animal tissue. Processing of up to 2 x 96 samples takes as quickly as 2–5 min.

Disruption and homogenization using the TissueLyser II produce yields comparable or better than traditional rotor–stator homogenization methods. With rotor–stator homogenization, the samples must be processed individually, and the rotor–stator homogenizer must be cleaned

* This is not a complete list of suppliers and does not include many important vendors of biological supplies.

after each sample to prevent cross-contamination. In contrast, the TissueLyser II provides simultaneous disruption for high-throughput processing of a variety of animal tissues.

The TissueLyser II includes a number of different accessories for ease of use with different sample sizes and throughputs. In the miRNeasy 96 Tissue/Cell Advanced procedure, the TissueLyser Adapter Set 2 x 96 allows simultaneous processing of up to 192 samples in Collection Microtubes. Stainless steel beads with a diameter of 5 mm are optimal to use for animal tissues in combination with the miRNeasy 96 Tissue/Cell Advanced Kit. The TissueLyser 5 mm Bead Dispenser (96-Well) is also available to conveniently deliver 96 beads in parallel with Collection Microtubes. See page 62 for Ordering Information.

Centrifuge 4–16K

All miRNeasy 96 Tissue/Cell Advanced protocols utilize a streamlined centrifugation procedure that allows preparation of RNA from up to 2 x 96 samples in parallel for direct use in any downstream application. For optimal handling, QIAGEN, in cooperation with the centrifuge manufacturer Sigma Laborzentrifugen GmbH, has developed a centrifugation system consisting of the Plate Rotor 2 x 96 and the refrigerated table-top Centrifuge 4–16K (see Ordering Information, page 62). A wide range of other rotors can be used with Centrifuge 4–16K in addition to the Plate Rotor 2 x 96.

Standard table-top centrifuges and 96-well–microplate rotors are not suitable for the miRNeasy 96 Tissue/Cell Advanced procedure. Usually, 96-well–microplate buckets are not deep enough to carry the complete RNeasy assembly and may not swing out properly during centrifugation. Furthermore, high *g*-forces (>5500 x *g*) are required for optimal performance of the miRNeasy 96 Tissue/Cell Advanced procedure.

For further information about the centrifuge and rotor, please contact QIAGEN or your local distributor.

WARNING 	Do not centrifuge the Plate Rotor 2 x 96 metal holders without the RNeasy 96 plates and S-Blocks or elution microtubes. If unsupported, the holders will collapse under high *g*-force. Therefore, remove the holders during test runs. Standard 96-well microplates may be centrifuged in the holders if a *g*-force of 500 x *g* is not exceeded.

QIAvac 96 handling guidelines

QIAvac 96 facilitates the miRNeasy 96 Tissue/Cell Advanced procedure by providing a convenient, modular vacuum manifold for use with the miRNeasy 96 Tissue/Cell Advanced Kit. The following recommendations should be followed when handling the QIAvac 96 vacuum manifold.

- QIAvac 96 operates with a house vacuum or a vacuum pump. If the house vacuum is weak or inconsistent, we recommend using a vacuum pump with a capacity of 18 liter/min. Use of insufficient vacuum pressure may reduce RNA yield and purity.
- A vacuum pressure of –800 to –900 mbar should develop when an RNeasy 96 plate sealed with tape is used on the QIAvac 96. Vacuum pressures exceeding –900 mbar should be avoided. The vacuum pressure is the pressure difference between the inside of the manifold and the atmosphere (standard atmospheric pressure: 1013 millibar or 760 mm Hg) and can be regulated and measured using a pressure gauge or vacuum regulator (see Ordering Information, page 62). Vacuum recommendations are given in negative units to indicate the required reduction in pressure with respect to the atmosphere. Table 1 (page 16) provides pressure conversions to other units.
- Between loading steps, the vacuum must be switched off and the manifold ventilated to maintain uniform conditions for each sample. This can be done with a vacuum regulator (see Ordering Information, page 62) inserted between the vacuum source and the QIAvac 96 vacuum manifold.
- Wear safety glasses when working near a manifold under pressure.

- For safety reasons, do not use 96-well plates that have been damaged in any way.
- Always place the QIAvac 96 vacuum manifold on a secure bench top or work area. If dropped, the manifold may crack.
- Always store the QIAvac 96 vacuum manifold clean and dry. To clean, simply rinse all components with water and dry with paper towels. Do not air dry, as the screws may rust and need to be replaced. Do not use abrasives. Finally, wipe manifold components with paper towels wetted with 70% ethanol, and dry with fresh paper towels.
- The QIAvac 96 vacuum manifold and components are not resistant to ethanol, methanol or other organic solvents when exposed for long periods. If solvents are spilled on the unit, rinse thoroughly with distilled water after the RNeasy preparation. Ensure that no residual buffers remain in the vacuum manifold.
- To ensure consistent performance, do not apply silicone or vacuum grease to any part of the QIAvac 96 vacuum manifold. The spring lock on the top plate and the self-sealing gasket provide an airtight seal when vacuum is applied to the assembled unit. To maximize gasket life, rinse the gasket free of salts and buffers after each use and dry with paper towels before storage.

Table 1. Pressure conversions

To convert from millibars (mbar) to:	Multiply by:
Millimeters of mercury (mm Hg)	0.75
Kilopascals (kPa)	0.1
Inches of mercury (inch Hg)	0.0295
Torrs (Torr)	0.75
Atmospheres (atmos)	0.000987
Pounds per square inch (psi)	0.0145

Important Notes

Determining the amount of starting material

Using the correct amount of starting material is essential to obtain high yields of pure RNA with the miRNeasy 96 Tissue/Cell Advanced Kit. The maximum amount that can be used is limited by:

- The volume of Buffer RLT required for efficient lysis
- The RNA binding capacity of the RNeasy 96 plate wells (100 µg)
- The type of sample and its RNA content

The miRNeasy 96 Tissue/Cell Advanced procedure is optimized for use with a maximum of 30 mg animal tissue (flash frozen). With liver, thymus, spleen or RNAprotect-Tissue-stabilized tissues*, only 15 mg should be used to avoid clogging the RNeasy 96 plate.

Table 2 provides specifications for the RNeasy 96 plate. Each well of the RNeasy 96 plate has a maximum binding capacity of 100 µg of RNA, but actual RNA yields depend on the sample type used. Table 4 (page 20) provides examples of expected RNA yields from various sample types.

Table 2. RNeasy 96 Plate specifications

Preps per plate	96
Amount of starting material	Up to 50 mg tissue (up to 100 mg for adipose tissue) Up to 1 x 10^7 cells
Binding capacity per well	100 µg RNA*
Maximum loading volume per well	1 mL
Minimum elution volume	45 µL
Preps per plate	96

* See the *RNAprotect Handbook* for more information about RNAprotect Tissue Reagent.

Note: If the binding capacity of the RNeasy 96 plate is exceeded, yields of total RNA will not be consistent and may be less than expected. If lysis of the starting material is incomplete, yields of total RNA will be lower than expected, even if the binding capacity of the RNeasy 96 plate is not exceeded.

Determining the correct amount of starting material – cells

Use of gDNA Eliminator plate in the miRNeasy 96 Tissue/Cell Advanced procedure removes most of the genomic DNA. For this reason, up to 3 x 10^6 cells can be processed by vortexing without further homogenization of the cell lysates. In general, a mechanical disruption always helps in releasing RNA, and reduce viscosity of the lysate which will reduce clogging probability

Counting cells is the most accurate way to quantitate the amount of starting material. As a guide, the number of HeLa cells obtained in various culture vessels after confluent growth is given in Table 3.

Table 3. Growth area and number of HeLa cells in various culture vessels

Cell-culture vessel	Growth area (cm_2)*	Number of cells†
Multiwell-plates		
96-well	0.32–0.6	4–5 x 10^4
48-well	1	1 x 10^5
24-well	2	2.5 x 10^5
12-well	4	5 x 10^5
6-well	9.5	1 x 10^6
Dishes		
35 mm	8	1 x 10^6
60 mm	21	2.5 x 10^6
100 mm	56	7 x 10^6
145–150 mm	145	2 x 10^7
Flasks		
40–50 mL	25	3 x 10^6
250–300 mL	75	1 x 10^7
650–750 mL	162–175	2 x 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

Generally, this protocol can be used with a maximum of:

- 30 mg flash-frozen tissue
- 15 mg liver, thymus, spleen or RNAprotect-Tissue-stabilized tissue

Average RNA yields from various sources are given in Table 4.

Table 4. Average yields of total RNA with the miRNeasy 96 Tissue/Cell Advanced Kit

Sample	Average RNA yield* (µg)
Cell culture (1 x 10^6 cells)	
NIH/3T3	10
HeLa	15
COS-7	35
LMH	12
Huh	15
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

* Amounts can vary due to species, age, gender, physiological state, growth conditions, 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 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 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. Therefore, it is 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.

The procedures for harvesting and RNA protection should be carried out as quickly as possible. Frozen samples should not be allowed to thaw during handling or weighing. After disruption and homogenization in Buffer RLT, samples can be stored at –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. Incomplete homogenization results in inefficient binding of RNA to the RNeasy membrane, significantly reducing RNA yields.

Some disruption methods simultaneously homogenize the sample, while others require an additional homogenization step. Table 5 gives an overview of different disruption and homogenization methods and is followed by a detailed description of each. This information can be used as a guide to choose the appropriate methods for your starting material.

Table 5. Disruption and homogenization methods

Sample	Disruption method	Homogenization method	Comments
Animal cells	Addition of lysis buffer	Vortexing (for ≤3 x 10^6 cells), TissueLyser II or rotor–stator homogenizer	If processing ≤3 x 10^6 cells, lysate can be homogenized by vortexing.
Animal tissues	TissueLyser II	TissueLyser II	Simultaneously disrupts and homogenizes up to 192 samples in parallel. The TissueLyser II gives results comparable to using a rotor–stator homogenizer.
	Rotor–stator homogenization	Rotor–stator homogenization	Simultaneously disrupts and homogenizes individual samples.

Disruption and homogenization using the TissueLyser II

In bead-milling, tissues can be disrupted by rapid agitation in the presence of beads and lysis buffer. Disruption and simultaneous homogenization occur by the shearing and crushing action of the beads as they collide with the cells. 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 5 mm diameter are optimal for use with animal tissues in combination with the miRNeasy 96 Tissue/Cell Advanced Kit. All other disruption parameters should be determined empirically for each application. The protocols in this handbook provide guidelines for disruption and homogenization of tissues using the TissueLyser II and stainless-steel beads. For other bead mills, please refer to suppliers' guidelines for further details.

Disruption and homogenization using rotor–stator homogenizers

Rotor–stator homogenizers thoroughly disrupt and simultaneously homogenize animal tissues in the presence of lysis buffer in 15–90 s depending on the toughness and size of the sample. Rotor–stator homogenizers can also be used to homogenize cell lysates.

The rotor turns at a very high speed, causing the sample to be disrupted and homogenized by a combination of turbulence and mechanical shearing. Foaming of the sample should be kept to a minimum by using properly sized vessels, keeping the tip of the homogenizer submerged, and holding the immersed tip to the side of the tube. Rotor–stator homogenizers are available in different sizes and operate with differently sized probes.

S-Blocks

S-Blocks are supplied with the kit. If several RNeasy 96 plates are processed per day, it may be convenient to keep extra S-Blocks on hand. See Ordering Information on page 62.

Fresh S-Blocks must be used to take up the flow through after gDNA elimination. S-Blocks may be cleaned and reused as waste trays.

Important: Do not reuse cleaned S-Blocks to collect the flow-through.

To reuse the S-Blocks as waste trays, rinse them thoroughly with tap water and incubate for 2 hours or overnight in 0.1 N NaOH, 1 mM EDTA*. Rinse in distilled water and dry at 50°C.

Note: Do not use bleach. Bleach may react with residual amounts of Buffer RLT and RWT on the S-Blocks.

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

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

Important points before starting

- Buffers RLT and RWT contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach.
- Add ethanol (96–100%) to Buffer RWT and Buffer RPE as indicated on the bottle label.
- Equilibrate buffers to room temperature.
- All centrifugation steps in the protocol are performed in a Centrifuge 4–16K.
- All steps should be performed at room temperature and at a fast pace.
- If purifying RNA from cell lines rich in RNases or from tissue, we recommend adding either β-mercaptoethanol (β-ME) or 2 M dithiothreitol (DTT) to Buffer RLT before use (10 µL β-ME or 20 µL DTT per 1 mL Buffer RLT). Buffer RLT containing DTT or β-ME can be stored at room temperature for up to 1 month.
- If using the miRNeasy 96 Tissue/Cell Advanced Kit for the first time, read "Important Notes" (page 17).
- Do not overload the RNeasy plate; overloading will significantly reduce RNA yield and quality, and may cause clogging of the plate. Read "Determining the amount of starting material" (page 17).
- If working with RNA for the first time, read Appendix C (page 55).
- Cell pellets can be stored at –70°C for either 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 3. Homogenized cell lysates from step 4 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 RNeasy technologies in combination with the gDNA eliminator efficiently removes most of the DNA without DNase treatment. Additionally, miRCURY Assays and most other assays for mature miRNA are not affected by the presence of small amounts of genomic DNA.. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA. In these cases, small residual amounts of DNA can be removed by 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.
- Buffer RLT and Buffer RWT contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 5 for Safety Information.
- Use of a multichannel pipette is recommended (see page 11). Pour buffers and RNase-free water into reagent reservoirs for multichannel pipettes. Use reservoirs from a freshly opened package.
- All centrifugation steps in the protocol are performed in a Centrifuge 4–16K (see page 14).
- All steps of the miRNeasy 96 Tissue/Cell Advanced protocol should be performed at room temperature. Avoid interruptions during the procedure.

Things to do before starting

- Buffer RWT and Buffer 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.
- Ensure that all the buffers are at room temperature.

Procedure

1. Harvest cells as a cell pellet or, for cells grown in a monolayer, aspirate the cell-culture medium from the cell-culture vessel. Add 300 µL Buffer RLT to either the pellet or the cell-culture vessel, vortex, or pipet to mix and homogenize.
2. Harvest cells according to step 2a or 2b:

   2a. **Cells grown in a monolaye**r:
   Cells grown in a monolayer in multiwell cell-culture plates can be lysed directly in the wells (do not use more than 1 x 10^7 cells per well). Completely remove medium by pipetting, and continue with step 3.

   Note: Incomplete removal of the supernatant will dilute the Buffer RLT. This may lead to reduced RNA yield.

   2b. **Cells grown in suspension**:
   Transfer aliquots of up to 1 x 10^7 cells into Collection Microtubes (supplied). Spin cells for 5 min at 300 x *g*. Completely remove supernatant by pipetting and continue with step 3.

   Note: Incomplete removal of the supernatant will dilute the Buffer RLT. This may lead to reduced RNA yield.
3. Disrupt the cells by adding Buffer RLT. For pelleted cells, loosen the cell pellet thoroughly by flicking the tube. Add 300 µL Buffer RLT to each plate well/Collection Microtube. Pipet up and down 3 times. If the lysates are in plate wells, transfer to Collection Microtubes (cat. no. 19560 not provided) or another suitable 96 well plate. .
4. Close the Collection Microtubes using Collection Microtubes Caps (both not provided). Vortex for 1 min at maximum speed. Vortexing for 1 min is sufficient for homogenization of up to 3 x 10^6 cells. If the cell number is greater than 3 x 10^6, the TissueLyser II should be used to homogenize cells (as described in the protocols for animal tissue).

   Note: Homogenized cell lysates can be stored at –70°C for several months.
5. Centrifuge at 6000 x *g* for 1 min to collect residual liquid from the caps of the tubes.

6. Add 80 µL Buffer AL to each sample and mix by pipetting. Incubate at room temperature for 3 min.
7. Put the gDNA Eliminator 96 plate on top of a new S-Block (provided), transfer the lysate to the gDNA Eliminator 96 plate.

   Note: Take care not to wet the rims of the wells, as this could lead to cross contamination
8. Seal the gDNA Eliminator 96 plate with an AirPore tape sheet. Place the S-Block and gDNA Eliminator 96 plate into the metal holder, and place the whole assembly into the rotor bucket. Centrifuge at 6000 rpm (~5600 x *g*) for 4 min at 20–25°C. Discard the gDNA Eliminator 96 plate, and save the flow-through. Centrifugation with sealed plates prevents cross-contamination.

   Note: Make sure that no liquid remains on the membranes after centrifugation. If necessary, repeat the centrifugation until all liquid has passed through the membranes.
9. Add 75 µL RNase-free Water and 25 µL ProtK, to the flow through in the S-Block, mix and incubate for 10min at room temperature.
10. Place an RNeasy 96 plate on top of a square-well block (either new or reused). Mark the plate for later identification. If reusing an S-Block, make sure it is cleaned as described on page 16.
11. Add 1 volume (approximately 480 µL) of 100% isopropanol to each well of the S-Block containing the flow-through from step 5. Mix well by pipetting up and down 3 times.
12. Pipet the samples into the wells of the RNeasy® 96 plate.

    Note: Take care not to wet the rims of the wells, as this could lead to cross contamination.
14. Seal the RNeasy 96 plate with an AirPore tape sheet. Place the S-Block and RNeasy 96 plate into the metal holder, and place the whole assembly into the rotor bucket. Centrifuge at 6000 rpm (~5600 x *g*) for 4 min at 20–25°C. Centrifugation with sealed plates prevents cross-contamination.

15. Empty the S-Block* and remove the AirPore tape sheet. Add 800 µL Buffer RWT to each well of the RNeasy 96 plate, and seal the plate with a new AirPore tape sheet. Centrifuge at 6000 rpm (~5600 x *g*) for 4 min at 20–25°C.
16. Empty the S-Block* and remove the AirPore tape sheet. Add 800 µL Buffer RPE to each well of the RNeasy 96 plate, and seal the plate with a new AirPore tape sheet. Centrifuge at 6000 rpm (~5600 x *g*) for 4 min at 20–25°C.

    Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see "Things to do before starting")
17. Empty the S-Block* and remove the AirPore tape sheet. Add 800 µL Buffer RPE to each well of the RNeasy 96 plate, and seal the plate with a new AirPore tape sheet. Centrifuge at 6000 rpm (~5600 x *g*) for 10 min at 20–25°C to dry the membranes. It is important to dry the RNeasy membranes, since residual ethanol may interfere with downstream reactions. The 10 min centrifugation ensures that residual traces of salt are removed and that no ethanol is carried over during RNA elution.
18. Remove the AirPore tape sheet. Place the RNeasy 96 plate on top of a rack of Elution Microtubes CL. Add 70–100 µL RNase-free water to each well, and seal the plate with a new AirPore tape sheet. Incubate for 1 min at room temperature. Then centrifuge at 6000 rpm (~5600 x *g*) for 4 min at 20–25°C to elute the RNA.

    Note: Be sure to pipet the RNase-free water directly onto the RNeasy membranes. Elution will be incomplete if some of the water sticks to the walls or the O-rings of the RNeasy 96 plate.

    The eluate volume will be approximately 15 µL less than the volume of RNase-free water added to the membrane (the 15 µL corresponds to the membrane dead volume). Use the caps provided with the kit to seal the microtubes for storage. Store RNA at –20°C or at –70°C.

* S-Block can be reused. See page 26 for its cleaning instructions.

Protocol: Purification of Total RNA, Including Small RNAs, from Animal Cells using Vacuum/Spin Technology

Important points before starting

- Buffers RLT and RWT contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach.
- Add ethanol (96–100%) to Buffer RWT and Buffer RPE as indicated on the bottle label.
- Equilibrate buffers to room temperature.
- All centrifugation steps in the protocol are performed in a Centrifuge 4–16K.
- All steps should be performed at room temperature and at a fast pace.
- If purifying RNA from cell lines rich in RNases or from tissue, we recommend adding either β-mercaptoethanol (β-ME) or 2 M dithiothreitol (DTT) to Buffer RLT before use (10 µL β-ME or 20 µL DTT per 1 mL Buffer RLT). Buffer RLT containing DTT or β-ME can be stored at room temperature for up to 1 month.
- If using the miRNeasy 96 Tissue/Cell Advanced Kit for the first time, read "Important Notes" (page 17).
- Do not overload the RNeasy plate; overloading will significantly reduce RNA yield and quality, and may cause clogging of the plate. Read "Determining the amount of starting material" (page 17).
- If working with RNA for the first time, read Appendix C (page 58).
- 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 3.
  Homogenized cell lysates from step 4 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 RNeasy technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA. In these cases, small residual amounts of DNA can be removed 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.
- Buffer RLT and Buffer RWT contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 5 for Safety Information.
- Use of a multichannel pipette is recommended (see page 11). Pour buffers and RNase-free water into reagent reservoirs for multichannel pipettes. Use reservoirs from a freshly opened package.
- A vacuum source capable of generating a vacuum pressure of –800 to –900 mbar is necessary (see pages 15–18). The vacuum must be switched off and the manifold ventilated between pipetting steps to maintain uniform conditions for each sample.
- All centrifugation steps in the protocol are performed in a Centrifuge 4–16K (see page 14).
- All steps of the miRNeasy 96 Tissue/Cell Advanced protocol should be performed at room temperature. Avoid interruptions during the procedure.

Things to do before starting

- Buffer RWT and Buffer 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.
- Check that all the buffers are at room temperature.

Procedure

1. **Preparation of QIAvac 96 vacuum manifold**: Place the waste tray inside the QIAvac base. Place the top plate squarely over the base. Place the RNeasy 96 plate in the QIAvac top plate, making sure that the plate is seated tightly. Attach the QIAvac 96 manifold to the vacuum source. Keep vacuum switched off.

   Note: Always place the RNeasy 96 plate into the vacuum manifold with the beveled edges pointing to the right-hand side.

1. Harvest cells as a cell pellet or, for cells grown in a monolayer, aspirate the cell-culture medium from the cell-culture vessel. Add 300 µL Buffer RLT to either the pellet or the cell-culture vessel, vortex, or pipet to mix and homogenize.
2. Harvest cells according to step 2a or 2b:

   2a. Cells grown in a monolayer:
   Cells grown in a monolayer in multiwell cell-culture plates can be lysed directly in the wells (do not use more than 1 x 10^7 cells per well). Completely remove medium by pipetting, and continue with step 3.

   Note: Incomplete removal of the supernatant will dilute the Buffer RLT. This may lead to reduced RNA yield.

   2b. Cells grown in suspension:
   Transfer aliquots of up to 1 x 10^7 cells into Collection Microtubes (supplied). Spin cells for 5 min at 300 x *g*. Completely remove supernatant by pipetting and continue with step 3.

   Note: Incomplete removal of the supernatant will dilute the Buffer RLT. This may lead to reduced RNA yield.

3. Disrupt the cells by adding Buffer RLT.

   For pelleted cells, loosen the cell pellet thoroughly by flicking the tube. Add300 µL Buffer RLT to each plate well/Collection Microtube. Pipet up and down 3 times.

   If the lysates are in plate wells, transfer to Collection Microtubes (not provided) or another suitable 96 well plate. .

4. Close the Collection Microtubes using Collection Microtubes Caps (both not provided). Vortex for 1 min at maximum speed.

   Note: Vortexing for 1 min is sufficient for homogenization of up to 3 x 10^6 cells. If the cell number is greater than 3 x 10^6, the TissueLyser II should be used to homogenize cells (as described in the protocols for animal tissue).

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

5. Centrifuge at 6000 x *g* for 1 min to collect residual liquid from the caps of the tubes.
6. Add 80 µL Buffer AL to each sample and mix by pipetting. Incubate at room temperature for 3 min.
7. Put the gDNA Eliminator 96 plate on top of a new S-Block (provided), transfer the lysate to the gDNA Eliminator 96 plate.

   Note: Take care not to wet the rims of the wells, as this could lead to cross contamination.

8. Seal the gDNA Eliminator 96 plate with an AirPore tape sheet. Place the S-Block and gDNA Eliminator 96 plate into the metal holder, and place the whole assembly into the rotor bucket. Centrifuge at 6000 rpm (~5600 x *g*) for 4 min at 20–25°C. Discard the gDNA Eliminator 96 plate, and save the flow-through. Centrifugation with sealed plates prevents cross-contamination.

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

9. Add 75 µL RNase-free Water and 25 µL ProtK, to the flow through in the S-Block, mix and incubate for 10 min at room temperature.
10. **Assemble the QIAvac 96 vacuum manifold**: First, place the waste tray inside the QIAvac base, then place the QIAvac 96 top plate squarely over the QIAvac base. Place an RNeasy 96 plate in the QIAvac 96 top plate, making sure that the plate is seated tightly. Attach the vacuum manifold to a vacuum source. Keep the vacuum switched off.

    Note: Always place the RNeasy 96 plate into the vacuum manifold with the beveled edges pointing to your right-hand side.

11. Add 1 volume (approximately 480 µL) of 100% isopropanol to each well of the S-Block containing the flow-through from step. Mix well by pipetting up and down 3 times.
12. Transfer the samples (600 µL) to the wells of the RNeasy 96 plate, and switch on the vacuum. Apply the vacuum until the samples have completely passed through the membranes (15–60 s). Switch off the vacuum, and ventilate the manifold.

    Note: Make sure the QIAvac 96 vacuum manifold is assembled correctly before loading the samples. The flow-through is collected in the waste tray.

    Note: Take care not to wet the rims of the wells, as this could lead to cross-contamination.

    Note: Tape unused wells with adhesive tape or Tape Pads (cat. no. 19570). Do not use the AirPore tape sheets supplied with the RNeasy Plus 96 Kit.

    Note: The vacuum must be switched off and the manifold ventilated between pipetting steps to maintain uniform conditions for each sample.
13. Add 800 µL Buffer RWT to each well of the RNeasy 96 plate, and switch on the vacuum. Apply the vacuum until the buffer has completely passed through the membranes (10–30 s). Switch off the vacuum, and ventilate the manifold. The flow-through is collected in the same waste tray from step 12.
14. Lift the QIAvac 96 top plate carrying the RNeasy 96 plate from the QIAvac base, and empty the waste tray*. Reassemble the QIAvac 96 vacuum manifold.
15. Add 800 µL Buffer RPE to each well of the RNeasy 96 plate, and switch on the vacuum. Apply the vacuum until the buffer has completely passed through the membranes (10–30 s). Switch off the vacuum, and ventilate the manifold.

    Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see "Things to do before starting").
16. Place the RNeasy 96 plate on top of an S-Block (either new or reused). Mark the plate for later identification. If reusing an S-Block, make sure it is cleaned as described on page 24.
17. Add 800 µL Buffer RPE to each well of the RNeasy 96 plate, and seal the plate with an AirPore tape sheet. Place the S-Block and RNeasy 96 plate into the metal holder, and

place the whole assembly into the rotor bucket. Centrifuge at 6000 rpm (~5600 x *g*) for 10 min at 20–25°C to dry the membranes.

Note: Centrifugation with sealed plates prevents cross-contamination. It is important to dry the RNeasy membranes, since residual ethanol may interfere with downstream reactions. The 10 min centrifugation ensures that residual traces of salt are removed and that no ethanol is carried over during RNA elution.

18. Remove the AirPore tape sheet. Place the RNeasy 96 plate on top of a rack of Elution Microtubes CL. Add 70–100 µL RNase-free water to each well, and seal the plate with a new AirPore tape sheet. Incubate for 1 min at room temperature. Then centrifuge at 6000 rpm (~5600 x *g*) for 4 min at 20–25°C to elute the RNA.

    Note: Be sure to pipette the RNase-free water directly onto the RNeasy membranes. Elution will be incomplete if some of the water sticks to the walls or the O-rings of the RNeasy 96 plate

    Note: The eluate volume will be approximately 15 µL less than the volume of RNase-free water added to the membrane (the 15 µL corresponds to the membrane dead volume). Use the caps provided. Use elution microtube caps provided to seal the microtubes for storage. Store RNA at –15 to –30°C or at –70°C.

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

Important points before starting

- Buffers RLT and RWT contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach.
- Add ethanol (96–100%) to Buffer RWT and Buffer RPE as indicated on the bottle label.
- Equilibrate buffers to room temperature.
- All centrifugation steps in the protocol are performed in a Centrifuge 4–16K.
- All steps should be performed at room temperature and at a fast pace.
- If purifying RNA from cell lines rich in RNases or from tissue, we recommend adding either β-mercaptoethanol (β-ME) or 2 M dithiothreitol (DTT) to Buffer RLT before use (10 µL β-ME or 20 µL DTT per 1 mL Buffer RLT). Buffer RLT containing DTT or β-ME can be stored at room temperature for up to 1 month.
- If using the miRNeasy 96 Tissue/Cell Advanced Kit for the first time, read "Important Notes" (page 17).
- It is important not to overload the RNeasy plate, as overloading will significantly reduce RNA yield and quality and may cause clogging of the plate. Read "Determining the amount of starting material" (page 17).
- If working with RNA for the first time, read Appendix C (page 58).
- Buffer RWT may form a precipitate upon storage. If necessary, redissolve by warming and then place at room temperature.
- Use of a multichannel pipette is recommended (see page 11). Pour buffers and RNase-free water into reagent reservoirs for multichannel pipettes. Use reservoirs from a freshly opened package.
- All centrifugation steps in the protocol are performed in a Centrifuge 4–16K (see page 14).

- All centrifugation steps of the miRNeasy 96 Tissue/Cell Advanced protocol should be performed at room temperature. Avoid interruptions during the procedure.
- Flash-frozen or RNAprotect-Tissue-stabilized tissue can be used. To freeze tissue for long-term storage, flash-freeze in liquid nitrogen and immediately transfer to –70°C. Tissue can be stored for several months at –70°C. To process, do not allow tissue to thaw during weighing or handling prior to disruption in Buffer RLT. Homogenized tissue lysates can also be stored at –70°C for several months. To process frozen homogenized lysates, thaw samples at room temperature or at 37°C in a water bath until they are completely thawed and salts in the Buffer RLT are dissolved. Avoid extended treatment at 37°C, which can cause chemical degradation of the RNA.

Things to do before starting

- Buffer RWT and Buffer 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.
- Ensure that all the buffers are at room temperature.

Procedure

1. Place 5 mm stainless steel beads into the Collection Microtubes (1 bead per tube), and transfer the Collection Microtube rack (not provided) to a box with dry ice.

   Note: We recommend using the TissueLyser II for disruption and homogenization of up to 192 samples in parallel. Alternatively, a rotor–stator homogenizer or a different disruption device can be used for tissue disruption and homogenization. This protocol describes RNA purification from flash-frozen tissue using the TissueLyser II. For RNAprotect-Tissue-stabilized tissue, cooling the Collection Microtube rack on dry ice is not necessary.
2. Excise the tissue sample from the animal or remove it from storage. Do not allow unstabilized tissue to thaw before it is placed in Buffer RLT.

3. Determine the amount of tissue. Do not use more than 30 mg flash-frozen tissue, 15 mg liver, thymus, spleen or RNAprotect-Tissue-stabilized tissue. Transfer it immediately to a cooled Collection Microtube. Repeat this until all required pieces of tissues are placed in the Collection Microtubes. See page 17 for guidelines to determine the amount of starting material.

   Note: RNA in unstabilized tissues is not protected after harvesting until the sample is flash-frozen or disrupted and homogenized in protocol steps 6 and 7. Frozen animal tissue should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible.

4. Remove the Collection Microtube rack from the dry ice and immediately pipet 300 µL Buffer RLT into each collection microtube.
5. Close the Collection Microtube rack using the supplied strips of collection microtube caps and homogenize on the TissueLyser II for 5 min at 25 Hz.
6. Rotate the TissueLyser rack to allow even homogenization and homogenize for another 5 min at 25 Hz. Some exceptionally tough tissues (e.g., pig skin) may not be completely homogenized after 2 x 5 min. This does not affect the protocol, however, since undisrupted pieces of tissue are removed after phase separation.

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

7. Centrifuge at 6000 x *g* for 1 min to collect residual liquid from the caps of the tubes.
8. Add 80 µL Buffer AL to each sample and mix by pipetting. Incubate at room temperature for 3 min.
9. Put the gDNA Eliminator 96 plate on top of a new S-Block (provided), transfer the lysate to the gDNA Eliminator 96 plate.

   Note: Take care not to wet the rims of the wells, as this could lead to cross contamination

10. Seal the gDNA Eliminator 96 plate with an AirPore tape sheet. Place the S-Block and gDNA Eliminator 96 plate into the metal holder, and place the whole assembly into the rotor bucket. Centrifuge at 6000 rpm (~5600 x *g*) for 4 min at 20–25°C. Discard the gDNA Eliminator 96 plate, and save the flow-through. Centrifugation with sealed plates prevents cross-contamination.

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

11. Add 75 µL RNase-free Water and 25 µL ProtK, to the flow through in the S-Block, mix and incubate for 10 min at room temperature.
12. Place an RNeasy 96 plate on top of a square-well block (either new or reused). Mark the plate for later identification. If reusing an S-Block, make sure it is cleaned as described on page 24.
13. Add 1 volume (approximately 480 µL) of 100% isopropanol to each well of the S-Block containing the flow-through from step. Mix well by pipetting up and down 3 times.

    Note: Take care not to wet the rims of the wells, as this could lead to cross contamination.
14. Seal the RNeasy 96 plate with an AirPore tape sheet. Place the S-Block and RNeasy 96 plate into the metal holder, and place the whole assembly into the rotor bucket. Centrifuge at 6000 rpm (~5600 x *g*) for 4 min at 20–25°C. Centrifugation with sealed plates prevents cross-contamination.15. Empty the S-Block* and remove the AirPore tape sheet. Add 800 µL Buffer RWT to each well of the RNeasy 96 plate, and seal the plate with a new AirPore tape sheet. Centrifuge at 6000 rpm (~5600 x *g*) for 4 min at 20–25°C.
15. Empty the S-Block* and remove the AirPore tape sheet. Add 800 µL Buffer RPE to each well of the RNeasy 96 plate, and seal the plate with a new AirPore tape sheet. Centrifuge at 6000 rpm (~5600 x *g*) for 4 min at 20–25°C.

    Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see "Things to do before starting")
16. Empty the S-Block and remove the AirPore tape sheet. Add 800 µL Buffer RPE to each well of the RNeasy 96 plate, and seal the plate with a new AirPore tape sheet. Centrifuge at 6000 rpm (~5600 x *g*) for 10 min at 20–25°C to dry the membranes. It is important to dry the RNeasy membranes, since residual ethanol may interfere with

* S-Block can be reused. See page 26 for its cleaning instructions.

downstream reactions. The 10 min centrifugation ensures that residual traces of salt are removed and that no ethanol is carried over during RNA elution.

17. Remove the AirPore tape sheet. Place the RNeasy 96 plate on top of a rack of Elution Microtubes CL. Add 70–100 µL RNase-free water to each well, and seal the plate with a new AirPore tape sheet. Incubate for 1 min at room temperature. Then centrifuge at 6000 rpm (~5600 x *g*) for 4 min at 20–25°C to elute the RNA.

    Note: Be sure to pipet the RNase-free water directly onto the RNeasy membranes. Elution will be incomplete if some of the water sticks to the walls or the O-rings of the RNeasy 96 plate.

    Note: The eluate volume will be approximately 15 µL less than the volume of RNase-free water added to the membrane (the 15 µL corresponds to the membrane dead volume). Use the caps provided with the kit to seal the microtubes for storage. Store RNA at –20°C or at –70°C

Protocol: Purification of Total RNA, Including Small RNAs, from Animal Tissues using Vacuum/Spin Technology

Important points before starting

- Buffers RLT and RWT contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach.
- Add ethanol (96–100%) to Buffer RWT and Buffer RPE as indicated on the bottle label.
- Equilibrate buffers to room temperature.
- All centrifugation steps in the protocol are performed in a Centrifuge 4–16K.
- All steps should be performed at room temperature and at a fast pace.
- If purifying RNA from cell lines rich in RNases or from tissue, we recommend adding either β-mercaptoethanol (β-ME) or 2 M dithiothreitol (DTT) to Buffer RLT before use (10 µL β-ME or 20 µL DTT per 1 mL Buffer RLT). Buffer RLT containing DTT or β-ME can be stored at room temperature for up to 1 month.
- If using the miRNeasy 96 Tissue/Cell Advanced Kit for the first time, read "Important Notes" (page 17).
- Do not to overload the RNeasy plate; overloading will significantly reduce RNA yield and quality and may cause clogging of the plate. Read "Determining the amount of starting material" (page 17).
- If working with RNA for the first time, read Appendix C (page 58).
- Buffer RWT may form a precipitate upon storage. If necessary, redissolve by warming and then place at room temperature.
- Use of a multichannel pipette is recommended (see page 11). Pour buffers and RNase-free water into reagent reservoirs for multichannel pipets. Use reservoirs from a freshly opened package.
- A vacuum source capable of generating a vacuum pressure of –800 to –900 mbar is necessary (see pages 15–18). The vacuum must be switched off and the

manifold ventilated between pipetting steps to maintain uniform conditions for each sample.

- All centrifugation steps in the protocol are performed in a Centrifuge 4–16K (see page 14).
- All centrifugation steps of the miRNeasy 96 Tissue/Cell Advanced protocol should be performed at room temperature. Avoid interruptions during the procedure.
- Flash-frozen or RNAprotect-Tissue-stabilized tissue can be used. To freeze tissue for long-term storage, flash-freeze in liquid nitrogen and immediately transfer to –70°C. Tissue can be stored for several months at –70°C. To process, do not allow tissue to thaw during weighing or handling prior to disruption in Buffer RLT. Homogenized tissue lysates can also be stored at –70°C for several months. To process frozen homogenized lysates, thaw samples at room temperature or at 37°C in a water bath until they are completely thawed and salts in the Buffer RLT are dissolved. Avoid extended treatment at 37°C, which can cause chemical degradation of the RNA.

Things to do before starting

- Buffer RWT and Buffer 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.
- Check that all the buffers are at room temperature.

Procedure

1. Place 5 mm stainless steel beads into the collection microtubes (1 bead per tube), and transfer the Collection Microtube rack (cat. no. 19560 not provided) to a box with dry ice.

   Note: We recommend using the TissueLyser II for disruption and homogenization of up to 192 samples in parallel. Alternatively, a rotor–stator homogenizer or a different disruption device can be used for tissue disruption and homogenization. This protocol

describes RNA purification from flash-frozen tissue using the TissueLyser II. For RNAprotect-Tissue-stabilized tissue, cooling the Collection Microtube rack on dry ice is not necessary.

2. Excise the tissue sample from the animal or remove it from storage. Do not allow unstabilized tissue to thaw before it is placed in Buffer RLT.
3. Determine the amount of tissue. Do not use more than 30 mg flash-frozen tissue, 15 mg liver, thymus, spleen or RNAprotect-Tissue-stabilized tissue. Transfer it immediately to a cooled Collection Microtube. Repeat this until all required pieces of tissues are placed in the collection microtubes. See page 17 for guidelines to determine the amount of starting material.

   Note: RNA in unstable tissues are not protected after harvesting until the sample is flash-frozen or disrupted and homogenized in protocol steps 6 and 7. Frozen animal tissue should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible.
4. Remove the Collection Microtube rack from the dry ice and immediately pipet 300 µL Buffer RLT into each Collection Microtube.
5. Close the Collection Microtube rack using the supplied strips of Collection Microtube Caps (cat. no. 19566) and homogenize on the TissueLyser II for 5 min at 25 Hz.
6. Rotate the TissueLyser rack to allow even homogenization and homogenize for another 5 min at 25 Hz. Some exceptionally tough tissues (e.g., pig skin) may not be completely homogenized after 2 x 5 min. This does not affect the protocol, however, since undisrupted pieces of tissue are removed after phase separation.

   Note: Homogenized tissue lysates can be stored at –70°C for several months.
7. Centrifuge at 6000 x *g* for 1 min to collect residual liquid from the caps of the tubes.
8. Add 80 µL Buffer AL to each sample and mix by pipetting. Incubate at room temperature for 3 min.
9. Put the gDNA Eliminator 96 plate on top of a new S-Block (provided), transfer the lysate to the gDNA Eliminator 96 plate.

Note: Take care not to wet the rims of the wells, as this could lead to cross contamination.

10. Seal the gDNA Eliminator 96 plate with an AirPore tape sheet. Place the S-Block and gDNA Eliminator 96 plate into the metal holder, and place the whole assembly into the rotor bucket. Centrifuge at 6000 rpm (~5600 x *g*) for 4 min at 20–25°C. Discard the gDNA Eliminator 96 plate, and save the flow-through. Centrifugation with sealed plates prevents cross-contamination.

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

11. Add 75 µL RNase-free Water and 25 µL ProtK, to the flow through in the S-Block, mix and incubate for 10 min at room temperature.

12. **Assemble the QIAvac 96 vacuum manifold**: First, place the waste tray inside the QIAvac base, then place the QIAvac 96 top plate squarely over the QIAvac base. Place an RNeasy 96 plate in the QIAvac 96 top plate, making sure that the plate is seated tightly. Attach the vacuum manifold to a vacuum source. Keep the vacuum switched off.

    Note: Always place the RNeasy 96 plate into the vacuum manifold with the beveled edges pointing to your right-hand side.

13. Add 1 volume (approximately 480 µL) of 100% isopropanol to each well of the S-Block containing the lysate from step 11. Mix well by pipetting up and down 3 times.

14. Transfer the samples (600 µL) to the wells of the RNeasy 96 plate, and switch on the vacuum. Apply the vacuum until the samples have completely passed through the membranes (15–60 s). Switch off the vacuum, and ventilate the manifold.

15. Make sure the QIAvac 96 vacuum manifold is assembled correctly before loading the samples. The flow-through is collected in the waste tray.

    Note: Take care not to wet the rims of the wells, as this could lead to cross-contamination.

    Note: Tape unused wells with adhesive tape or Tape Pads (5) (cat. no. 19570). Do not use the AirPore tape sheets supplied with the RNeasy Plus 96 Kit.

Note: The vacuum must be switched off and the manifold ventilated between pipetting steps to maintain uniform conditions for each sample.

16. Add 800 µL Buffer RWT to each well of the RNeasy 96 plate, and switch on the vacuum. Apply the vacuum until the buffer has completely passed through the membranes (10–30 s). Switch off the vacuum, and ventilate the manifold. The flow-through is collected in the same waste tray from step 15.
17. Lift the QIAvac 96 top plate carrying the RNeasy 96 plate from the QIAvac base, and empty the waste tray*. Reassemble the QIAvac 96 vacuum manifold.
18. Add 800 µL Buffer RPE to each well of the RNeasy 96 plate, and switch on the vacuum. Apply the vacuum until the buffer has completely passed through the membranes (10–30 s). Switch off the vacuum, and ventilate the manifold.

    Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see "Things to do before starting").
19. Place the RNeasy 96 plate on top of an S-Block (either new or reused). Mark the plate for later identification. If reusing an S-Block, make sure it is cleaned as described on page 24.
20. Add 800 µL Buffer RPE to each well of the RNeasy 96 plate, and seal the plate with an AirPore tape sheet. Place the S-Block and RNeasy 96 plate into the metal holder, and place the whole assembly into the rotor bucket. Centrifuge at 6000 rpm (~5600 x *g*) for 10 min at 20–25°C to dry the membranes.
21. Centrifugation with sealed plates prevents cross-contamination. It is important to dry the RNeasy membranes, since residual ethanol may interfere with downstream reactions. The 10 min centrifugation ensures that residual traces of salt are removed and that no ethanol is carried over during RNA elution.
22. Remove the AirPore tape sheet. Place the RNeasy 96 plate on top of a rack of Elution Microtubes CL. Add 70–100 µL RNase-free water to each well, and seal the plate with a new AirPore tape sheet. Incubate for 1 min at room temperature. Then centrifuge at 6000 rpm (~5600 x *g*) for 4 min at 20–25°C to elute the RNA.

Note: Be sure to pipet the RNase-free water directly onto the RNeasy membranes. Elution will be incomplete if some of the water sticks to the walls or the O-rings of the RNeasy 96 plate

23. The eluate volume will be approximately 15 µL less than the volume of RNase-free water added to the membrane (the 15 µL corresponds to the membrane dead volume). Use the caps provided. Use elution microtube caps provided to seal the microtubes for storage. Store RNA at –15 to –30°C or at –70°C.

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
Clogged plate wells	
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 17).
b) Inefficient disruption and/or homogenization	See "Disrupting and homogenizing starting materials" (pages 21–22) 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 17) and/or increase the homogenization time.
c) Centrifugation temperature too low	Except for the preliminary centrifugation step and the phase separation step, all centrifugation steps should be performed at room temperature. 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 96 plate. If this happens, set the centrifugation temperature to 25°C. Warm the ethanol-containing lysate to 37°C before transferring to the RNeasy 96 plate.
Little or no RNA eluted	
a) Too much starting material	Overloading significantly reduces yield. Reduce the amount of starting material (see page 17). Make sure to use the ethanol concentrations specified in the protocol steps.
b) Inefficient disruption and/or homogenization	In subsequent preparations, reduce the amount of starting material (see "Determining the amount of starting material", page 14) and/or increase the volume of lysis buffer and the homogenization time
c) Buffer temperatures too low	All buffers must be at room temperature throughout the procedure.
d) RNA still bound to the membrane	Repeat elution, but incubate the RNeasy 96 plate on the benchtop for 10 min with RNase-free water before centrifuging.

		Comments and suggestions
e)	ethanol carryover	During the second wash with Buffer RPE, be sure to centrifuge at 6000 rpm (~5600 x *g*) for 10 min at 20–25°C to dry the membranes of the RNeasy 96 plate
f)	Incomplete removal of cell-culture medium	Ensure complete removal of cell-culture medium after harvesting cells (see protocols, pages 17 and 21).
Low miRNA yield or poor performance of miRNA in downstream experiments		
a)	Incorrect isopropanol concentration	Be sure to use the isopropanol concentrations specified in the protocol steps.
Low A_{260}/A_{280} value		
a)	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 C, page 58).
RNA degraded		
a)	Inappropriate handling of starting material	Ensure that tissues have been properly handled and that the protocol has been performed without interruptions, especially the initial steps involving tissue lysis and homogenization. Some tissues (e.g., pancreas or intestine) contain high amounts of RNases. Care must be taken to excise these tissues from animals as fast as possible and to stabilize them either by freezing in liquid nitrogen or by immersing them in RNAprotect Tissue Reagent† immediately after excision.
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 55).
DNA contamination in downstream experiments		
a)	Too much starting material	For some cell types, the efficiency of DNA removal by the gDNA Eliminator 96 plate may be reduced when processing larger amounts. If the eluted RNA contains substantial DNA contamination, try processing smaller samples.
b)	Incomplete removal of cell-culture medium cell-culture medium or stabilization reagent	Be sure to remove any excess cell-culture medium or stabilization reagent to prevent significant dilution of the lysis buffer. The gDNA Eliminator 96 plate will not remove DNA effectively if the lysis buffer is diluted.
RNA does not perform well in downstream experiments		
a)	Salt carryover during elution	Ensure that Buffer RPE is at room temperature.

* 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.

† See the *RNAprotect Tissue Reagent Handbook* for more information about RNAprotect Tissue Reagent.

	Comments and suggestions
b) Ethanol carryover	For spin protocols only: during the second Buffer RPE wash, be sure to dry the plate-well membranes by centrifugation at 6000 rpm (approximately 5600 x *g*) for 10 min at room temperature.
c) Vacuum/spin protocol: Vacuum pressure too low	A vacuum source capable of generating a vacuum pressure of –800 to –900 mbar is necessary to achieve efficient RNA binding and washing.
Low well-to-well reproducibility	
a) Elution volume too low	Use elution volumes of 2 x 50 µL or 2 x 75 µL to improve well-to-well reproducibility.
b) Vacuum pressure too low	A vacuum source capable of generating a vacuum pressure of –800 to –900 mbar is necessary to achieve efficient RNA binding and washing.
c) Incomplete homogenization	Some types of tissues are more difficult to homogenize, resulting in greater variability from sample to sample.
d) Variability between samples	RNA yields from tissue samples can vary more than cultured cells due to the heterogeneous nature of most tissues and donor-to-donor variability.

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 this protocol, an additional RNeasy 96 plate is required. This will reduce the number of possible preparations from one miRNeasy 96 Tissue/Cell Advanced Kit from 4 x 96 to 2 x 96. As an economical alternative, we recommend purchase of an RNeasy 96 Kit (for Ordering Information, see page 62).

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 all protocol steps until after Proteinase K digest. Instead of continuing with the next step of the protocol, follow steps 1–15 below to isolate the miRNA-enriched fraction only or steps 1–22 to isolate separate fractions of small RNA and total RNA >200 nt.

Note: If the vacuum/spin protocol is used, centrifugation in steps 8–12 and 17–19 can be replaced by use of the vacuum. In this case, plates should be dried by centrifuging at 6000 rpm for 10 min prior to elution to avoid carryover of trace amounts of ethanol.

1. Transfer the lysate after the proteinase K digest to a new S-Block. Add 1 volume of 70% ethanol (usually 350 µL) and mix by pipetting up and down. Do not centrifuge. Proceed immediately to step 2.
2. Place the RNeasy 96 plate on top of a new S-Block.
3. Pipet the samples (approx. 700 µL), including any precipitates that may have formed, into the wells of the RNeasy 96 plate.
4. Seal the RNeasy 96 plate with an AirPore Tape Sheet. Load the S-Block and RNeasy 96 plate into the holder and place the whole assembly in the rotor bucket. Centrifuge at 6000 rpm (approximately 5600 x *g*) for 4 min at room temperature.
5. Keep the S-Block with the flow-through for later purification of the miRNA-enriched fraction.
6. If purifying the miRNA-enriched fraction only, discard the RNeasy 96 plate and follow steps 7–15 only.

   If purifying both miRNA-enriched fraction and larger RNAs (>200 nt), save the RNeasy 96 plate for use in step 16 (the plate can be stored at 4°C or at room temperature, but not for long periods). Follow steps 7–15 to purify miRNA and then steps 16–22 to purify large RNAs.

Purifying the miRNA-enriched fraction

7. Add 0.65 volumes of 100% ethanol (usually 450 µL) to the S-Block containing the flow-through from step 4 and mix by pipetting up and down. Do not centrifuge. Proceed immediately to step 8.
8. Place a new RNeasy 96 plate on top of an S-Block.

9. Pipet 900 µL of each sample into the wells of the new RNeasy 96 plate. Seal the RNeasy 96 plate with an AirPore Tape Sheet. Load the S-Block and RNeasy 96 plate into the holder, and place the whole assembly in the rotor bucket. Centrifuge at 6000 rpm (approximately 5600 x g) for 4 min at room temperature (15–25°C).
10. Empty the S-Block*, remove the AirPore Tape Sheet, and repeat step 8 with the remaining sample.

    Optional: Empty the S-Block* and remove the AirPore Tape Sheet. Add 800 µL Buffer RWT to each well of the RNeasy 96 plate. Seal the RNeasy 96 plate with a new AirPore Tape Sheet. Centrifuge at 6000 rpm (approximately 5600 x g) for 4 min at room temperature.

    Important: Do not perform this step if you are purifying both the miRNA-enriched fraction and larger RNAs (>200 nt).
11. Empty the S-Block* and remove the AirPore Tape Sheet. Add 800 µL Buffer RPE to each well of the RNeasy 96 plate. Seal the RNeasy 96 plate with a new AirPore Tape Sheet. Centrifuge at 6000 rpm (approximately 5600 x *g*) for 4 min at room temperature.
12. Empty the S-Block and remove the AirPore Tape Sheet. Add another 800 µL Buffer RPE to each well of the RNeasy 96 plate. Seal the RNeasy 96 plate with a new AirPore Tape Sheet. Centrifuge at 6000 rpm (approximately 5600 x *g*) for 10 min at room temperature.

    Important: It is important to dry the RNeasy membrane since residual ethanol may interfere with subsequent reactions. The 10 min spin ensures that residual traces of salt are removed and that no ethanol is carried over during elution.
13. Remove the AirPore Tape Sheet. Place the RNeasy 96 plate on top of a clean elution microtube rack containing elution microtubes.
14. To elute the small RNA fraction, add 45–70 µL of RNase-free water to each well and seal the RNeasy 96 plate with a new AirPore Tape Sheet. Incubate for 1 min at room

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

temperature. Then centrifuge at 6000 rpm (approximately 5600 x *g*) for 4 min at room temperature.

15. Remove the AirPore Tape Sheet. Repeat the elution step (step 14) with a second volume of 45–70 µL RNase-free water.

    Note: Repeating the elution step is required for complete recovery of RNA.

Purifying total RNA (>200 nt)

16. Place the RNeasy 96 plate from step 5 on top of an S-Block.
17. Add 800 µL Buffer RWT to each well of the RNeasy 96 plate. Seal the RNeasy 96 plate with a new AirPore Tape Sheet. Centrifuge at 6000 rpm (approximately 5600 x *g*) for 4 min at room temperature.
18. Empty the S-Block* and remove the AirPore Tape Sheet. Add 800 µL Buffer RPE to each well of the RNeasy 96 plate. Seal the RNeasy 96 plate with a new AirPore Tape Sheet. Centrifuge at 6000 rpm (approximately 5600 x *g*) for 4 min at room temperature.
19. Empty the S-Block and remove the AirPore Tape Sheet. Add another 800 µL Buffer RPE to each well of the RNeasy 96 plate. Seal the RNeasy 96 plate with a new AirPore Tape Sheet. Centrifuge at 6000 rpm (approximately 5600 x *g*) for 10 min at room temperature.

    Important: It is important to dry the RNeasy membrane since residual ethanol may interfere with subsequent reactions. The 10 min spin ensures that residual traces of salt are removed and that no ethanol is carried over during elution.
20. Remove the AirPore Tape Sheet. Place the RNeasy 96 plate on top of a clean elution microtube rack containing elution microtubes.
21. To elute the RNA, add 45–70 µL of RNase-free water to each well and seal the RNeasy 96 plate with a new AirPore Tape Sheet. Incubate for 1 min at room temperature. Then centrifuge at 6000 rpm (approximately 5600 x *g*) for 4 min at room temperature.

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

22. Remove the AirPore Tape Sheet. Repeat the elution step with a second volume of 45–70 µL RNase-free water.

    Note: Repeating the elution step is required for complete recovery of RNA.

Appendix B: General Remarks on Handling RNA

Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to destroy RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Great care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the purification procedure. To create and maintain an RNase-free environment, the following precautions must be taken during pretreatment and use of disposable and non-disposable 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, non-disposable plasticware, and laboratory equipment (e.g., pipettes 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 56), or rinse with chloroform* if the plasticware is chloroform-resistant. To decontaminate electrophoresis tanks, clean with detergent (e.g., 0.5% SDS),* rinse with RNase-free water, then rinse with ethanol (if the tanks are ethanol resistant) and allow to dry.

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

Disposable plasticware

The use of sterile, disposable polypropylene tubes is recommended throughout the procedure. These tubes are generally RNase-free and do not require pretreatment to inactivate RNases*. When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier. Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent*, thoroughly rinsed and oven baked at 240°C for at least 4 hours (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware can be treated with diethyl pyrocarbonate (DEPC)*, as described in "Solutions" below.

Solutions

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

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

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

Storage of RNA

Purified RNA may be stored at –70°C to –15°C in RNase-free water. Under these conditions, no degradation of RNA is detectable after 1 year.

Quantification of RNA

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

Using a standard spectrophotometer

To ensure significance, A_{260} readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 44 µg of RNA per mL (A_{260} = 1 → 4 µg/mL). This relation is valid only for measurements at a neutral pH. Therefore, if it is necessary to dilute the RNA sample, this should be done in a buffer with neutral pH*. As discussed below, the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity. When measuring RNA samples, be certain that cuvettes are RNase-free, especially if the RNA is to be recovered after spectrophotometry. This can be accomplished by washing cuvettes with 0.1 M NaOH, 1 mM EDTA,* followed by washing with RNase-free water (see "Solutions", page 56). Use

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the buffer in which the RNA is diluted to zero the spectrophotometer. An example of the calculation involved in RNA quantification is shown below:

Volume of RNA sample	= 100 µL
Dilution	= 10 µL of RNA sample + 490 µL of 10 mM Tris·Cl,* pH 7.0 (1/50 dilution)

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

A_{260}	= 0.2
Concentration of RNA sample	= 44 µg/mL x A_{260} x dilution factor
	= 44 µg/mL x 0.2 x 50
	= 440 µg/mL
Total amount	= concentration x volume in milliliters
	= 440 µg/mL x 0.1 mL
	= 44 µg of RNA

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

* 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.

and concentration (A_{260} reading of 1 = 44 µg/mL RNA) is based on an extinction coefficient calculated for RNA at neutral pH.

DNA contamination

No currently available purification method can guarantee that RNA is completely free of DNA, even when it is not visible on an agarose gel. RNeasy Kits will, however, remove the vast majority of cellular DNA. gDNA Eliminator Solution helps to further reduce genomic DNA contamination; however, trace amounts of genomic DNA may still remain, depending on the amount and nature of the sample. For analysis of very low abundance targets, any interference by residual DNA contamination can be detected by performing real-time RT-PCR control experiments in which no reverse transcriptase is added prior to the PCR step.

To prevent any interference by DNA in real-time RT-PCR applications, such as with Applied Biosystems®, Rotor-Gene®, Rotor-Gene Q®, and QIAquant® 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 62).

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

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as sharp bands or peaks. The apparent ratio of 28S rRNA to 18S rRNA should be approximately 2:1. If the ribosomal bands or peaks of a specific sample are not sharp, but appear as a smear towards smaller sized RNAs, it is likely that the sample suffered major degradation either before or during RNA purification. As a useful measure of RNA integrity, the QIAxcel Advanced system and the Agilent 2100 Bioanalyzer provide an RNA integrity score (RIS) and an RNA integrity number (RIN), respectively. Ideally, the value should be close to 10, but in many cases (particularly with tissue samples), RNA quality is greatly influenced by how well the original sample was preserved.

Ordering Information

Product	Contents	Cat. no.
miRNeasy 96 Tissue/Cell Advanced Kit (4)	For 4 x 96 preps: 4 RNeasy 96 plates, Collection Microtubes (racked), Elution Microtubes CL, Caps, S-Blocks, AirPore Tape Sheets, RNase-Free Reagents and Buffers	217661
Related products		
miRNeasy Tissue/Cells Advanced Mini Kit (50)	For 50 preps: RNeasy Mini Spin Columns, gDNA Eliminator Spin Columns, Collection Tubes, RNase-Free Water and Buffers	217604
RNeasy 96 Kit (4)*	For 4 x 96 preps: 4 RNeasy 96 Plates, Elution Microtubes CL, Caps, S-Blocks, AirPore Tape Sheets, RNase-Free Reagents and Buffers	74181
miRNeasy 96 Tissue/Cell Advanced QIAcube HT Kit (5)	For 480 preps: RNeasy 96 plates, RNase-free water, buffers	217261
Accessories		
TissueLyser II	Bead mill; requires the TissueLyser Adapter Set 2 x 24 or TissueLyser Adapter Set 2 x 96 (available separately)	Various
TissueLyser Adapter Set 2 x 96	2 sets of Adapter Plates for use with collection microtubes (racked) on the TissueLyser	69984
Stainless Steel Beads, 5 mm (200)	Stainless Steel Beads, suitable for use with the TissueLyser system	69989
TissueLyser 5 mm Bead Dispenser, 96-Well	For dispensing 96 beads (5 mm diameter) in parallel	69975
Centrifuge 4–16 S	Universal laboratory centrifuge with brushless motor (120 V, 60 Hz)	81510
Centrifuge 4–16 KS	Refrigerated universal laboratory centrifuge with brushless motor (220–240 V, 50/60Hz)	81610
Plate Rotor 2 x 96	Rotor for 2 QIAGEN 96 plates, for use with QIAGEN Centrifuges	81031

Product	Contents	Cat. no.
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
RNAprotect Tissue Tubes (50 x 1.5 mL)	For stabilization of RNA in 50 x 50 mg tissue samples: 50 screw-top tubes containing 1.5 mL RNAprotect Tissue Reagent each	76154
RNAprotect Tissue Tubes (20 x 5 mL)	For stabilization of RNA in 20 x 500 mg tissue samples: 20 screw-top tubes containing 5 mL RNAprotect Tissue Reagent each	76163
QIAvac 96	Vacuum manifold for processing QIAGEN 96-well plates: includes QIAvac 96 Top Plate, Base, Waste Tray, Plate Holder, Rack of Collection Microtubes (1.2 mL)	19504
Vacuum Regulator	For use with QIAvac manifolds	19530
Vacuum Pump	Universal vacuum pump (capacity 34 L/min, 8 mbar vacuum abs.)	Various
RNase-Free DNase Set (50)	1500 units RNase-free DNase I, RNase-Free Buffer RDD and RNase-Free Water for 50 RNA minipreps	79254
QIAzol Lysis Reagent (200 mL)	200 mL QIAzol Lysis Reagent	79306
S-Blocks (24)	96-well blocks with 2.2 mL wells, 24 per case	19585
Elution Microtubes CL (24 x 96)	Nonsterile polypropylene tubes (0.85 mL maximum capacity, less than 0.7 mL storage capacity, 0.4 mL elution capacity); 2304 in racks of 96; includes cap strips	19588
Collection Microtubes (racked)	Nonsterile polypropylene tubes (1.2 mL), 960 in racks of 96	19560
Collection Microtube Caps	Nonsterile polypropylene caps for collection microtubes (1.2 mL) and round-well blocks, 960 in strips of 8	19566
Tape pads (5)	Adhesive tape sheets for sealing multiwell plates and blocks: 25 sheets per pad, 5 pads per pack	19570

Product	Contents	Cat. no.
AirPore Tape Sheets (50)	Microporous tape sheets for covering 96-well blocks: 50 sheets per pack	19571
QIAxcel Advanced system	Operates in conjunction with the QIAxcel DNA Kits and QIAxcel RNA QC Kit v2.0.	9002123
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
miRCURY LNA SYBR® Green PCR Kit (200)	For 200 reactions: 2X miRCURY SYBR Green Master Mix, RNase-Free Water	339345
miRCURY LNA miRNA PCR Assay	Contains forward and reverse primers for 200 reactions	339306
miRCURY LNA miRNA Focus PCR Panels	miRCURY LNA miRNA PCR Panels for application-based miRNome profiling, available in 96-well or 384-well format	339325
miRCURY LNA miRNA miRNome PCR Panels	miRCURY LNA miRNA PCR Panels for PCR-based miRNome profiling, available in 384-well format	339322
QuantiTect Primer Assay (200)	Lyophilized primer mix of forward and reverse primers for SYBR Green based real-time RT-PCR.	Varies†

* Other kit sizes are available; see www.qiagen.com.

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

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.

Notes

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Document Revision History

Date	Changes
02 / 2023	Initial release.

Limited License Agreement for miRNeasy 96 Tissue/Cell Advanced

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