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RNeasy® Plus 96 Handbook

For purification of total RNA from animal and human cells in 96-well format using gDNA Eliminator 96 plates

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Sample & Assay Technologies

QIAGEN Sample and Assay Technologies

QIAGEN is the leading provider of innovative sample and assay technologies, enabling the isolation and detection of contents of any biological sample. Our advanced, high-quality products and services ensure success from sample to result.

QIAGEN sets standards in:

- Purification of DNA, RNA, and proteins
- Nucleic acid and protein assays
- microRNA research and RNAi
- Automation of sample and assay technologies

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Contents

Kit Contents

RNeasy Plus 96 Kit	**(12)**
Catalog no.	**74192**
Number of preps	**12 x 96**
gDNA Eliminator 96 Plates	12
RNeasy 96 Plates	12
Register Cards (96-well)	12
S-Blocks*	12
Elution Microtubes CL	12 x 96
Caps for Elution Microtubes CL	150 x 8
AirPore Tape Sheets	2 x 25
Buffer RLT Plus†	2 x 220 ml
Buffer RW1†	4 x 400 ml
Buffer RPE‡ (concentrate)	8 x 65 ml
RNase-Free Water	12 x 30 ml
Handbook	1

* Reusable; see page 16 for cleaning instructions.

† Contains a guanidine salt. Not compatible with disinfecting reagents containing bleach. See page 6 for safety information.

‡ Before using for the first time, add the appropriate volume of ethanol (96–100%) as indicated on the bottle to obtain a working solution.

The following kit components are also available separately: S-Blocks, Elution Microtubes CL (including caps), and AirPore Tape Sheets. See page 40 for ordering information.

Storage

The RNeasy Plus 96 Kit should be stored dry at room temperature (15–25°C) and is stable for at least 9 months under these conditions.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of RNeasy Plus 96 Kit is tested against predetermined specifications to ensure consistent product quality.

Product Use Limitations

The RNeasy Plus 96 Kit is intended for research use. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

Product Warranty and Satisfaction Guarantee

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

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see back cover or visit www.qiagen.com).

Technical Assistance

At QIAGEN, we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of QIAGEN products. If you have any questions or experience any difficulties regarding the RNeasy Plus 96 Kit or QIAGEN products in general, please do not hesitate to contact us.

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

For technical assistance and more information, please see our Technical Support Center at www.qiagen.com/Support or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Safety Information

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

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

Buffer RLT Plus contains guanidine thiocyanate, and Buffer RW1 contains a small amount of guanidine thiocyanate. Guanidine salts can form highly reactive compounds when combined with bleach. If liquid containing these buffers is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

Introduction

The RNeasy Plus 96 Kit is designed for high-throughput purification of total RNA from cultured cells in 96-well format, and can purify RNA from up to 2 x 10^6 cells per sample. Genomic DNA contamination is effectively removed using a specially designed gDNA Eliminator 96 plate.

The RNeasy Plus 96 Kit allows the parallel processing of 96 samples in less than 50 minutes. The RNeasy Plus procedure replaces methods involving the use of toxic substances such as phenol and/or chloroform, or time-consuming and tedious methods such as alcohol precipitation.

With the RNeasy Plus procedure, all RNA molecules longer than 200 nucleotides are purified. The procedure provides an enrichment for mRNA, since most RNAs <200 nucleotides (such as 5.8S rRNA, 5S rRNA, and tRNAs, which together comprise 15–20% of total RNA) are selectively excluded.* The purified RNA is ready to use and is ideally suited for downstream applications that are sensitive to low amounts of DNA contamination, such as quantitative, real-time RT-PCR.† The purified RNA can also be used in other applications, including:

- RT-PCR
- Differential display
- cDNA synthesis
- Northern, dot, and slot blot analyses
- Primer extension
- Poly A^+ RNA selection
- RNase/S1 nuclease protection
- Microarrays

Optionally, the RNeasy Plus procedure can be modified to allow the purification of total RNA containing **small RNAs, such as miRNA**, from cultured cells (see Appendices D and E, pages 35 and 38).

Principle and procedure

The RNeasy Plus procedure integrates QIAGEN's patented technology for selective binding of double-stranded DNA with well-established RNeasy technology. Efficient purification of high-quality RNA is guaranteed, without the need for additional DNase digestion.

* For purification of miRNA and other small RNAs from cells and tissues, we recommend using the miRNeasy 96 Kit. For details, visit www.qiagen.com/miRNA .

† Visit www.qiagen.com/geneXpression for information on standardized solutions for gene expression analysis from QIAGEN.

Cells are first lysed and homogenized in a highly denaturing guanidine-isothiocyanate–containing buffer, which immediately inactivates RNases to ensure isolation of intact RNA. The lysates are then passed through a gDNA Eliminator 96 plate. This 96-well plate, in combination with the high-salt buffer, selectively and efficiently removes genomic DNA.

Ethanol is added to the flow-throughs from the gDNA Eliminator 96 plate to provide appropriate binding conditions for RNA, and the samples are then applied to an RNeasy 96 plate. Total RNA binds to the membranes of this 96-well plate, and contaminants are efficiently washed away. High-quality RNA is then eluted in 45–70 µl water.

Description of protocols

The RNeasy Plus 96 procedure is carried out using either a vacuum/spin protocol or a spin protocol. With a vacuum/spin protocol, certain binding and washing steps are performed on the QIAvac 96 vacuum manifold for faster and easier sample processing; all other steps are performed in the Centrifuge 4-15C or Centrifuge 4K15C. With a spin protocol, all steps are performed in the Centrifuge 4-15C or Centrifuge 4K15C, and twice as much starting material can be used compared with the vacuum/spin protocol.

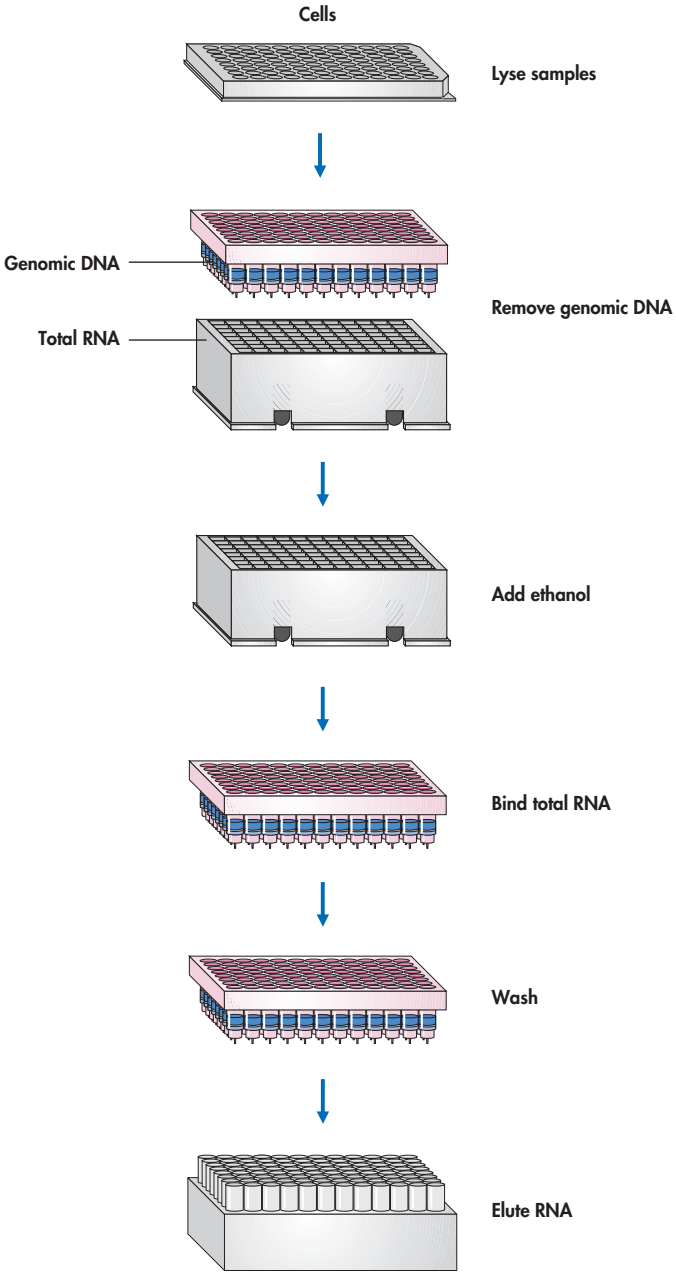
Purification of Total RNA from Cells Using Vacuum/Spin Technology

In this protocol, total RNA is purified from up to **1 x 10^6 cells**. The DNA removal step, the final wash step including membrane drying, and the elution step are performed in the Centrifuge 4-15C or Centrifuge 4K15C (see page 13). All other steps are performed on the QIAvac 96 vacuum manifold (see page 11). Residual traces of salt are removed by centrifugation in the final wash step. The Plate Rotor 2 x 96 holds 2 gDNA Eliminator 96 plates or 2 RNeasy 96 plates, allowing up to 192 cultured-cell samples to be processed in parallel.

Purification of Total RNA from Cells Using Spin Technology

In this protocol, total RNA is purified from up to **2 x 10^6 cells**. All protocol steps are performed in the Centrifuge 4-15C or Centrifuge 4K15C (see page 13). The Plate Rotor 2 x 96 holds 2 gDNA Eliminator 96 plates or 2 RNeasy 96 plates, allowing up to 192 cultured-cell samples to be processed in parallel.

RNeasy Plus 96 Procedure



Equipment and Reagents to Be Supplied by User

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

For all protocols

- Multichannel pipet with tips. For efficient liquid handling, we recommend using an electric multichannel pipet with a minimum capacity of 650 µl per pipet tip. Matrix Technologies Corporation (www.matrixtechcorp.com) provides cordless electronic multichannel pipets with a unique expandable tip-spacing system, allowing transfer of liquid between different types of multiwell plate.*
- Reagent reservoirs for multichannel pipets
- Centrifuge 4-15C or Centrifuge 4K15C†
- Plate Rotor 2 x 96 (cat. no. 81031)
- 96–100% ethanol and 70% ethanol in water‡
- Collection microtubes (cat. no. 19560)
- Collection microtube caps (cat. no. 19566)
- Optional:14.3 M β-mercaptoethanol (β-ME) (commercially available solutions are usually 14.3 M) or, alternatively, 2 M dithiothreitol (DTT) in water
- Optional: Tape Pads (cat. no. 19570) (for sealing unused wells)
- Optional: S-Blocks (cat. no. 19585) (if performing several 96-well preps per day, it may be convenient to have additional S-Blocks)
- Vortexer

For the vacuum/spin protocol

- QIAvac 96 vacuum manifold (cat. no. 19504)
- QIAGEN Vacuum Pump† or other vacuum pump capable of generating a vacuum pressure of –800 to –900 mbar
- QIAGEN Vacuum Regulator (cat. no. 19530) or pressure gauge

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

† For ordering information, please inquire (www.qiagen.com).

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

QIAvac 96 vacuum manifold

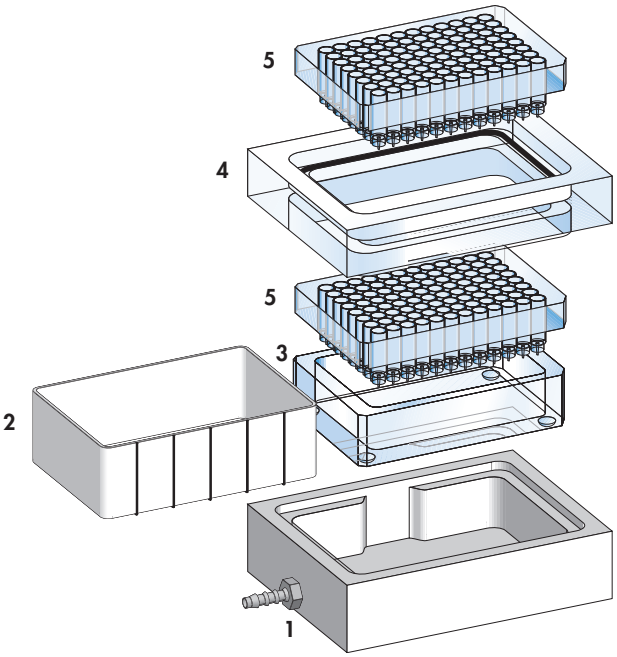


Figure 1. Components of the QIAvac 96 vacuum manifold.
1. QIAvac base, which holds a waste tray, a plate holder, or a microtube rack.
2. Waste tray.
3. Plate holder (shown with 96-well plate) — not used in RNeasy Plus 96 protocol.
4. QIAvac 96 top plate with aperture for 96-well plate.
5. 96-well plate (i.e., gDNA Eliminator 96 plate or RNeasy 96 plate).*

QIAvac 96 handling guidelines

The QIAvac 96 vacuum manifold facilitates vacuum processing of gDNA Eliminator 96 plates and RNeasy 96 plates through its convenient, modular design. The following recommendations should be followed when handling the manifold.

- Operation of the manifold requires 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 liters per minute. Use of insufficient vacuum pressure may reduce nucleic acid yield and purity.

* Not included with QIAvac 96. Included in the RNeasy Plus 96 Kit.

- A vacuum pressure of –800 to –900 mbar should develop when a gDNA Eliminator 96 plate or RNeasy 96 plate sealed with tape is used on the QIAvac 96 vacuum manifold. 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 mbar or 760 mm Hg) and can be regulated and measured using a pressure gauge or vacuum regulator. Vacuum recommendations are given in negative units to indicate the required reduction in pressure with respect to the atmosphere. Table 1 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 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 benchtop 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. After rinsing and drying, 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 at the end of the RNeasy Plus procedure. 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

Centrifuge 4-15C and Centrifuge 4K15C

For optimal centrifugation of gDNA Eliminator 96 plates and RNeasy 96 plates, 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 table-top Centrifuge 4-15C. The plates can also be centrifuged with the Plate Rotor 2 x 96 in the refrigerated table-top Centrifuge 4K15C; cooling of the centrifuge is necessary for certain QIAGEN purification procedures, but must be avoided when carrying out the RNeasy Plus 96 procedure. A wide range of other rotors can be used with the Centrifuge 415C and Centrifuge 4K15C in addition to the Plate Rotor 2 x 96.

Standard table-top centrifuges and 96-well–microplate rotors are not suitable for use with the RNeasy Plus 96 Kit. Usually, 96-well–microplate buckets are not deep enough to carry the complete plate assembly without interfering with how the buckets swing out. Furthermore, high *g*-forces (>5500 x *g*) are required for optimal performance of the RNeasy Plus 96 Kit.

For further information about the Centrifuge 4-15C, the Centrifuge 4K15C, and the Plate Rotor 2 x 96, please contact QIAGEN or your local distributor.

Important: Do not centrifuge the Plate Rotor 2 x 96 metal holders without the complete plate assembly, which can be a gDNA Eliminator 96 plate or RNeasy 96 plate on top of an S-Block or elution microtubes rack. 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.

Important Notes

Determining the amount of starting material

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

- The type of sample and its RNA content
- The volume of Buffer RLT Plus required for efficient lysis
- The DNA removal capacity of the gDNA Eliminator 96 plate
- The RNA binding capacity of the RNeasy 96 plate

Table 2 shows the specifications for the RNeasy 96 plates supplied with the RNeasy Plus 96 Kit. Each well of an RNeasy 96 plate has a maximum binding capacity of 100 µg RNA, but actual yields depend on the sample. Table 3 shows typical RNA yields from various cultured cells.

Table 2. Specifications of the RNeasy 96 plate

Specification	RNeasy 96 plate
Preps per plate	96
Maximum binding capacity	100 µg RNA
Maximum loading volume	1 ml
Nucleic acid size distribution	RNA >200 nucleotides*
Minimum elution volume	45 µl
Maximum amount of starting material using vacuum/spin protocol	Up to 1 x 10^6 cells
Maximum amount of starting material using spin protocol	Up to 2 x 10^6 cells

* Purification of total RNA containing small RNAs from cultured cells is possible through a modification of the RNeasy Plus procedure. For details, see Appendices D and E, pages 35 and 38.

Note: Although the gDNA Eliminator 96 plate can bind a maximum of 100 µg DNA, using starting materials containing more than 20 µg DNA may lead to small amounts of DNA passing through the plate, resulting in DNA contamination of the RNA eluates. When using the RNeasy 96 plate, do not exceed the binding capacity of 100 µg RNA, otherwise RNA yields will not be consistent and will be less than expected.

Note: Do not overload the gDNA Eliminator 96 plate, as this may lead to clogging of the wells and to copurification of DNA with RNA. Do not overload the RNeasy 96 plate, as this will significantly reduce RNA yield and purity.

Table 3. Typical yields of total RNA with the RNeasy Plus 96 Kit

Cell cultures (5 x 10^5 cells)	Typical yield of total RNA* (µg)
NIH/3T3	5
HeLa, Jurkat	8
COS-7	15

* Amounts can vary due to factors such as species, developmental stage, and growth conditions. Since the RNeasy Plus procedure enriches for mRNA and other RNA species >200 nucleotides, the total RNA yield does not include 5.8S rRNA, tRNA, and other low-molecular-weight RNAs, which make up 15–20% of total cellular RNA.

The RNeasy Plus procedure is optimized for use with 100 to 1 x 10^6 cells (vacuum/spin protocol) or 100 to 2 x 10^6 cells (spin protocol). Direct counting is the most accurate way to quantify the number of cells. As a guide, the number of HeLa cells obtained in various cell-culture plates after confluent growth is given in Table 4.

Table 4. Growth area and number of HeLa cells in various multiwell cell-culture plates

Cell-culture plate	Growth area per well (cm^2)†	Number of cells per well‡
96-well	0.32–0.60	4–5 x 10^4
48-well	1.0	1.3 x 10^5
24-well	2.0	2.5 x 10^5
12-well	4.0	5.0 x 10^5
6-well	9.5	1.2 x 10^6

† Growth area varies slightly depending on the supplier.

‡ Confluent growth is assumed.

Handling and storing starting material

After harvesting, cells should be immediately lysed in Buffer RLT Plus to prevent unwanted changes in the gene expression profile. This highly denaturing lysis buffer inactivates RNases and other proteins to prevent RNA degradation as well as downregulation or upregulation of transcripts.

If the cells are to be shipped to another lab for nucleic acid purification, they should be pelleted, frozen in liquid nitrogen, and transported on dry ice.* Alternatively, the cells can be mixed with RNAprotect® Cell Reagent (see page 40 for ordering information) at room temperature and then shipped at ambient temperature.

S-Blocks

The kit contains 12 S-Blocks. If carrying out several 96-well preps per day, it may be convenient to have extra S-Blocks available (cat. no. 19585; case of 24). Fresh S-Blocks must be used to collect the flow-through from the gDNA Eliminator 96 plate (the flow-through contains RNA). After use, S-Blocks may be cleaned and reused to collect waste liquid from RNeasy 96 plates. Do not reuse cleaned S-Blocks to collect the flow-through from gDNA Eliminator 96 plates. To clean S-Blocks, rinse them thoroughly with tap water, incubate for 2 hours or overnight in 0.1 M NaOH, 1 mM EDTA,* rinse in distilled water, and dry at 50°C.

Note: Used S-Blocks contain residual amounts of Buffer RLT Plus or Buffer RW1 and should therefore not be cleaned with bleach. See page 6 for safety information.

* 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 from Cells Using Vacuum/Spin Technology

Important points before starting

- If using the RNeasy Plus 96 Kit for the first time, read "Important Notes" (page 14).
- If preparing RNA for the first time, read Appendix A (page 28).
- All vacuum steps are performed on the QIAvac 96 vacuum manifold. If using the manifold for the first time, read "QIAvac 96 vacuum manifold" (page 11).
- All centrifugation steps are performed in the Centrifuge 4-15C or Centrifuge 4K15C with the Plate Rotor 2 x 96. If using the centrifuge for the first time, read "Centrifuge 4-15C and Centrifuge 4K15C" (page 13).
- Use of a multichannel pipet is recommended (see page 10). Pour buffers and RNase-free water into reagent reservoirs for multichannel pipets. Use reservoirs from a freshly opened package or clean them as described for S-Blocks (see page 16).
- Cell pellets can be stored at –70°C for later use or used directly in the procedure. Frozen cell pellets should be thawed slightly before starting the procedure.
- Cell lysates in Buffer RLT Plus from step 2 can be stored at –70°C for several months. Frozen lysates should be incubated at 37°C in a water bath until completely thawed and salts are dissolved. Avoid prolonged incubation, which may compromise RNA integrity. Mix by pipetting up and down 3 times before continuing with step 3.
- Cells stored in RNAprotect Cell Reagent can also be used in the procedure. Be sure to pellet the cells and carefully remove the supernatant as described in steps 1–3 of the RNA purification protocol in the *RNAprotect Cell Reagent Handbook*. Add 300 µl Buffer RLT Plus to each sample, and transfer the samples to a rack of collection microtubes (cat. no. 19560). Seal the tubes with collection microtube caps (cat. no. 19566), and proceed immediately to step 2.
- Buffer RLT Plus and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 6 for safety information.
- Perform all steps of the procedure at room temperature (15–25°C). During the procedure, work quickly.
- Perform all centrifugation steps at 20–25°C. Ensure that the centrifuge does not cool below 20°C.

Things to do before starting

- If purifying RNA from cell lines rich in RNases, we recommend adding β-mercaptoethanol (β-ME) to Buffer RLT Plus before use. Add 10 µl β-ME per 1 ml Buffer RLT Plus. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT Plus containing β-ME can be stored at room temperature (15–25°C) for up to 1 month. Alternatively, add 20 µl of 2 M dithiothreitol (DTT) per 1 ml Buffer RLT Plus. The stock solution of 2 M DTT in water should be prepared fresh, or frozen in single-use aliquots. Buffer RLT Plus containing DTT can be stored at room temperature for up to 1 month.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add the appropriate volume of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- Buffer RLT Plus may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature.

Procedure

1. Harvest cells (up to 1 x 10^6 cells) according to step 1a or 1b.

1a. Cells grown in a monolayer:
Completely remove the cell-culture medium by pipetting, and add 300 µl Buffer RLT Plus to each well. Transfer the lysates to a rack of collection microtubes (cat. no. 19560), and seal the tubes with collection microtube caps (cat. no. 19566).

As an alternative to collection microtubes, a 96-well plate with caps can be used.

Note: Incomplete removal of cell-culture medium will inhibit lysis and dilute the lysate, affecting the conditions for DNA removal and RNA purification. Both effects may reduce RNA yield and purity.

Note: The volume of Buffer RLT Plus may be reduced to 200 µl if the well volume is too low and there is a risk of cross-contamination during handling.

1b. Cells grown in suspension:
Transfer up to 1 x 10^6 cells from each sample to a rack of collection microtubes (cat. no. 19560). Pellet the cells by centrifuging for 5 min at 300 x *g*. Completely remove all supernatant by pipetting, and add 300 µl Buffer RLT Plus to each tube. Seal the tubes with collection microtube caps (cat. no. 19566).

As an alternative to collection microtubes, a 96-well plate with caps can be used.

Note: Incomplete removal of cell-culture medium will inhibit lysis and dilute the lysate, affecting the conditions for DNA removal and RNA purification. Both effects may reduce RNA yield and purity.

Note: The volume of Buffer RLT Plus may be reduced to 200 µl if the well volume is too low and there is a risk of cross-contamination during handling.

2. **Homogenize the lysates by vortexing the rack of collection microtubes at full speed for at least 30 s.**
3. **Place a gDNA Eliminator 96 plate on top of a new S-Block. Mark the plate for later identification.**
4. **Transfer the lysates from step 2 to the wells of the gDNA Eliminator 96 plate.**

   Note: Take care not to wet the rims of the wells, as this could lead to cross-contamination.
5. **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.
6. **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.
7. **Add 1 volume (300 µl) of 70% ethanol to each well of the S-Block containing the flow-through from step 5. Mix well by pipetting up and down 3 times.**

   Note: Add 200 µl of 70% ethanol if 200 µl Buffer RLT Plus was used in step 1.
8. **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.**

   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.

9. **Add 800 µl Buffer RW1 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 8.

10. **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.**

11. **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").

12. **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 16.

13. **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.**

    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.

14. **Remove the AirPore tape sheet. Place the RNeasy 96 plate on top of a rack of Elution Microtubes CL. Add 45–70 µl RNase-free water to each well, and seal the plate with a new AirPore tape sheet. Incubate for 1 min at room temperature (15–25°C). 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.

15. **Remove the AirPore tape sheet. Repeat step 14 with a second volume of 45–70 µl RNase-free water.**

    Note: Repeating step 14 is required for complete recovery of RNA. 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.

* The waste liquid contains Buffer RLT Plus and Buffer RW1 and is therefore not compatible with bleach. See page 6 for safety information.

Protocol: Purification of Total RNA from Cells Using Spin Technology

Important points before starting

- If using the RNeasy Plus 96 Kit for the first time, read "Important Notes" (page 14).
- If preparing RNA for the first time, read Appendix A (page 28).
- All centrifugation steps are performed in the Centrifuge 4-15C or Centrifuge 4K15C with the Plate Rotor 2 x 96. If using the centrifuge for the first time, read "Centrifuge 4-15C and Centrifuge 4K15C" (page 13).
- Use of a multichannel pipet is recommended (see page 10). Pour buffers and RNase-free water into reagent reservoirs for multichannel pipets. Use reservoirs from a freshly opened package or clean them as described for S-Blocks (see page 16).
- Cell pellets can be stored at –70°C for later use or used directly in the procedure. Frozen cell pellets should be thawed slightly before starting the procedure.
- Cell lysates in Buffer RLT Plus from step 2 can be stored at –70°C for several months. Frozen lysates should be incubated at 37°C in a water bath until completely thawed and salts are dissolved. Avoid prolonged incubation, which may compromise RNA integrity. Mix by pipetting up and down 3 times before continuing with step 3.
- Cells stored in RNAprotect Cell Reagent can also be used in the procedure. Be sure to pellet the cells and carefully remove the supernatant as described in steps 1–3 of the RNA purification protocol in the *RNAprotect Cell Reagent Handbook*. Add 300 µl Buffer RLT Plus to each sample, and transfer the samples to a rack of collection microtubes (cat. no. 19560). Seal the tubes with collection microtube caps (cat. no. 19566), and proceed immediately to step 2.
- Buffer RLT Plus and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 6 for safety information.
- Perform all steps of the procedure at room temperature (15–25°C). During the procedure, work quickly.
- Perform all centrifugation steps at 20–25°C. Ensure that the centrifuge does not cool below 20°C.

Things to do before starting

- If purifying RNA from cell lines rich in RNases, we recommend adding β-mercaptoethanol (β-ME) to Buffer RLT Plus before use. Add 10 µl β-ME per 1 ml Buffer RLT Plus. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT Plus containing β-ME can be stored at room temperature (15–25°C) for up to 1 month. Alternatively, add 20 µl of 2 M dithiothreitol (DTT) per 1 ml Buffer RLT Plus. The stock solution of 2 M DTT in water should be prepared fresh, or frozen in single-use aliquots. Buffer RLT Plus containing DTT can be stored at room temperature for up to 1 month.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add the appropriate volume of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- Buffer RLT Plus may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature.

Procedure

1. Harvest cells (up to 2 x 10^6 cells) according to step 1a or 1b.

1a. Cells grown in a monolayer:
Completely remove the cell-culture medium by pipetting, and add 300 µl Buffer RLT Plus to each well. Transfer the lysates to a rack of collection microtubes (cat. no. 19560), and seal the tubes with collection microtube caps (cat. no. 19566).

As an alternative to collection microtubes, a 96-well plate with caps can be used.

Note: Incomplete removal of cell-culture medium will inhibit lysis and dilute the lysate, affecting the conditions for DNA removal and RNA purification. Both effects may reduce RNA yield and purity.

Note: The volume of Buffer RLT Plus may be reduced to 200 µl if the well volume is too low and there is a risk of cross-contamination during handling.

1b. Cells grown in suspension:
Transfer up to 2 x 10^6 cells from each sample to a rack of collection microtubes (cat. no. 19560). Pellet the cells by centrifuging for 5 min at 300 x *g*. Completely remove all supernatant by pipetting, and add 300 µl Buffer RLT Plus to each tube. Seal the tubes with collection microtube caps (cat. no. 19566).

As an alternative to collection microtubes, a 96-well plate with caps can be used.

Note: Incomplete removal of cell-culture medium will inhibit lysis and dilute the lysate, affecting the conditions for DNA removal and RNA purification. Both effects may reduce RNA yield and purity.

Note: The volume of Buffer RLT Plus may be reduced to 200 µl if the well volume is too low and there is a risk of cross-contamination during handling.

2. **Homogenize the lysates by vortexing the rack of collection microtubes at full speed for at least 30 s.**
3. **Place a gDNA Eliminator 96 plate on top of a new S-Block. Mark the plate for later identification.**
4. **Transfer the lysates from step 2 to the wells of the gDNA Eliminator 96 plate.**

   Note: Take care not to wet the rims of the wells, as this could lead to cross-contamination.
5. **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.
6. **Place an 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 16.
7. **Add 1 volume (300 µl) of 70% ethanol to each well of the S-Block containing the flow-through from step 5. Mix well by pipetting up and down 3 times.**

   Note: Add 200 µl of 70% ethanol if 200 µl Buffer RLT Plus was used in step 1.
8. **Transfer the samples (600 µl) to 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.
9. **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.
10. **Empty the S-Block* and remove the AirPore tape sheet. Add 800 µl Buffer RW1 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.**
11. **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").

* The waste liquid contains Buffer RLT Plus or Buffer RW1 and is therefore not compatible with bleach. See page 6 for safety information.

12. **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.

13. **Remove the AirPore tape sheet. Place the RNeasy 96 plate on top of a rack of Elution Microtubes CL. Add 45–70 µl RNase-free water to each well, and seal the plate with a new AirPore tape sheet. Incubate for 1 min at room temperature (15–25°C). 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.

14. **Remove the AirPore tape sheet. Repeat step 13 with a second volume of 45–70 µl RNase-free water.**

    Note: Repeating step 13 is required for complete recovery of RNA. 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.

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)	Inefficient disruption and/or homogenization	Use the spin protocol instead of the vacuum/spin protocol. Increase *g*-force and centrifugation time if necessary. In subsequent preparations, reduce the amount of starting material (see "Determining the amount of starting material", page 14) and/or increase the homogenization time.
b)	Too much starting material	Reduce the amount of starting material. It is essential to use the correct amount of starting material (see page 14).
c)	Centrifugation temperature too low	The centrifugation temperature should be 20–25°C. Some centrifuges may cool to below 20°C even when set at 20°C. This can cause formation of precipitates that can clog the 96-well plates. If this happens, set the centrifugation temperature to 25°C. Warm the lysates to 37°C before transferring them to the gDNA Eliminator 96 plate.
Low RNA yield		
a)	Insufficient disruption and 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.
b)	Too much starting material	Overloading the gDNA Eliminator 96 plate significantly reduces RNA yields. Reduce the amount of starting material (see page 14).

		Comments and suggestions
c)	RNA still bound to RNeasy 96 plate membrane	Repeat RNA elution, but incubate the RNeasy 96 plate on the benchtop for 10 min with RNase-free water before centrifuging.
d)	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.
e)	Incomplete removal of cell-culture medium	Ensure complete removal of cell-culture medium after harvesting cells (see protocols, pages 17 and 21).
Low A_{260}/A_{280} value in RNA eluate		
	Water used to dilute RNA for A_{260}/A_{280} measurement	Use 10 mM Tris·Cl, pH 7.5, not RNase-free water, to dilute the sample before measuring purity (see Appendix B, page 30).
Contamination of RNA with DNA affects downstream applications		
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 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 degraded		
a)	Inappropriate handling of starting material	Ensure that the samples have been properly handled and that the protocol has been performed without interruptions. For details, see Appendix A (page 28), "Handling and storing starting material" (page 16), and "Important points before starting" for each protocol.
b)	RNase contamination	Although all buffers have been tested and are guaranteed RNase-free, RNases can be introduced during use. Be certain not to introduce any RNases during the RNeasy Plus procedure or later handling. See Appendix A (page 28) for general remarks on handling RNA.

		Comments and suggestions
RNA does not perform well in downstream experiments		
a)	Salt carryover during elution	Ensure that buffers are at 20–30°C. Ensure that the correct buffer is used for each step of the procedure. Ensure that all liquid has completely passed through the membranes in each step of the protocol. When reusing S-Blocks between washing steps, remove residual flow-through from the rim by blotting on clean paper towels.
b)	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.

Appendix A: General Remarks on Handling RNA

Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to destroy RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Great care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the purification procedure. In order to create and maintain an RNase-free environment, the following precautions must be taken during pretreatment and use of disposable and nondisposable vessels and solutions while working with RNA.

General handling

Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible. Keep purified RNA on ice when aliquots are pipetted for downstream applications.

Disposable plasticware

The use of sterile, disposable polypropylene tubes is recommended throughout the procedure. These tubes are generally RNase-free and do not require pretreatment to inactivate RNases.

Nondisposable plasticware

Nondisposable plasticware should be treated before use to ensure that it is RNase-free. Plasticware should be thoroughly rinsed with 0.1 M NaOH, 1 mM EDTA* followed by RNase-free water (see "Solutions", page 29). Alternatively, chloroform-resistant plasticware can be rinsed with chloroform* to inactivate RNases.

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

Glassware

Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent,* thoroughly rinsed, and oven baked at 240°C for at least 4 hours (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware can be treated with DEPC* (diethyl pyrocarbonate). Fill glassware with 0.1% DEPC (0.1% in water), allow to stand overnight (12 hours) at 37°C, and then autoclave or heat to 100°C for 15 minutes to eliminate residual DEPC.

Electrophoresis tanks

Electrophoresis tanks should be cleaned with detergent solution (e.g., 0.5% SDS),* thoroughly rinsed with RNase-free water, and then rinsed with ethanol† and allowed to dry.

Solutions

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

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

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

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

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

Storage of RNA

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

Quantification of RNA

The concentration of RNA can 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 not be possible to accurately determine amounts photometrically. Small amounts of RNA can be quantified using the QIAxcel system or Agilent® 2100 bioanalyzer, fluorometric quantification, or quantitative, real-time RT-PCR. When purifying RNA from particularly small samples (e.g., laser-microdissected samples), quantitative, real-time RT-PCR should be used for quantification.

Spectrophotometric quantification of RNA

To ensure significance, A_{260} readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 44 µg of RNA per ml (A_{260}=1 → 44 µg/ml). This relation is valid only for measurements at a neutral pH. Therefore, if it is necessary to dilute the RNA sample, this should be done in a buffer with neutral pH.* As discussed below (see "Purity of RNA", page 31), 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 29). Use the buffer in which the RNA is diluted to zero the spectrophotometer. An example of the calculation involved in RNA quantification is shown below:

Volume of RNA sample = 100 µl

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

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

A_{260} = 0.2

* 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.

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

Purity of RNA

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

For determination of RNA concentration, however, we recommend dilution of the sample in a buffer with neutral pH since the relationship between absorbance and concentration (A_{260} reading of 1 = 44 µg/ml RNA) is based on an extinction coefficient calculated for RNA at neutral pH (see "Spectrophotometric quantification of RNA", page 30).

DNA contamination

No currently available purification method can guarantee that RNA is completely free of DNA, even when it is not visible on an agarose gel. While the vast majority of cellular DNA will be removed by the gDNA Eliminator 96 plate, trace amounts may still remain in the purified RNA, depending on the amount and nature of the sample.

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

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

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

To prevent any interference by DNA in real-time RT-PCR applications, such as with Applied Biosystems® and LightCycler® instruments, we recommend designing primers that anneal at intron splice junctions so that genomic DNA will not be amplified. QuantiTect® Primer Assays from QIAGEN (www.qiagen.com/GeneGlobe) are designed for SYBR® Green-based real-time RT-PCR analysis of RNA sequences (without detection of genomic DNA) where possible. For real-time RT-PCR assays where amplification of genomic DNA cannot be avoided, the QuantiTect Reverse Transcription Kit provides fast cDNA synthesis with integrated removal of genomic DNA contamination (see ordering information, page 41).

Integrity of RNA

The integrity and size distribution of total RNA purified with the RNeasy Plus 96 Kit can be checked by denaturing agarose gel electrophoresis and ethidium bromide staining* or by using the QIAxcel system or Agilent 2100 bioanalyzer. The respective ribosomal RNAs should appear as sharp bands or peaks. The apparent ratio of 28S rRNA to 18S rRNA should be approximately 2:1. If the ribosomal bands or peaks of a specific sample are not sharp, but appear as a smear towards smaller sized RNAs, it is likely that the sample suffered major degradation either before or during RNA purification.

The Agilent 2100 bioanalyzer also provides an RNA Integrity Number (RIN) as a useful measure of RNA integrity. Ideally, the RIN should be close to 10, but in many cases (particularly with tissue samples), how well the original sample is preserved greatly influences RNA quality.

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

Appendix C: Protocol for Formaldehyde Agarose Gel Electrophoresis

The following protocol for formaldehyde agarose (FA) gel electrophoresis is routinely used at QIAGEN and gives enhanced sensitivity for gel and subsequent analysis (e.g., northern blotting). A key feature is the concentrated RNA loading buffer that allows a larger volume of RNA sample to be loaded onto the gel than conventional protocols (e.g., Sambrook et al., eds. [1989] *Molecular cloning — a laboratory manual,* 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

FA gel preparation

To prepare FA gel (1.2% agarose) of size 10 x 14 x 0.7 cm, mix:

1.2 g agarose*

10 ml 10x FA gel buffer (see composition below)

Add RNase-free water to 100 ml

If smaller or larger gels are needed, adjust the quantities of components proportionately.

Heat the mixture to melt agarose. Cool to 65°C in a water bath. Add 1.8 ml of 37% (12.3 M) formaldehyde* and 1 µl of a 10 mg/ml ethidium bromide* stock solution. Mix thoroughly and pour onto gel support. Prior to running the gel, equilibrate in 1x FA gel running buffer (see composition below) for at least 30 min.

RNA sample preparation for FA gel electrophoresis

Add 1 volume of 5x RNA loading buffer (see composition below) to 4 volumes of RNA sample (for example, 10 µl of loading buffer and 40 µl of RNA) and mix.

Incubate for 3–5 min at 65°C, chill on ice, and load onto the equilibrated FA gel.

Gel running conditions

Run gel at 5–7 V/cm in 1x FA gel running buffer.

* 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.

Composition of FA gel buffers

10x FA gel buffer

200 mM	3-[N-morpholino]propanesulfonic acid (MOPS) (free acid)*
50 mM	sodium acetate*
10 mM	EDTA*

pH to 7.0 with NaOH*

1x FA gel running buffer

100 ml	10x FA gel buffer
20 ml	37% (12.3 M) formaldehyde
880 ml	RNase-free water

5x RNA loading buffer

16 µl	saturated aqueous bromophenol blue solution*†
80 µl	500 mM EDTA, pH 8.0
720 µl	37% (12.3 M) formaldehyde
2 ml	100% glycerol*
3.084 ml	formamide*
4 ml	10x FA gel buffer

RNase-free water to 10 ml

Stability: approximately 3 months at 4°C

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

† To make a saturated solution, add solid bromophenol blue to distilled water. Mix and continue to add more bromophenol blue until no more will dissolve. Centrifuge to pellet the undissolved powder, and carefully pipet the saturated supernatant.

Appendix D: Purification of Total RNA Containing Small RNAs from Cells Using Vacuum/Spin Technology

The following procedure allows the purification of total RNA containing small RNAs such as miRNA from animal and human cells using vacuum/spin technology.

Reagents to be supplied by user

- Ethanol (100%)*

Important points before starting

- Perform all steps of the procedure at room temperature (15–25°C). During the procedure, work quickly.
- Perform all centrifugation steps at 20–25°C. Ensure that the centrifuge does not cool below 20°C.

Things to do before starting

- 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.

Procedure

Carry out the protocol starting on page 17 up to and including step 5. Instead of performing steps 6–15 (purification of total RNA >200 nucleotides), follow steps D1–D8 below (purification of total RNA containing small RNAs).

D1. 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.

D2. Add 1.5 volumes (450 µl) of 100% ethanol to each well of the S-Block containing the flow-through from step 5. Mix well by pipetting up and down 3 times.

Note: Add 300 µl of 100% ethanol if 200 µl Buffer RLT Plus was used in step 1.

* 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.

D3. Transfer the samples (750 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.

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.

D4. 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.

The flow-through is collected in the same waste tray from step D3.*

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

D5. 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 16.

D6. 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 20–30°C to dry the membranes.

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.

D7. Remove the AirPore tape sheet. Place the RNeasy 96 plate on top of a rack of Elution Microtubes CL. Add 45–70 µl RNase-free water to each well, and seal the plate with a new AirPore tape sheet. Incubate for 1 min at room temperature (15–25°C). Then centrifuge at 6000 rpm (~5600 x *g*) for 4 min at 20–30°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 waste liquid contains Buffer RLT Plus and is therefore not compatible with bleach. See page 6 for safety information.

D8. Remove the AirPore tape sheet. Repeat step D7 with a second volume of 45–70 µl RNase-free water.

Note: Repeating step D7 is required for complete recovery of RNA. 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.

Appendix E: Purification of Total RNA Containing Small RNAs from Cells Using Spin Technology

The following procedure allows the purification of total RNA containing small RNAs such as miRNA from animal and human cells using spin technology.

Reagents to be supplied by user

- Ethanol (100%)*

Important points before starting

- Perform all steps of the procedure at room temperature (15–25°C). During the procedure, work quickly.
- Perform all centrifugation steps at 20–25°C. Ensure that the centrifuge does not cool below 20°C.

Things to do before starting

- 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.

Procedure

Carry out the protocol starting on page 21 up to and including step 5. Instead of performing steps 6–14 (purification of total RNA >200 nucleotides), follow steps E1–E8 below (purification of total RNA containing small RNAs).

E1. Place an 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 16.

E2. Add 1.5 volumes (450 µl) of 100% ethanol to each well of the S-Block containing the flow-through from step 5. Mix well by pipetting up and down 3 times.

Note: Add 300 µl of 100% ethanol if 200 µl Buffer RLT Plus was used in step 1.

E3. Transfer the samples (750 µl) to 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.

* 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.

E4. 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.

E5. 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").

E6. 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.

E7. Remove the AirPore tape sheet. Place the RNeasy 96 plate on top of a rack of Elution Microtubes CL. Add 45–70 µl RNase-free water to each well, and seal the plate with a new AirPore tape sheet. Incubate for 1 min at room temperature (15–25°C). 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.

E8. Remove the AirPore tape sheet. Repeat step E7 with a second volume of 45–70 µl RNase-free water.

Note: Repeating step E7 is required for complete recovery of RNA. 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.

* The waste liquid contains Buffer RLT Plus and is therefore not compatible with bleach. See page 6 for safety information.

Ordering Information

Product	Contents	Cat. no.
RNeasy Plus 96 Kit (12)	For 12 x 96 preps: gDNA Eliminator 96 Plates, RNeasy 96 Plates, Elution Microtubes CL, Caps, S-Blocks, AirPore Tape Sheets, RNase-Free Water and Buffers	74192
Accessories		
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
AirPore Tape Sheets (50)	Microporous tape sheets for covering 96-well blocks; 50 sheets per pack	19571
Tape Pads (5)	Adhesive tape sheets for sealing multiwell plates and blocks; 25 sheets per pad, 5 pads per pack	19570
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 Pump	Universal vacuum pump (capacity 34 liters per minute, 8 mbar vacuum abs.)	Inquire
Vacuum Regulator	For use with QIAvac manifolds	19530
Centrifuge 4-15C	Universal laboratory centrifuge with brushless motor	Inquire
Centrifuge 4K15C	Universal refrigerated laboratory centrifuge with brushless motor	Inquire
Plate Rotor 2 x 96	Rotor for 2 QIAGEN 96-well plates; for use with QIAGEN centrifuges	81031
RNAprotect Cell Reagent (250 ml)	250 ml RNAprotect Cell Reagent	76526

Ordering Information

Product	Contents	Cat. no.
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
QIAxcel system	Capillary electrophoresis device, including computer, and BioCalculator Analysis software; 1-year warranty on parts and labor	9001421
Related products		
QuantiTect Primer Assays — for use in real-time RT-PCR with SYBR Green detection (search for and order assays at www.qiagen.com/GeneGlobe)		
QuantiTect Primer Assay (200)	For 200 x 50 µl reactions or 400 x 25 µl reactions: 10x QuantiTect Primer Assay (lyophilized)	Varies
QuantiTect Reverse Transcription Kit — for fast cDNA synthesis for sensitive real-time two-step RT-PCR		
QuantiTect Reverse Transcription Kit (50)	For 50 x 20 µl reactions: gDNA Wipeout Buffer, Quantiscript® Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-Free Water	205311
QuantiTect Reverse Transcription Kit (200)	For 200 x 20 µl reactions: gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-Free Water	205313

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Notes

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The Plate Rotor 2 x 96 is available exclusively from QIAGEN and its distributors. Under the current liability and warranty conditions, the rotor may only be used in Centrifuges 4-15C and 4K15C from QIAGEN, and freely programmable models of centrifuges 4-15, 4K15, 6-10, 6K10, 6-15, and 6K15 from Sigma Laborzentrifugen GmbH.

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