June 2023

RNeasy® 96 Handbook

For RNA isolation from animal and human cells and for RNA cleanup

Sample to Insight

Contents

Kit Contents

Kit Catalog no. Number of preps	RNeasy 96 Kit (4) 74181	RNeasy 96 Kit (12) 74182
Preparations per kit	4 x 96	12 x 96
RNeasy 96 Plates	4	12
Register Cards (96-well)	4	12
S- Blocks (2.2 mL)*	2	2
Elution Microtubes (1.2 mL), racked	4 x 96	12 x 96
Caps for Elution Microtubes, in strips	55 x 8	165 x 8
AirPore™ Tape Sheets	3 x 5 sheets	2 x 25 sheets
Buffer RLT†	220 mL	2 x 220 mL
Buffer RW1†	2 x 220 mL	4 x 400 mL
Buffer RPE‡	4 x 55 mL	8 x 65 mL
RNase-free water	2 x 50 mL	12 x 30 mL

* Reusable; see page 23 for cleaning instructions.

† Not compatible with disinfecting containing bleach. Contains guanidine isothiocyanate, which is an irritant. Take appropriate safety measures, and wear gloves when handling.

‡ Buffer RPE is supplied as a concentrate. Add 4 volumes of ethanol (96–100%) before use to obtain a working solution of Buffer RPE.

Additional Buffer RLT, 6, Square-Well Blocks, Elution Microtubes (1.2 mL), and AirPore Tape Sheets are available separately. See ordering information (page 70).

Shipping and Storage

The RNeasy 96 Kit is shipped at ambient temperature. All components can be stored dry at room temperature (15–25°C). Under these conditions, the components are stable for 9 months without showing any reduction in performance and quality, unless otherwise indicated on the label.

Intended Use

The RNeasy 96 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 http://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.

Buffers RLT and RW1 contain guanidine thiocyanate, which 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.

Quality Control

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

Introduction

The RNeasy 96 Kit is ideal for simultaneous isolation of 96 or 192 RNA samples from up to 5 x 10^5 animal or human cells per sample. The RNeasy 96 Kit facilitates efficient, high-throughput RNA sample preparation for research use (see Intended Use, page 6) in fields such as:

- Drug screening
- Molecular Diagnostics
- Therapy monitoring
- Basic research

In less than 1 hour, 192 high-purity RNA samples can be obtained when processing 2 RNeasy 96 plates in parallel (15–20 s per RNA sample). The RNeasy 96 procedure replaces current time-consuming and tedious methods involving alcohol-precipitation steps, large numbers of washing steps, or the use of toxic substances such as phenol and/or chloroform. The purified RNA is ready to use in any downstream application including:

- RT-PCR
- RNA-seq
- Quantitative RT-PCR, including TaqMan® and LightCycler® technology
- Differential display
- cDNA synthesis
- Northern, dot, and slot blot analysis
- Primer extension
- Poly A+ RNA selection
- RNase/S1 nuclease protection
- Microarrays

In addition, the RNeasy 96 Kit can be used to desalt or to purify RNA from enzymatic reactions such as DNase digestions, proteinase digestions, RNA ligation, or labeling reactions.

Principle and procedure

The RNeasy 96 system provides a fast and efficient procedure for high-throughput RNA preparation. This technology combines the selective binding properties of a silica-gel–based membrane with the speed of vacuum and/or spin technology. Cells are first lysed under highly denaturing conditions with guanidine isothiocyanate (GITC) to immediately inactivate RNases and ensure isolation of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to the wells of the RNeasy 96 plate. 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.

With the RNeasy 96 procedure (Figure 1), all RNA molecules longer than 200 nucleotides are isolated. 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 size distribution of purified RNA is comparable to that obtained by centrifugation through a CsCl cushion, where small RNAs do not sediment efficiently.

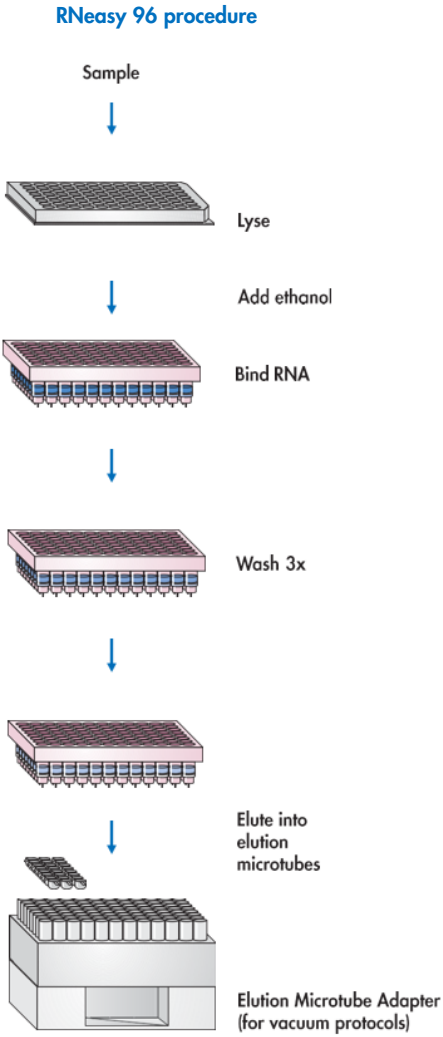

Figure 1. RNA isolation with the RNeasy 96 Kit. Protocol steps can be performed on a QIAvac 96 vacuum manifold (with the Elution Microtube Adapter shown) or in a specially designed centrifuge system (see page 11).

Description of protocols

Isolation of Total RNA from Animal Cells

The RNeasy 96 procedure is optimized for processing up to 5 x 10^5 animal cells per sample (see "Sample size", page 14 for details). In the RNeasy 96 Protocol for Isolation of Total RNA from Animal Cells (Page 12), cells are lysed in a buffer containing guanidine isothiocyanate (GITC). Ethanol is then added to the lysates, creating conditions that promote selective binding of RNA to the RNeasy membrane. The samples are then applied to the wells of the RNeasy 96 plate. Total RNA binds to the membrane at the bottom of each well. Contaminants are efficiently washed away by wash buffers, and the RNeasy membrane is dried. High-quality RNA is then eluted in a small volume of water.

RNA Cleanup

The RNeasy 96 Protocol for RNA Cleanup (page 38) is used to purify RNA from enzymatic reactions (e.g., RNA labeling, DNase digestion) or for desalting RNA samples (maximum 100 µg of RNA per well). GITC-containing lysis buffer and ethanol are added to the sample to create conditions that promote selective binding of RNA to the RNeasy membrane. The samples are then applied to the wells of the RNeasy 96 plate. RNA binds to the membrane at the bottom of each well. Contaminants are efficiently washed away by wash buffers, and the RNeasy membrane is dried. High-quality RNA is then eluted in a small volume of water.

Handling options

Each of the protocols in this handbook is provided with three different handling options, using vacuum technology, a combination of vacuum and spin technology, or spin technology alone. Each handling option provides high yields of high-quality RNA. The requirements of the downstream application determine which option should be used.

Vacuum technology

Using the QIAvac 96 vacuum manifold (see pages 14–15) is the quickest way to carry out the RNeasy 96 RNA isolation. Up to 96 RNA samples can be processed in 30–40 minutes. Use of two vacuum manifolds to process two RNeasy 96 plates in parallel allows processing of up to 192 RNA samples in 35–45 minutes. RNA isolated using vacuum technology can be used in any non-enzymatic application (e.g., northern, dot, and slot blot analysis). The RNA may also be used in enzymatic applications. However, because RNA samples prepared using vacuum technology may still contain trace amounts of salt, we recommend preliminary experiments with the application required. If RNA performance is unsatisfactory, the RNeasy 96 vacuum/spin or spin options should be used.

Vacuum/spin technology

Using vacuum/spin technology, all protocol steps up to the final wash step are performed on the QIAvac 96 vacuum manifold (see pages 14–15). The final wash step, including membrane drying, and all the elution steps are performed in the Centrifuge 4–16 KS or Centrifuge 4–16 S (see page 22). The Plate Rotor 2 x 96 holds two RNeasy 96 plates, allowing up to 192 RNA samples to be prepared in only 1 hour. Residual traces of salt are removed by centrifugation in the final wash step. RNA isolated using vacuum/spin technology can be used

for any non-enzymatic or enzymatic downstream application including quantitative RT-PCR analysis by TaqMan technology.

Spin technology

Using spin technology, all protocol steps are performed in the Centrifuge 4–16 KS or Centrifuge 4–16 S (see page 22). The Plate Rotor 2 x 96 holds two RNeasy 96 plates, allowing up to 192 RNA samples to be prepared in only 1.5 hours. RNA isolated using spin technology can be used for any non-enzymatic or enzymatic downstream application including quantitative RT-PCR analysis by TaqMan technology.

Important Notes

Sample size

The RNeasy 96 procedure is optimized for use with 10 to 5 x 10^5 animal cells. Direct counting is the most accurate way to quantify the number of cells. However, Table 1 may be used as a guide.

Table 2 gives 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 yields depend on the sample. The RNA amounts for the recommended numbers of cells are significantly less than the binding capacity of the RNeasy membrane and expected yields are therefore less than the RNeasy 96 binding capacity. Table 3 gives examples of expected RNA yields from various cultured animal cells.

If more than 5 x 10^5 cells are to be processed, the volume of lysis buffer and other reagents added to the sample before loading must be doubled. The volumes of the wash and elution buffers need not be increased. Depending on the cell line, the lysates may become viscous

when starting with more than 5 x 10^5 cells. This may lead to significantly lower yield and purity and may cause the RNeasy 96 plate wells to clog. If using more than 5 x 10^5 cells, we recommend performing preliminary experiments, increasing the number of cells step-by-step (e.g., 5 x 10^5, 7.5 x 10^5, 1 x 10^6 cells) and analyzing RNA yield and purity for each cell number. More than 1 x 10^6 cells should not be processed as lysates become too viscous.

Note: Additional Buffer RLT can be purchased separately (see ordering information on page 70).

Table 1. Growth area and number of HeLa cells in various multiwell cell-culture plates

Cell culture vessel	Growth area (cm2)*	Number of cells†
Multiwell plates		
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. Values are reported per well.

† Confluent growth is assumed. Values are reported per well.

Table 2. RNeasy 96 plate specifications

Preps per plate	96
Amount of starting material	10 to 5 x 10^5 cells*
Binding capacity per well	100 µg RNA†
Maximum loading volume per well	1 mL
RNA size distribution	All RNA >200 nucleotides

* The RNeasy 96 procedure is optimized for processing up to 5 x 10^5 animal cells. Depending on the cells used it may be possible to increase the maximum amount of starting cells up to 1 x 10^6 cells (see text). Please call QIAGEN Technical Services for guidelines to purify RNA from 10–100 cells.

† Yields are limited by cell type and number. The maximum binding capacity of 100 µg RNA is usually not reached (see text).

Table 3. Average total RNA yields obtained from a variety of cell lines using the RNeasy 96 kit

Animal cell line	Source	Yield* (µg) for 1 x 10^5 cells
HeLa	Human cervical carcinoma	1.6
LMH	Chicken hepatoma	1.3
COS-7	Monkey kidney, SV-40 transformed	3.1
Huh7	Human hepatoma	2.0
Jurkat	Human T-cell leukemia	1.4
K-562	Human chronic myelogenous	1.9

*Amounts can vary due to development stage, growth conditions used, etc. Since the RNeasy procedure enriches for RNA >200 bases long, 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.

Handling and storage of starting material

RNA is not protected until the sample material is flash frozen or disrupted in the presence of RNase- inhibiting or denaturing agents. It is therefore important that cell samples are immediately frozen and stored at –70°C or processed as soon as harvested. The relevant procedures should be carried out as quickly as possible. Samples can also be stored at –70°C in lysis buffer (RLT) after disruption. Frozen samples are stable for months.

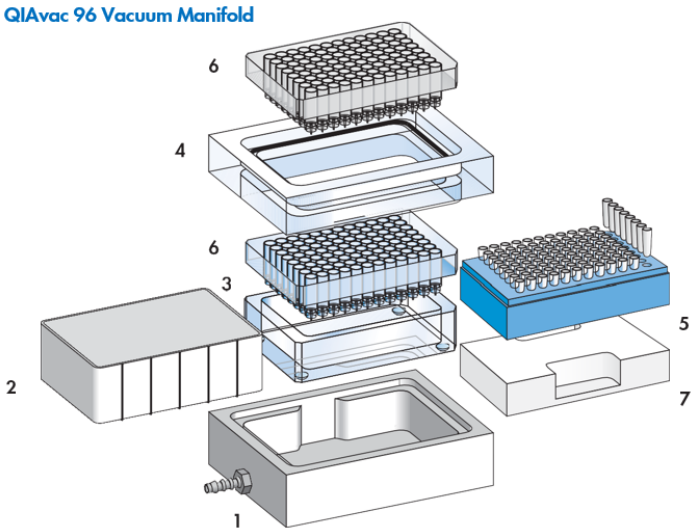

Figure 2. Components of the QIAvac 96 vacuum manifold

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 96 Protocol
4. QIAvac 96 top plate with aperture for 96-well plate
5. Disposable microtube rack*
6. 96-well plate*
7. Elution Microtube Adapter*

* Not included with QIAvac 96.

QIAvac 96 handling guidelines

QIAvac 96 facilitates RNeasy minipreparation by providing a convenient, modular vacuum manifold (Figure 2) for use with the RNeasy 96 Kit. The following recommendations should be followed when handling the QIAvac 96 vacuum manifold.

- QIAvac 96 operates with 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 differential 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 70). Vacuum recommendations are given in negative units to indicate the required reduction in pressure with respect to the atmosphere. Table 4 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 70) inserted between the vacuum source and the QIAvac 96 vacuum manifold.
- Wear safety glasses when working near a manifold under pressure.
- Always place the RNeasy 96 plate into the vacuum manifold with the beveled edges pointing to the right-hand side. For safety reasons, do not use 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 the 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 4. 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)	0.0394
Torrs (Torr)	0.75
Atmospheres (atmos)	0.000987
Pounds per square inch (psi)	0.0145

Centrifuge 4–16K and Centrifuge 4–16 KS

RNeasy 96 protocols using vacuum/spin and spin technology 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 table-top Centrifuge 4–16Kor Centrifuge 4–16KS (see ordering information, page 70). A wide range of other rotors can be used with Centrifuge 4–16K or Centrifuge 4–16KS in addition to the Plate Rotor 2 x 96.

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

Note: If Centrifuge 4–16KSis used, set the temperature at room temperature (20 to 30°C) for all remaining centrifugation steps.

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

CAUTION 	Do not centrifuge the Plate Rotor 2 x 96 metal holders without the RNeasy 96 plates and Square-Well 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.

S-Blocks

Four S-Blocks are supplied per kit. If several RNeasy 96 plates are processed per day, it may be convenient to keep extra Square-Well Blocks on hand. See ordering information on page 70.

To reuse the S- Blocks, rinse them thoroughly with tap water, incubate for 2 h 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 Buffers RLT and RW1 on the S-Blocks.

Equipment and Reagents to Be Supplied by User

- Multichannel pipet with tips
- For the most efficient sample processing in the RNeasy 96 protocol, we recommend the use of an electric multichannel pipet with a minimum capacity of 650 µL per pipet tip.
- Reagent reservoirs for multichannel pipets
- Disposable gloves
- S–Blocks (Cat. No. 19585, optional)*
- 96–100% ethanol
- 14.5 M β-mercaptoethanol (β-ME, optional)†

Using vacuum technology

- QIAvac 96 vacuum manifold (see pages 18)
- Vacuum source capable of generating a vacuum pressure of –800 to –900 mbar (see Ordering Info
- Elution Microtube Adapter (available from QIAGEN Technical Services)

Using vacuum/spin technology

- Centrifuge 4–16 S or Centrifuge 4–16 KS (see page 22)
- Plate Rotor 2 x 96 (see page 22)
- QIAvac 96 vacuum manifold (see pages 18)
- Vacuum source capable of generating a vacuum pressure of –800 to –900 mbar (see page 15)

* Four S- Blocks are supplied with the kit. They can be reused (see page 25). If several plates are processed per day it may be convenient to have extra S- Blocks available.

† Addition of -ME to Buffer RLT is optional for the RNeasy 96 protocols (see protocols for detailed information).

Using spin technology

- Centrifuge 4–16 Centrifuge 4–16 KS(see page 22).
- Plate Rotor 2 x 96 (see page 22)

For optional DNase treatment on RNeasy 96 plates

Generally, DNase digestion is not required since the RNeasy 96 silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). In these cases, an optional on-column DNase step allows digestion of the small residual amounts of DNA remaining (see Appendix F, page 68) The DNase is efficiently removed in the following wash step of the protocol.

The RNase-Free DNase Set (cat. no. 79254) provides efficient on-plate digestion of DNA during RNA purification. The DNase is efficiently removed in subsequent wash steps. The RNeasy 96 procedure requires 2 RNase-Free DNase Sets per 96-well plate

Protocol: Isolation of Total RNA from Animal Cells

Important notes before starting

- If preparing RNA for the first time, please read Appendix A (page 49). If using the RNeasy 96 Kit for the first time, please read "Important Notes before Using the RNeasy 96 Kit" (Page 14).
- All centrifugation steps in the vacuum/spin protocol and in the spin protocol are performed in a Centrifuge 4-15C or Centrifuge 4K15C (see page 22).
- Use of a multichannel pipet is recommended (see page 23). 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 23).
- A vacuum source capable of generating a vacuum pressure of –800 to –900 mbar is necessary for the vacuum and vacuum/spin protocol (see page 13). The vacuum must be switched off and the manifold ventilated between pipetting steps to maintain uniform conditions for each sample.
- Cell pellets can be stored at –70°C for later use or used directly in the procedure. Frozen cell pellets should be thawed slightly. Continue with steps 1 and 3 (using vacuum or vacuum/spin technology) or with step 2 (using spin technology).
- Cell lysates in Buffer RLT can be stored at –70°C for several months. To process frozen lysates, thaw and incubate at 37°C for 10 min to ensure that the chaotropic salt has dissolved. Mix by pipetting up and down 3 times. Continue with steps 1 and 4 (using vacuum or vacuum/spin technology) or with step 3 (using spin technology).
- Buffer RLT may form a precipitate upon storage. If necessary, warm to 37°C to redissolve.
- When isolating RNA from cells containing high amounts of RNases, it may be necessary to add

- β-mercaptoethanol (β-ME) to Buffer RLT to avoid degradation of RNA. β-ME supports the inactivation of RNases by GITC. Add 10 µL of 14.5 M β-ME per 1 mL of Buffer RLT. Buffer RLT is stable for
- 1 month after addition of β-ME. In most cases it will not be necessary to add β-ME to Buffer RLT.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) to obtain a working solution.

All steps of the RNeasy 96 protocol for isolation of total RNA should be performed at room temperature (20–30°C). Avoid interruptions during the procedure.

Generally, DNase digestion is not required since the RNeasy 96 silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA (See Using vacuum technology, page 2613).

I. Using vacuum technology

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 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. Harvesting cells

   1a. Cells grown in a monolayer

   Cells grown in a monolayer in multiwell cell-culture plates can be lysed directly in the wells. Completely remove medium by pipetting, and continue with step 3.

Note: Incomplete removal of the supernatant will dilute Buffer RLT in subsequent steps, inhibiting lysis and the binding of RNA to the RNeasy membrane. This will lead to reduced yield.

1b. Cells grown in suspension

Transfer aliquots of up to 5 x 10^5 cells into the wells of a 96-well microplate. Spin cells for 5 min at 300 x *g*, and completely remove supernatant by pipetting. Proceed with step 3.

Note: Incomplete removal of the supernatant will dilute Buffer RLT in subsequent steps, inhibiting lysis and the binding of RNA to the RNeasy membrane. This will lead to reduced yield.

2. Add 150 µL of Buffer RLT to each microplate well. Keeping the microplate flat on the bench, shake it vigorously back and forth for 10 s. While continuing to keep the plate flat on the bench, rotate the plate by 90° and shake it for an additional 10 s.

   Note: After the addition of ethanol in step 4, total volume will be 300 µL. If the surface of the liquid is too high for convenient use in the 96-well microplate used, the volume of Buffer RLT may be reduced to 100 µL.

3. Add 1 volume (150 µL) of 70% ethanol. Mix by pipetting up and down 3 times.

   Note: Add 100 µL of 70% ethanol if 100 µL of Buffer RLT has been used in step 3.

4. Apply the samples (300 µL) from step 4 into the wells of the RNeasy 96 plate, and switch on vacuum source. Apply vacuum until transfer is complete (15–60 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

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

   Note: Tape unused wells with adhesive tape. Do not use the AirPore Tape Sheets supplied with the RNeasy 96 Kit. Use either adhesive tape or Tape Pads (cat. no. 19570) from QIAGEN.

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

5. DNase digestion (optional)

Note: Generally, DNase digestion is not required since the RNeasy 96 silica-membrane, spin-column technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA. See page 48 for more information and page 18 for details to prepare the DNase I incubation mix.

5a. Pipet 80 µL of the DNase I incubation mix (see page 18) directly onto the RNeasy membrane in each well of the RNeasy 96 plate. Seal the plate with an AirPore Tape Sheet.

Note: Make sure to pipet the DNase I incubation mix directly onto the RNeasy membrane. DNase digestion will be incomplete if some of the mix sticks to the walls or the O-rings of the RNeasy 96 plate.

5b. Place at room temperature for 15 min.

5c. Remove the AirPore Tape from the RNeasy 96 plate. Proceed with step 7.

6. Add 1 mL of Buffer RW1 to each well of the RNeasy 96 plate. (If on-membrane DNase digestion was performed in the previous step, wait 5 min before proceeding.) Switch on vacuum source, and apply vacuum until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

   Note: To efficiently remove the DNase following DNase digestion, allow Buffer RW1 to remain on the RNeasy membrane for 5 min before vacuuming the buffer through the membrane.

   Collect wash fraction in the same waste tray used in step 5.

7. Lift the top plate carrying the RNeasy 96 plate from the base, and empty the waste tray. Reassemble the QIAvac 96 vacuum manifold.

   Add 1 mL of Buffer RPE to each well of the RNeasy 96 plate, and switch on vacuum source. Apply vacuum until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

   Note: Ensure that ethanol is added to Buffer RPE (see “Important notes before starting”, page 27).

8. Add another 1 mL of Buffer RPE to each well of the RNeasy 96 plate, and apply vacuum until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.
9. Lift the RNeasy 96 plate from the QIAvac top plate, and strike the bottom side of the RNeasy 96 plate on a stack of paper towels (~4 cm high). Repeat several times until no further liquid is released onto the paper towels.

   Residual Buffer RPE from the collars and nozzles of each well will be absorbed by the paper towels. Droplets adhering to the nozzles and collars should be removed with a tissue.
10. Place the RNeasy 96 plate back in the QIAvac top plate. Apply vacuum for 10 min. Switch off vacuum, and ventilate QIAvac 96 manifold.

    It is important to dry the RNeasy membrane, since residual ethanol may interfere with subsequent reactions. The 10 min vacuum application ensures that no ethanol is carried over during elution.
11. Replace the waste tray with an elution microtube rack containing 1.2 mL elution microtubes assembled on top of the Elution Microtube Adapter.
12. To elute, pipet 60–70 µL of RNase-free water directly onto the membrane in each well. Let stand for 1 min. Then switch on vacuum source until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

    Note: Make sure to pipet the RNase-free water directly onto the RNeasy membrane. Elution will be incomplete if some of the water sticks to the walls or the O-rings of the RNeasy 96 plate.
13. Repeat the elution step (step 14) once, as described, with a second volume of 60–70 µL RNase-free water.

    Note: Repeating the elution step is required for complete recovery of RNA. The elution volume will be approximately 30 µL less than the volume of RNase-free water added to the membrane, corresponding to the membrane dead volume.

    Use caps provided to seal the microtubes for storage. Store RNA at –20°C or at –70°C.

II. Using vacuum/spin technology

1. Preparation of QIAvac 96 vacuum manifold

   Place the waste tray inside the QIAvac base. Place the top plate squarely over the base. Place RNeasy 96 plate in the QIAvac top plate, making sure that the plate is seated tightly. Attach 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.

2. Harvesting cells

   2a. Cells grown in a monolayer

   Cells grown in a monolayer in multiwell cell-culture plates can be lysed directly in the wells. Completely remove medium by pipetting, and continue with step 3.

   Note: Incomplete removal of the supernatant will dilute Buffer RLT in subsequent steps, inhibiting lysis and the binding of RNA to the RNeasy membrane. This will lead to reduced yield.

   2b. Cells grown in suspension

   Transfer aliquots of up to 5 x 10^5 cells into a 96-well microplate. Spin cells for 5 min at 300 x *g*, and completely remove supernatant by pipetting. Proceed with step 3.

   Note: Incomplete removal of the supernatant will dilute Buffer RLT in subsequent steps, inhibiting lysis and the binding of RNA to the RNeasy membrane. This will lead to reduced yield.

3. Add 150 µL of Buffer RLT to each well of the microplate. Keeping the microplate flat on the bench, shake it vigorously back and forth for 10 s. While continuing to keep the plate flat on the bench, rotate the plate by 90° and shake it for an additional 10 s.

   Note: After the addition of ethanol in step 4, total volume will be 300 µL. If the surface of the liquid is too high for convenient use in the 96-well microplate used, the volume of Buffer RLT may be reduced to 100 µL.

4. Add 1 volume (150 µL) of 70% ethanol. Mix by pipetting up and down 3 times. Note: Add 100 µL of 70% ethanol if 100 µL of Buffer RLT has been used in step 3.

5. Apply the samples (300 µL) from step 4 into the wells of the RNeasy 96 plate, and switch on vacuum source. Apply vacuum until transfer is complete (15–60 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

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

   Note: Tape unused wells with adhesive tape. Do not use the AirPore Tape Sheets supplied with the RNeasy 96 Kit. Use either adhesive tape or Tape Pads (cat. no. 19570) from QIAGEN.

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

6. DNase digestion (optional)

   Note: Generally, DNase digestion is not required since the RNeasy 96 silica-membrane, spin-column technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA. See page 48 for more information and page 18 for details to prepare the DNase I incubation mix.

   6a. Pipet 80 µL of the DNase I incubation mix (see page 18) directly onto the RNeasy membrane in each well of the RNeasy 96 plate. Seal the plate with an AirPore Tape Sheet.

   Note: Make sure to pipet the DNase I incubation mix directly onto the RNeasy membrane. DNase digestion will be incomplete if some of the mix sticks to the walls or the O-rings of the RNeasy 96 plate.

   6b. Place at room temperature for 15 min.

   6c. Remove the AirPore Tape from the RNeasy 96 plate. Proceed with step 7.

7. Add 1 mL of Buffer RW1 to each well of the RNeasy 96 plate. (If on-membrane DNase digestion was performed in the previous step, wait 5 min before proceeding.) Switch on vacuum source, and apply vacuum until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

Note: To efficiently remove the DNase following DNase digestion, allow Buffer RW1 to remain on the RNeasy membrane for 5 min before vacuuming the buffer through the membrane.

Collect wash fraction in the same waste tray used in step 5.

8. Lift the top plate carrying the RNeasy 96 plate from the base, and empty the waste tray. Reassemble the QIAvac 96 vacuum manifold.
9. Add 1 mL of Buffer RPE to each well of the RNeasy 96 plate, and switch on vacuum source. Apply vacuum until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

   Note: Ensure that ethanol is added to Buffer RPE (see "Important notes before starting", page 27).
10. Place the RNeasy 96 plate on top of a S-Block. Mark the RNeasy plate for later identification.
11. Add another 1 mL of Buffer RPE to each well of the 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 (~5600 x *g*) for 10 min at room temperature to dry the plate membranes.

    Centrifugation with sealed plates prevents cross-contamination.

    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.
12. Remove the AirPore Tape. Place RNeasy 96 plate on top of an elution microtube rack containing 1.2 mL elution microtubes.
13. To elute the RNA, add 45–70 µL of RNase-free water to each well, and seal the RNeasy 96 plate with a new sheet of AirPore Tape. Incubate for 1 min at room temperature. Then centrifuge at 6000 rpm (~5600 x g) for 4 min at room temperature.

    Note: Make sure to pipet the RNase-free water directly onto the RNeasy membrane. 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. Repeat the elution step (step 13) once with a second volume of 45–70 µL RNase-free water.

    Note: Repeating the elution step is required for complete recovery of RNA. The elution volume will be approximately 15 µL less than the volume of RNase-free water added to the membrane, corresponding to the membrane dead volume.

    Use caps provided to seal the microtubes for storage. Store RNA at –20°C or at –70°C.

III. Using spin technology

1. Harvesting cells

    1a. Cells grown in a monolayer

    Cells grown in a monolayer in multiwell cell-culture plates can be lysed directly in the wells. Completely remove medium by pipetting, and continue with step 2.

    Note: Incomplete removal of the supernatant will dilute Buffer RLT in subsequent steps, inhibiting lysis and the binding of RNA to the RNeasy membrane. This will lead to reduced yield.

    1b. Cells grown in suspension

    Transfer aliquots of up to 5 x 10^5 cells into a 96-well microplate. Spin cells for 5 min at 300 x *g*, and completely remove supernatant by pipetting. Proceed with step 2.

    Note: Incomplete removal of the supernatant will dilute Buffer RLT in subsequent steps, inhibiting lysis and the binding of RNA to the RNeasy membrane. This will lead to reduced yield.

2. Add 150 µL of Buffer RLT to each well of the microplate. Keeping the microplate flat on the bench, shake it vigorously back and forth for 10 s. While continuing to keep the plate flat on the bench, rotate the plate by 90° and shake it for an additional 10 s.

    Note: After the addition of ethanol in step 4, total volume will be 300 µL. If the surface of the liquid is too high for convenient use in the 96-well microplate used, the volume of Buffer RLT may be reduced to 100 µL.

3. Add 1 volume (150 µL) of 70% ethanol to each microplate well. Mix by pipetting up and down 3 times.

   Note: Add 100 µL of 70% ethanol if 100 µL of Buffer RLT has been used in step 2.

4. Place an RNeasy 96 plate on top of a S- Block. Mark the RNeasy 96 plate for later identification.

5. Apply the samples from step 3 into the wells of the RNeasy 96 plate.

   Take care not to wet the rims of the wells to avoid cross-contamination in subsequent steps.

6. 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 (~5600 x *g*) for 4 min at room temperature.

   Centrifugation with sealed plates prevents cross-contamination.

7. DNase digestion (optional)

   Note: Generally, DNase digestion is not required since the RNeasy 96 silica-membrane, spin- column technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA. See page 48 for more information and page 18 for details to prepare the DNase I incubation mix.

   7a. Remove the AirPore Tape. Pipet 80 µL of the DNase I incubation mix (see page 18) directly onto the RNeasy membrane in each well of the RNeasy 96 plate. Seal the plate with a new sheet of AirPore Tape.

   Note: Make sure to pipet the DNase I incubation mix directly onto the RNeasy membrane. DNase digestion will be incomplete if some of the mix sticks to the walls or the O-rings of the RNeasy 96 plate.

   7b. Place at room temperature for 15 min. Then proceed with step 8.

8. Remove AirPore Tape. Add 0.8 mL of Buffer RW1 to each well of the RNeasy 96 plate. Seal the RNeasy 96 plate with a new sheet of AirPore Tape. (If on-membrane DNase digestion was performed in the previous step, wait 5 min before proceeding.) Centrifuge at 6000 rpm (~5600 x *g*) for 4 min at room temperature.

Note: To efficiently remove the DNase following DNase digestion, allow Buffer RW1 to remain on the RNeasy membrane for 5 min before centrifuging the buffer through the membrane.

9. Place the RNeasy 96 plate on top of another clean S- Block. Remove AirPore Tape. Add 0.8 mL of Buffer RPE to each well of the RNeasy 96 plate. Seal the RNeasy 96 plate with a new sheet of AirPore Tape. Centrifuge at 6000 rpm (~5600 x *g*) for 4 min at room temperature.

   Note: Ensure that ethanol is added to Buffer RPE (see "Important notes before starting", page 27).

10. Remove AirPore Tape. Add another 0.8 mL of Buffer RPE to each well of the RNeasy 96 plate. Seal the RNeasy 96 plate with a new piece of AirPore Tape. Centrifuge at 6000 rpm (~5600 x *g*) for 10 min at room temperature.

    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.

    Remove AirPore Tape. Place RNeasy 96 plate on top of an elution microtube rack containing 1.2 mL elution microtubes.

11. To elute the RNA, add 45–70 µL of RNase-free water to each well, and seal the RNeasy 96 plate with a new piece of AirPore Tape. Incubate for 1 min at room temperature. Then centrifuge at 6000 rpm (~5600 x g) for 4 min at room temperature.

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

12. Remove AirPore Tape. Repeat the elution step (step 12) once with a second volume of 45–70 µL RNase-free water.

    Note: Repeating the elution step is required for complete recovery of RNA. The elution volume will be approximately 15 µL less than the volume of RNase-free water added to the membrane, corresponding to the membrane dead volume.

    Use caps provided to seal the microtubes for storage. Store RNA at –20°C or at –70°C.

Protocol: RNA Cleanup

Important notes before starting

- If preparing RNA for the first time, please read Appendix A (page 49). If using the RNeasy 96 Kit for the first time, please read "Important Notes before Using the RNeasy 96 Kit" (page 14).
- All centrifugation steps in the vacuum/spin protocol and in the spin protocol are performed in a Centrifuge 4-15C or Centrifuge 4K15C (see page 22).
- Use of a multichannel pipet is recommended (see page 23). 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 23).
- A vacuum source capable of generating a vacuum pressure of –800 to –900 mbar is necessary for the vacuum and vacuum/spin protocol (see page 20). The vacuum must be switched off and the manifold ventilated between pipetting steps to maintain uniform conditions for each sample.
- Buffer RLT may form a precipitate upon storage. If necessary, warm to redissolve.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) to obtain a working solution.
- All steps of the RNeasy 96 cleanup protocol should be performed at room temperature (20 to 30°C). Avoid interruptions during the procedure.
- Generally, DNase digestion is not required since the RNeasy 96 silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA. We recommend using the optional on-column DNase digestion step or a DNase digestion of the reaction mixture before starting the procedure. See page 48 for more information and page 18 for details to prepare the DNase I incubation mix.

I. Using vacuum technology

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 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.

2. Adjust each sample volume to 100 µL with RNase-free water. Add 350 µL Buffer RLT to each sample, and mix by pipetting up and down 3 times with a multichannel pipet.
3. Add 250 µL of ethanol (96–100%) to each sample, and mix by pipetting up and down 3 times with a multichannel pipet.
4. Apply the samples from step 3 (700 µL) into the wells of the RNeasy 96 plate, and switch on vacuum source. Apply vacuum until transfer is complete (15–60 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

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

   Note: Tape unused wells with adhesive tape. Do not use the AirPore Tape Sheets supplied with the RNeasy 96 Kit. Use either adhesive tape or Tape Pads (cat. no. 19570) from QIAGEN.

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

5. DNase digestion (optional)

   Note: Generally, DNase digestion is not required since the RNeasy 96 silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA. See page 23 for more information and page 26 for details to prepare the DNase I incubation mix.

5a. Pipet 80 µL of the DNase I incubation mix (see page 23) directly onto the RNeasy membrane in each well of the RNeasy 96 plate. Seal the plate with AirPore Tape.

Note: Make sure to pipet the DNase I incubation mix directly onto the RNeasy membrane. DNase digestion will be incomplete if some of the mix sticks to the walls or the O-rings of the RNeasy 96 plate.

5b. Place at room temperature for 15 min.

5c. Remove AirPore Tape from the RNeasy 96 plate. Proceed with step 6.

6. Add 1 mL of Buffer RW1 to each well of the RNeasy 96 plate. (If on-membrane DNase digestion was performed in the previous step, wait 5 min before proceeding.) Switch on vacuum source, and apply vacuum until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

   Note: To efficiently remove the DNase following DNase digestion, allow Buffer RW1 to remain on the RNeasy membrane for 5 min before vacuuming the buffer through the membrane.

   Collect wash fraction in the same waste tray used in step 4.

7. Lift the top plate carrying the RNeasy 96 plate from the base, and empty the waste tray. Reassemble the QIAvac 96 vacuum manifold.

8. Add 1 ml of Buffer RPE to each well of the RNeasy 96 plate, and switch on vacuum source. Apply vacuum until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

   Note: Ensure that ethanol is added to Buffer RPE (see “Important notes before starting”, page 40).

9. Add another 1 mL of Buffer RPE to each well of the RNeasy 96 plate, and apply vacuum until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

10. Lift the RNeasy 96 plate from the QIAvac top plate, and strike the bottom side of the RNeasy 96 plate on a stack of paper towels (~4 cm high). Repeat several times until no further liquid is released onto the paper towels.

Residual Buffer RPE from the collars and nozzles of each well will be absorbed by the paper towels. Droplets adhering to the nozzles and collars should be removed with a tissue.

11. Place the RNeasy 96 plate back in the QIAvac top plate. Apply vacuum for 10 min. Switch off vacuum, and ventilate QIAvac 96 manifold.

    It is important to dry the RNeasy membrane, since residual ethanol may interfere with subsequent reactions. The 10 min vacuum application ensures that no ethanol is carried over during elution.

12. Replace the waste tray with an elution microtube rack containing 1.2 mL elution microtubes assembled on top of the Elution Microtube Adapter.

13. To elute, pipet 60–70 µL of RNase-free water directly onto the membrane in each well. Let stand for 1 min. Then switch on vacuum source until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

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

14. Repeat the elution step (step 13) once, as described, with a second volume of 60–70 µL RNase-free water.

    Note: Repeating the elution step is required for complete recovery of RNA. The elution volume will be 30 µL less than the volume of RNase-free water added to the membrane, corresponding to the membrane dead volume.

    Use caps provided to seal the microtubes for storage. Store RNA at –20°C or at –70°C.

II. Using vacuum/spin technology

1. Preparation of QIAvac 96 vacuum manifold

   Place the waste tray inside the QIAvac base. Place the top plate squarely over the base. Place RNeasy 96 plate in the QIAvac top plate, making sure that the plate is seated tightly. Attach 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.

2. Adjust each sample volume to 100 µL with RNase-free water. Add 350 µL Buffer RLT to each sample, and mix by pipetting up and down 3 times with a multichannel pipet.
3. Add 250 µL of ethanol (96–100%) to each sample, and mix by pipetting up and down 3 times with a multichannel pipet.
4. Apply the samples from step 3 (700 µL) into the wells of the RNeasy 96 plate, and switch on vacuum source. Apply vacuum until transfer is complete (15–60 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

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

   Note: Tape unused wells with adhesive tape. Do not use the AirPore Tape Sheets supplied with the RNeasy 96 Kit. Use either adhesive tape or Tape Pads (cat. no. 19570) from QIAGEN.

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

5. DNase digestion (optional)

   Note: Generally, DNase digestion is not required since the RNeasy 96 silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA. See page 68 for more information and page 23 for details to prepare the DNase I incubation mix.

5a. Pipet 80 µL of the DNase I incubation mix (see page 18) directly onto the RNeasy membrane in each well of the RNeasy 96 plate. Seal the plate with AirPore Tape.

Note: Make sure to pipet the DNase I incubation mix directly onto the RNeasy membrane. DNase digestion will be incomplete if some of the mix sticks to the walls or the O-rings of the RNeasy 96 plate.

5b. Place at room temperature for 15 min.

5c. Remove AirPore Tape from the RNeasy 96 plate. Proceed with step 6.

6. Add 1 mL of Buffer RW1 to each well of the RNeasy 96 plate. (If on-membrane DNase digestion was performed in the previous step, wait 5 min before proceeding.) Switch on vacuum source, and apply vacuum until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

   Note: To efficiently remove the DNase following DNase digestion, allow Buffer RW1 to remain on the RNeasy membrane for 5 min before vacuuming the buffer through the membrane.

   Collect wash fraction in the same waste tray used in step 4.

7. Lift the top plate holding the RNeasy 96 plate from the base, and empty the waste tray. Reassemble the QIAvac 96 vacuum manifold.

8. Add 1 mL of Buffer RPE to each well of the RNeasy 96 plate, and switch on vacuum source. Apply vacuum until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

   Note: Ensure that ethanol is added to Buffer RPE (see "Important notes before starting", page 35).

9. Place the RNeasy 96 plate on top of a S-Block. Mark the RNeasy 96 plate for later identification.

10. Add another 1 mL of Buffer RPE to each well of the RNeasy 96 plate. Seal the RNeasy 96 plate with AirPore Tape. Load the S- Block and RNeasy 96 plate into the holder, and place the whole assembly in the rotor bucket. Centrifuge at 6000 rpm (~5600 x *g*) for 10 min at room temperature to dry the plate membranes.

    Centrifugation with sealed plates prevents cross-contamination.

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.

11. Remove AirPore Tape. Place RNeasy 96 plate on top of an elution microtube rack containing 1.2 mL elution microtubes.
12. To elute the RNA, add 45–70 µL of RNase-free water to each well, and seal the RNeasy 96 plate with a new sheet of AirPore Tape. Incubate for 1 min at room temperature. Then centrifuge at 6000 rpm (~5600 x *g*) for 4 min at room temperature.

    Note: Make sure to pipet the RNase-free water directly onto the RNeasy membrane. Elution will be incomplete if some of the water sticks to the walls or the O-rings of the RNeasy 96 plate.
13. Remove AirPore Tape. Repeat the elution step (step 12) once with a second volume of 45–70 µL RNase-free water.

    Note: Repeating the elution step is required for complete recovery of RNA. The elution volume will be 15 µL less than the volume of RNase-free water added to the membrane, corresponding to the membrane dead volume.

    Use caps provided to seal the microtubes for storage. Store RNA at –20°C or at –70°C.

III. Using spin technology

1. Adjust each sample volume to 100 µL with RNase-free water. Add 350 µL Buffer RLT to each sample, and mix by pipetting up and down 3 times with a multichannel pipet.
2. Add 250 µL of ethanol (96–100%) to each sample, and mix by pipetting 3 times up and down with a multichannel pipet.
3. Place an RNeasy 96 plate on top of a S-Block. Mark the RNeasy 96 plate for later identification.
4. Apply the samples from step 2 (700 µL) into the wells of the RNeasy 96 plate.

   Take care not to wet the rims of the wells to avoid aerosols during centrifugation.

5. Seal the RNeasy 96 plate with AirPore Tape. Load the S-Block and RNeasy 96 plate into the holder, and place the whole assembly in the rotor bucket. Centrifuge at 6000 rpm (~5600 x *g*) for 4 min at room temperature.

   Centrifugation with sealed plates prevents cross-contamination.

6. DNase digestion (optional)

   Note: Generally, DNase digestion is not required since the RNeasy 96 silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA. See page 68 for more information and page 23 for details to prepare the DNase I incubation mix.

   6a. Remove AirPore Tape. Pipet 80 µL of the DNase I incubation mix (see page 23) directly onto the RNeasy membrane in each well of the RNeasy 96 plate. Seal the plate with a new sheet of AirPore Tape.

   Note: Make sure to pipet the DNase I incubation mix directly onto the RNeasy membrane. DNase digestion will be incomplete if some of the mix sticks to the walls or the O-rings of the RNeasy 96 plate.

   6b. Place at room temperature for 15 min. Then proceed with step 7.

7. Remove AirPore Tape. Add 0.8 mL of Buffer RW1 to each well of the RNeasy 96 plate. Seal the RNeasy 96 plate with a new sheet of AirPore Tape. (If on-membrane DNase digestion was performed in the previous step, wait 5 min before proceeding.) Centrifuge at 6000 rpm (~5600 x *g*) for 4 min at room temperature.

   Note: To efficiently remove the DNase following DNase digestion, allow Buffer RW1 to remain on the RNeasy membrane for 5 min before centrifuging the buffer through the membrane.

8. Place the RNeasy 96 plate on top of another clean S-Block. Remove AirPore Tape. Add 0.8 mL of Buffer RPE to each well of the RNeasy 96 plate. Seal the RNeasy 96 plate with a new sheet of AirPore Tape. Centrifuge at 6000 rpm (~5600 x *g*) for 4 min at room temperature.

Note: Ensure that ethanol is added to Buffer RPE (see "Important notes before starting", page 35).

9. Remove AirPore Tape. Add another 0.8 ml of Buffer RPE to each well of the RNeasy 96 plate. Seal the RNeasy 96 plate with a new sheet of AirPore Tape. Centrifuge at 6000 rpm (~5600 x *g*) for 10 min at room temperature.

   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.

10. Remove AirPore Tape. Place RNeasy 96 plate on top of an elution microtube rack containing 1.2 mL elution microtubes.

11. To elute the RNA, add 45–70 µL of RNase-free water to each well, and seal the RNeasy 96 plate with a new sheet of AirPore Tape. Incubate for 1 min at room temperature. Then centrifuge at 6000 rpm (~5600 x *g*) for 4 min at room temperature.

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

12. Remove AirPore Tape. Repeat the elution step (step 11) once with a second volume of 45–70 µL RNase-free water.

    Note: Repeating the elution step is required for complete recovery of RNA. The elution volume will be 15 µL less than the volume of RNase-free water added to the membrane, corresponding to the membrane dead volume. Use caps provided 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 in 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 or protocols in this handbook (for contact information, visit **support.qiagen.com**).

	Comments and suggestions
Clogged plate wells	
Too much starting material	Reduce amount of starting material. It is essential to use the correct amount of starting material (see page 14)
Litter to no RNA eluted	
Too much starting material	Overloading significantly reduces yield. Reduce the amount of starting material (see page 14)
Incomplete removal of supernatant	Ensure complete removal of the supernatant after harvesting cells (see protocols).
Buffer temperatures too low	All buffers must be at room temperature throughout the procedure.
Low A_{260}/A_{280} value	Use 10 mM Tris·Cl, not RNase-free water, to dilute the sample before measuring purity (see Appendix B, page 52).
RNA Degraded	
Inappropriate handling of starting material	Ensure that cells have been properly handled and that the protocol has been performed without interruptions, especially the initial steps involving cell lysis. See Appendix A (page 49), "Handling and storage of starting material" (page 18), and the "Important notes before starting" for each protocol.
RNase contamination	Check for RNase contamination of buffers. Although all buffers have been tested and are guaranteed RNase-free, RNases can be introduced during use. Be certain not to introduce any RNase during the procedure or later handling. See Appendix A (page 49) for general remarks on handling RNA.
DNA contamination in downstream experiments	
	12
No incubation with Buffer RW1 after optional DNase treatment	In subsequent preparations, incubate plate wells for 5 min at room temperature with Buffer RW1 following the DNase- treatment step.

	Comments and suggestions
No DNase treatment	Follow the optional on-column DNase digest step in the Protocol (Appendix F, page 68). Alternatively, after the RNeasy 96 procedure, DNase digest the eluate containing the RNA. After inactivating DNase by heat treatment, the RNA can be either used directly in the subsequent application without further treatment, or repurified using the RNA cleanup protocol.
Elution volume too low	Use elution volumes of 60–70 µL if using vacuum technology or 45–70 µL if using vacuum/spin or spin technology. Repeat the elution step (see protocols).
RNA does not perform well in downstream experiments	
a. Salt carryover	Ensure that Buffer RPE is at room temperature (20 to 30°C). If using vacuum technology, be sure to strike the bottom side of the RNeasy 96 plate repeatedly on a stack of paper towels until no further liquid is released (see protocols). Follow the protocol using vacuum/spin or spin technology (see page 13).
b. Ethanol carryover	During the second Buffer RPE wash, be sure to dry the plate-well membranes by vacuum for 10 min (vacuum technology) or centrifuge the plate at 6000 rpm (~5600 x g) for 10 min at room temperature (vacuum/spin or spin technology).
c. Elution volume too low	Use elution volumes of 60–70 µL if using vacuum technology or 45–70 µL if using vacuum/spin or spin technology. Repeat the elution step (see protocols).
d. 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 to the membrane, washing, and elution when using vacuum or vacuum/spin technology (see page 20).
Low well-to-well reproducibility	
Low well-to-well reproducibility	
a) Elution volume too low	Use elution volumes of 60–70 µL if using vacuum technology or 45–70 µL if using vacuum/spin or spin technology. Repeat the elution step (see protocols).
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 to the membrane, washing, and elution when using vacuum or vacuum/spin technology (see page 20).

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 isolation procedure. In order 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 isolated 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.

Non-disposable plasticware

Non-disposable 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 50). Alternatively, chloroform-resistant plasticware can be rinsed with chloroform to inactivate RNases.

Glassware

Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent, thoroughly rinsed, and oven baked at 240°C for four or more 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

* DEPC is a suspected carcinogen and should be handled with great care. Wear gloves and use a fume hood when using this chemical.

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

‡ DEPC is a suspected carcinogen and should be handled with great care. Wear gloves and use a fume hood when using this chemical.

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 CO2. 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 carboxymethylation. Carboxymethylated 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.

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 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. To ensure significance, readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per ml (A_{260}=1 ⇒ 40 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"), 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 0.1M NaOH, 1 mM EDTA followed by washing with RNase-free water (see "Solutions", page 50). 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 = 20 µL of RNA sample + 180 µL of 10 mM Tris, pH 7.0 (1/10 dilution).

Measure absorbance of diluted sample in a 0.2 mL cuvette (RNase-free).

A_{260} = 0.2

Concentration or RNA sample	= 40 µg/mL x A_{260} x dilution factor = 40 µg/mL x 0.2 x 10 = 80 µg/mL
Total amount	= concentration x volume of sample in mL = 80 µg/ml x 0.1 mL = 8 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, 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.

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 = 40 µg/mL RNA) is based on an extinction coefficient calculated for RNA at neutral pH (see "Quantification of RNA").

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

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

DNA 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. To prevent any interference by DNA in RT-PCR applications, such as TaqMan and LightCycler RT-PCR analyses, we recommend working with intron-spanning primers so that genomic DNA will not be amplified. Alternatively, DNA contamination can be detected on agarose gels following RT-PCR by performing control experiments in which no reverse transcriptase is added prior to the PCR step. For sensitive applications, such as differential display, or if it is not practical to use intron-spanning primers in TaqMan RT-PCR analysis, DNase digestion of the purified RNA with RNase-free DNase is recommended.

An optional on-column DNase digest step is included in all protocols. The DNase is efficiently washed away in the subsequent wash steps. Alternatively, after the RNeasy 96 procedure, the eluate containing the RNA can be treated with DNase. The RNA can then be repurified with the RNeasy cleanup protocol, or after heat inactivation of the DNase, the RNA can be used directly in downstream applications.

Integrity of RNA

The integrity and size distribution of total RNA purified with RNeasy Kits can be checked by denaturing agarose gel electrophoresis and ethidium bromide staining (see “Appendix E: Protocol for Formaldehyde Agarose Gel Electrophoresis”, page 65). The respective ribosomal bands (Table 5) should appear as sharp bands on the stained gel. 28S ribosomal RNA bands should be present at approximately twice the amounts of the 18S RNA band. If the ribosomal bands in a given lane are not sharp, but appear as a smear towards smaller sized RNAs, it is likely that the RNA sample suffered major degradation during preparation.

Table 5. Size of ribosomal RNAs from various sources

Source	rRNA	Size (kb)
Mouse	18S	1.9
	28S	4.7
Human	18S	1.9
	28S	5.0

Appendix C: RNeasy 96 for Real-Time, Quantitative RT-PCR

Overview of real-time, quantitative RT-PCR technology

High-throughput RNA analysis is an increasingly important tool in biomedical research, diagnostics, and drug discovery. Quantitative, real-time, high-throughput RT-PCR is possible with RNA purified with the RNeasy 96 Kit in combination with QuantiTect™ RT-PCR Kits from QIAGEN. The QuantiTect SYBR® Green RT-PCR Kit is designed for RNA quantification on any real-time thermal cycler using the fluorescent dye SYBR® Green I. The QuantiTect Probe RT-PCR Kit provides minimal optimization on any real-time thermal cycler using sequence-specific probes. This includes the use of dual-labeled (TaqMan) probes, FRET probes (including LightCycler hybridization probes), and Molecular Beacons.

This appendix describes some of these technologies and provides guidelines for setting up standard curves for real-time quantitative RT-PCR analyses. For more information, see the QuantiTect Probe RT- PCR Handbook and the QuantiTect SYBR® Green RT-PCR Handbook.

Dual-labeled probes

Dual-labeled probes, including TaqMan probes, are sequence-specific oligonucleotides with a fluorophore and a quencher dye attached (Figure 3). The fluorophore is at the 5' end of the probe, and the quencher dye is usually located at the 3' end or internally. During the extension phase of PCR, the probe is cleaved by the 5'➔3' exonuclease activity of Taq DNA polymerase or HotStarTaq DNA Polymerase, separating the fluorophore and the quencher dyes. This results in detectable fluorescence that is directly proportional to the amount of accumulated target DNA. QIAGEN Operon offers a large number of dual-labeled probes with different fluorescent reporters and quenchers (see www.operon.com).

Dual-Labeled probe principlel

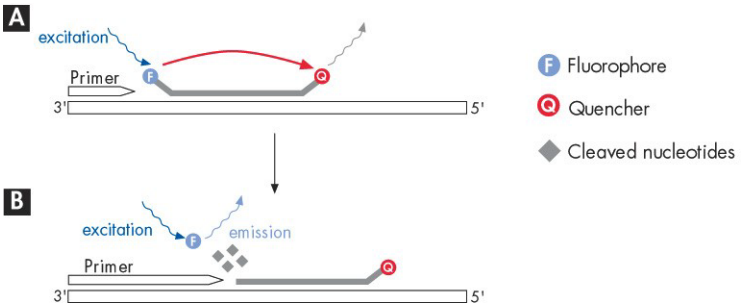

Figure 3. Schematic diagram of the principle of dual-labeled probes in quantitative, real-time RT-PCR.
A Both the dual-labeled probe and the PCR primers anneal to the target sequence during the PCR annealing step. The proximity of the fluorescent reporter with the quencher prevents the reporter from fluorescing. B During the PCR extension step, Taq DNA polymerase or HotStarTaq DNA Polymerase extend the primer. When the enzyme reaches the dual-labeled probe, its 5'➔3' exonuclease activity cleaves the fluorescent reporter from the probe. The fluorescent signal from the free reporter is measured.

FRET probes

PCR with fluorescent resonance energy transfer (FRET) probes, such as LightCycler hybridization probes, uses 2 labeled oligonucleotide probes that bind to the PCR product in a head-to-tail fashion (Figure 4). When the 2 oligonucleotides bind, their fluorophores come into close proximity, allowing energy transfer from a donor to an acceptor fluorophore. This causes fluorescence that is proportional to the amount of product. Unlike dual-labeled probes, FRET probes are not cleaved during the reaction; they can bind to target again in the next PCR cycle. QIAGEN Operon offers a large number of FRET probes with different fluorescent reporters and quenchers and is a licensed supplier of LC-Red 640 for FRET probes (see www.operon.com).

FRET Probe Principle

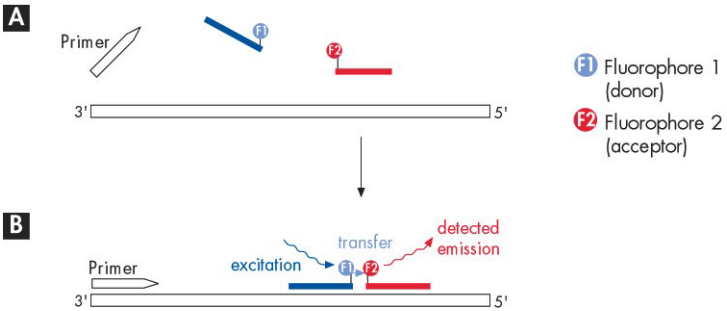

Figure 4. Schematic diagram of the principle of FRET probes in quantitative, real-time RT-PCR. A) When not bound to their target sequence, no fluorescent signal from the acceptor flurophore is detected. **B)** During the PCR annealing step, both FRET probes hybridize to the target sequence. This brings the donor and acceptor fluorophore into close proximity, allowing energy transfer between the fluorophores and resulting in a fluorescent signal from the acceptor fluorophore that is detected. The amount of signal is proportional to the amount of target sequence, and is measured in real time to allow quantification of the amount of target sequence.

Molecular Beacons

Molecular Beacons are dual-labeled with a 3' quencher and a 5' fluorophore. The probes are designed so that the ends are complementary. When the probe is in solution, the ends bind together and form a stem–loop structure with the fluorophore and the quencher near each other (Figure 5). This effectively quenches the fluorescent signal. When the probe finds its target and binds to it, the stem opens and the fluorophore and the quencher separate. This generates a fluorescent signal proportional to the amount of PCR product. QIAGEN Operon is a licensed supplier of Molecular Beacons, with a large number of different fluorescent reporters and quenchers (see www.operon.com).

Molecular Beacon Principle

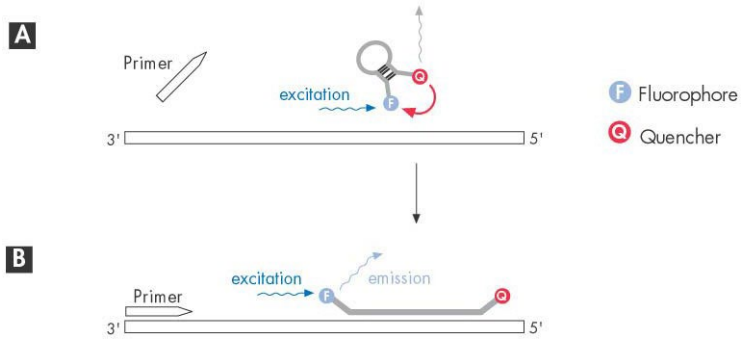

Figure 5. Schematic diagram of the principle of Molecular Beacons in quantitative, real-time RT-PCR. A) When not bound to its target sequence, the Molecular Beacon forms a hairpin structure. The proximity of the fluorescent reporter with the quencher prevents the reporter from fluorescing. B) During the PCR annealing step, the Molecular Beacon probe hybridizes to its target sequence. This separates the fluorescent dye and reporter, resulting in a fluorescent signal. The amount of signal is proportional to the amount of target sequence, and is measured in real time to allow quantification of the amount of target sequence.

Quantifications on real-time thermal cyclers

Quantification is based on the threshold cycle, where the amplification plot crosses a defined fluorescence threshold. Comparison of the threshold cycles provides a highly sensitive measure of relative template concentration in different samples. Figure 6 shows an example of real-time analysis using dual-labeled probes in TaqMan analysis. Monitoring during the early cycles, when PCR fidelity is at its highest, provides precise data for accurate quantification.

TaqMan analysis

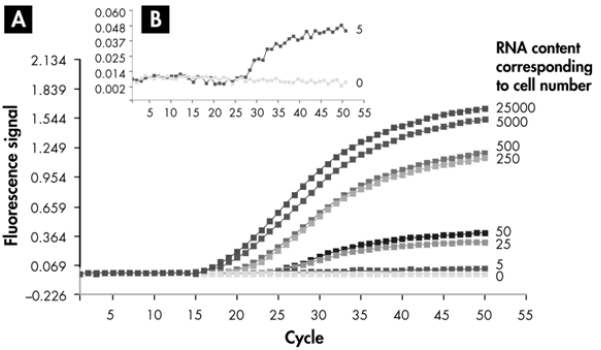

Figure 6. TaqMan quantitative RT-PCR analysis of RNA isolated from HeLa cells using the RNeasy 96 Kit. A) Real-time RT-PCR analysis of -actin mRNA was performed with RNA isolated from 100 to 5 x 105 HeLa cells, using 1/20 of each eluate (corresponding to RNA from 5 to 25,000 cells) in a single- tube protocol. The number of PCR cycles needed to detect the amplicon (threshold cycle) is a highly sensitive measure of relative template concentration. B) Detail of (A). Signals were analyzed with RNA corresponding to 5 cells.

For transcription analysis and quantification, quantitative RT-PCR assays require the highest-quality RNA. TaqMan technology was used in the development and evaluation of the RNeasy 96 Kit, and RNA purified with the RNeasy 96 Kit continues to be thoroughly tested by TaqMan and other real-time analyses. The RNeasy 96 Kit is the only high-throughput total cellular RNA purification system providing RNA that meets stringent TaqMan standards.

Guidelines for quantitative RT-PCR analysis

Quantitative, real-time RT-PCR analysis can be carried out in a two-tube or one-tube format. In two-tube RT-PCR analysis, the reverse-transcription reaction and PCR quantification are performed sequentially in two separate reactions. This can be carried out using Omniscript RT for reverse transcription, followed by PCR using the QuantiTect PCR Kits. For one-tube RT-PCR analysis, using the QuantiTect RT-PCR Kits, both reactions are performed in the same tube on a real-time thermal cycler. Generally, one-tube systems are more commonly used. Some guidelines for setting up quantitative, real-time RT-PCR analysis and determining the linear range of the system are given below.

1. Isolate RNA from cells following one of the RNeasy 96 Protocols for Isolation of Total RNA . For best results, we recommend using either vacuum/spin or spin technology. For the elution steps, elute twice with 45–60 µL RNase-free water. The final volume eluted should be approximately 75–105 µL.
2. For quantitative results, the amount of input RNA must be within the linear response range of the real-time assay, which may vary with the primers used and the transcripts assayed. In order to determine the optimal linear range of input RNA for a specific system, run a series of trial assays with 1, 2, 4, 6, 8, and 10 µL of an RNeasy 96 eluate in a 25 µL reaction volume.* For statistical significance, we recommend assaying each volume in triplicate and repeating the (triplicated) series of assays at least once.
3. Plot the resulting threshold cycle against the logarithm of the eluate volume. Figure 7 shows an example of such an experiment, with a linear response over the entire range. Note that, for some systems, the linear response will not cover the full range. Volumes outside the linear range will not yield quantitative results.

* The free sample volume in a 25 µL one-tube, real-time RT-PCR analysis is typically 9–10 µl. For a 50 µL assay, with approximately twice the free sample volume, we recommend using 1, 3, 6, 9, 12, 15, 18, and 20 µL.

Determination of Linear Range for TaqMan Analysis

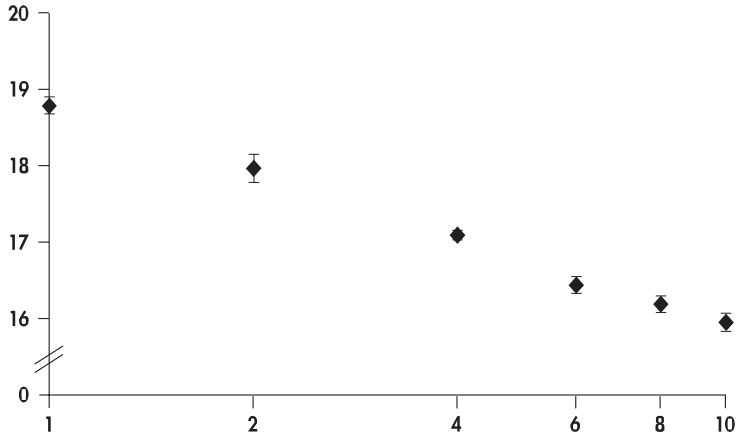

Figure 7. Linearity of RNeasy 96 RNA isolation for one-tube X-actin RT-PCR analysis using dual-labeled (TaqMan) probes. Total RNA was isolated from 5 x 104 HeLa cells with the RNeasy 96 Kit using vacuum/spin technology. RNA was eluted in 2 x 60 µL RNase-free water. RT-PCR TaqMan analysis of -actin mRNA was performed in triplicate using 1, 2, 4, 6, 8, and 10 µl of the RNeasy 96 eluate in a 25 µL reaction volume, and the entire triplicated series was repeated three times. The mean of the threshold cycle for each volume is presented here, plotted against the logarithm of the volume. Error bars represent the n – 1 standard deviation. The linear response range covers all volumes from 1 to 10 µL.

Appendix D: Guidelines for RT-PCR

To perform PCR using RNA as a starting template, the RNA must first be reverse transcribed into cDNA in a reverse transcription (RT) reaction. RT and PCR can either be carried out sequentially in the same tube (one-step RT-PCR), or an aliquot of the finished RT reaction can be used in a separate PCR (two- step RT-PCR).

Since the same primers are used in both the RT and the PCR reactions, one-step RT-PCR requires gene- specific primers. For this application, QIAGEN offers the QIAGEN OneStep RT-PCR Kit. Two-step RT-PCR is generally carried out using oligo-dT primers in the RT step and gene-specific primers in the PCR step. General guidelines for two-step RT-PCR are presented in Table 6 in the next page.

Table 6. General guidelines for performing two-step RT-PCR

Reverse transcription	**QIAGEN offers Omniscript™ and Sensiscript™ RT Kits for reverse transcription. Omniscript RT is specially designed for all reverse transcription with any amount of RNA from 50 ng to 2 µg per reaction. Sensiscript RT is optimized for use with very small amounts of RNA (1 pg – 50 ng).** **Follow the detailed protocol in the accompanying handbook, or, when using an enzyme from another supplier, follow the supplier's instructions. The following guidelines may be helpful.** • Mix the following reagents in a microcentrifuge tube: 2.0 µL 10x Buffer RT 2.0 µL dNTP Mix (5 mM each dNTP) 2.0 µL oligo-dT primer (10 µM) 1.0 µL RNase inhibitor (10 units/µL) 1.0 µL Omniscript or Sensiscript RT template RNA (up to 2 µg with Omniscript RT or up to 50 ng with Sensiscript RT) Add RNase-free water to a final volume of 20 µL. • Incubate at 37°C for 60 min.*
PCR	• Add an aliquot of the finished reverse-transcription reaction to the PCR mix. (No more than 1/5 of the final PCR volume should derive from the finished reverse-transcription reaction.)
	• Carry out PCR with Taq DNA polymerase as recommended by the supplier. (We have consistently obtained excellent results using Taq DNA Polymerase or HotStarTaq™ DNA Polymerase from QIAGEN.)

Appendix E: 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 (1.2% agarose, 10 x 14 x 0.7 cm)

1. 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 in the next page)
2. Add RNase-free water to 100 mL

   Note: If smaller or larger gels are needed, adjust the quantities of components proportionately.
3. 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.

* Toxic and/or mutagenic. Take appropriate safety measures.

RNA sample preparation for FA gel electrophoresis

1. Add 1 volume of 5x 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.
2. 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.

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

* Toxic and/or mutagenic. Take appropriate safety measures.

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 10 x FA gel buffer RNase-free water to 10 mL
- Stability: approximately 3 months at 4°C

Appendix F: Optional On-Plate DNase Digestion with the RNase-Free DNase Set

The RNase-Free DNase Set (cat. no. 79254) provides efficient on-plate digestion of DNA during RNA purification. The DNase is efficiently removed in subsequent wash steps. The RNeasy 96 procedure requires 2 RNase-Free DNase Sets per 96-well plate.

Note: Buffer RDD supplied with the RNase-Free DNase Set is specially optimized for on-plate DNase digestion. However, to prevent losses of small RNAs, a modified DNase digestion procedure is recommended for samples containing less than approximately 1 µg total RNA (equivalent to about 1 x 10^5 cells). In the modified procedure, the flow-through after on-plate digestion and washing is reapplied to the membrane. Buffer RWT used in this protocol should be prepared with isopropanol instead of ethanol, as is usually recommended (pages 27, 34, 40 and 47). Therefore, if not all preps will be performed using the procedure for DNase digestion for samples containing 1 µg total RNA approximately Carry out all protocol steps until the aqueous phase has been transferred through the RNeasy 96 plate (steps 1–11 of the animal cells-vacuum/spin protocol; steps 1–12 of the animal cellsspin protocol; steps 1–14 of the animal tissues-vacuum/spin protocol; steps 1–15 of the animal tissues-spin protocol; steps 1–16 of the miRNA-enriched fraction protocol). Then follow steps 1–4 below. If a vacuum/spin protocol is used, centrifugation in steps 1–4 can be replaced by use of the vacuum. 1. Pipet 400 µl Buffer RWT into each well of the RNeasy 96 plate and centrifuge for 4 min at 6000 rpm (approximately 5600 x *g*) to wash. Discard the flow-through.* Reuse the S-Block in step 4. 2. Add 670 µL DNase I stock solution to 7.3 ml Buffer RDD. Mix by gently inverting the tube. Do not vortex. Buffer RDD is supplied with the RNase-Free DNase Set. * Flow-through contains Buffer RWT and is therefore not compatible with bleach. See page 5 for safety information. miRNeasy 96 Handbook 11/2020 63 3. Pipet the DNase I incubation mix (80 µL per well) directly onto the membrane in each well of the RNeasy 96 plate and place on the benchtop at 20–30°C for 15 min. Note: Make sure to pipet the DNase I incubation mix directly

onto the RNeasy membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the RNeasy 96 plate wells. 4. Pipet 400 µl Buffer RWT into each well of the RNeasy 96 plate and centrifuge for 4 min at 6000 rpm (approx. 5600 x g). Discard the flow-through.* Continue with the protocol (at step 13 of the animal cells-vacuum/spin protocol; step 14 of the animal cells-spin protocol; step 16 of the animal tissues-vacuum/spin protocol; step 17 of the animal tissues-spin protocol; step 18 of the miRNA-enriched fraction protocol). Procedure: DNase digestion for samples containing

Ordering Information

Product	Contents	Cat. no.
RNeasy 96 Kit		
RNeasy 96 Kit (4)*	For 4 x 96 total RNA preps: 4 RNeasy 96 Plates, Elution Microtubes (1.2 mL), Caps, RNase-free Reagents and Buffers	74181
RNeasy 96 Kit* (12)	For 12 x 96 total RNA preps: 12 RNeasy 96 Plates, Elution Microtubes (1.2 mL), Caps, RNase-free Reagents and Buffers	74182
Accessories		
QIAvac 96	Vacuum manifold for processing QIAGEN 96-well plates: includes QIAvac 96 Top Plate, Base, Waste Tray, Plate Holder	19504
Vacuum Regulator	For use with QIAvac manifolds	19530
Vacuum Pump	Universal vacuum pump (capacity 34 L/min, 8 mbar vacuum abs.)	Various
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

* Requires use of either QIAvac 96 or the QIAGEN 96-Well-Plate Centrifugation System.

† Centrifuges 4-15C and 4K15C are not available in all countries. Specific formats are available in Japan. Please inquire.

Product	Contents	Cat. no.
Plate Rotor 2 x 96*	Rotor for 2 QIAGEN 96 plates, for use with QIAGEN Centrifuges	81031
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 screwtop tubes containing 5 mL RNAprotect Tissue Reagent e	76163
Buffer RLT	220 mL RNeasy Lysis Buffer for 6 RNeasy 96 plates	79216
S-Blocks (24)	96-well blocks with 2.2 mL wells, 24 blocks per case	19585
AirPore Tape Sheets (50)	Microporous tape sheets for covering 96-well blocks: 50 sheets per pack	19571

* 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.

Product	Contents	Cat. no.
Tape Pads (5)	Adhesive tape sheets for sealing multiwell plates and blocks: 25 sheets per pad, 5 pads per pack	19570
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	19560
Caps for Elution Microtubes (50 x 8)	Caps for Elution Microtubes (50x8)	19591
Collection Microtubes and Caps	Nonsterile polypropylene caps for collection microtubes (1.2 mL) and round-well blocks, 960 in strips of 8	19566
QIAxcel Advanced system	Operates in conjunction with the QIAxcel DNA Kits and QIAxcel RNA QC Kit v2.0.	9002123
Related Products		
RNeasy 96 BioRobot® Kits — for automated, high-throughput RNA isolation from cells		
RNeasy 96 BioRobot 8000 Kit (12)	For 12 x 96 total RNA preps on the BioRobot 8000 or BioRobot Universal System: 12 RNeasy 96 Plates, Elution Microtubes CL, Caps, S-Blocks, RNase-Free Reagents and Buffers	967152

Product	Contents	Cat. no.
DNeasy® 96 Tissue Kit* — for high-throughput DNA isolation from animal tissues and cells		
DNeasy 96 Tissue Kit (4)	For 4 x 96 DNA minipreps: 4 DNeasy 96 Plates, Proteinase K, Buffers, Square-Well Blocks, AirPore Tape Sheets, Collection Microtubes (1.2 mL), Caps, 96-well Plate Registers	69581
QIAamp 96 DNA Blood Kit† — for high-throughput DNA isolation from blood and body fluids		
QIAamp 96 DNA Blood Kit (4)	For 4 x 96 DNA preps: 4 QIAamp 96 Plates, QIAGEN Protease, Reagents, Buffers, Lysis Blocks, Tape Pads, Collection Vessels	51161

* Larger kit sizes available; please inquire. Requires use of the QIAGEN 96-Well-Plate Centrifugation System.

Product	Contents	Cat. no.
RNeasy Kits — for total RNA isolation from animal cells or tissues, yeast, or bacteria		
RNeasy Micro Kit (50)	50 RNeasy MinElute Spin Columns, Collection Tubes (1.5 mL and 2 mL), RNase-free DNase I, Carrier RNA, RNase-free Reagents and Buffers	74004
RNeasy Mini Kit (50)*	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 mL and 2 mL), RNase-free Reagents and Buffers	74104
RNeasy Mini QIAcube Kit (240)	For 240 RNA minipreps: RNeasy Mini Rotor Adapters (preloaded with spin columns and elution tubes); RNase-free reagents and buffers	74116
RNeasy Mini Kit (10)*	50 RNeasy Midi Spin Columns, Collection Tubes (15 ml), RNase-free Reagents and Buffers	75144
RNeasy Maxi Kit (12)	12 RNeasy Maxi Spin Columns, Collection Tubes (50 mL), RNase-free Reagents and Buffers	75162
RNeasy 96 QIAcube HT Kit	For 480 preps: RNeasy 96 plates, RNase-free water, buffers	74171
RNeasy Plant Mini Kit — for total RNA isolation from plants and fungi		
RNeasy Plant Mini Kit (50)*	50 RNeasy Mini Spin Columns, 50 QIAshredder Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	74903

* Larger kit sizes available; please inquire.

Product	Contents	Cat. no.
QIAamp® RNA Blood Mini Kit — for total RNA isolation from whole human blood		
QIAamp RNA Blood Mini Kit (50)*	For 50 RNA preps: 50 QIAamp Mini Spin Columns, 50 QIAshredder Spin Columns, Collection Tubes (1.5 mL and 2 mL), RNase-free reagents and buffers	52304
RNase-Free DNase Set — for DNase digestion during RNA purification		
RNase-Free DNase Set (50)	1500 units RNase-free DNase I, RNase-free Buffer, and RNase-free water for 50 RNA minipreps	79254
Omniscript RT Kit — for reverse transcription using ≥50 ng RNA		
Omniscript RT Kit (50)*	For 50 reverse-transcription reactions: 200 units Omniscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix,† RNase-free water	205111
Sensiscript RT Kit — for reverse transcription using <50 ng RNA		
Sensiscript RT Kit (50)*	For 50 reverse-transcription reactions: Sensiscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix,† RNase-free water	205211
QIAGEN OneStep RT-PCR Kit — for easy and sensitive one-step RT-PCR		
QIAGEN OneStep RT-PCR Kit (25)*	For 25 reactions: QIAGEN OneStep RT-PCR Enzyme Mix, 5x QIAGEN OneStep RT-PCR Buffer,‡ dNTP Mix,§§ 5x Q-Solution, RNase-free water	210210

* Larger kit sizes available; please inquire.
† Contains 5 mM of each dNTP
‡ Contains 12.5 mM $MgCl_2$
§ Contains 10 mM of each dNTP

Product	Contents	Cat. no.
Taq DNA Polymerase — for standard and specialized PCR applications		
Taq DNA Polymerase (250 U)	250 units Taq DNA Polymerase, 10x PCR Buffer,* 5x Q-Solution, 25 mM $MgCl_2$	201203
HotStarTaq DNA Polymerase — for highly specific hot-start PCR		
HotStarTaq DNA Polymerase (250 U)	250 units HotStarTaq DNA Polymerase, 10x PCR Buffer,† 5x Q-Solution, 25 mM $MgCl_2$	203203
QuantiTect SYBR® Green PCR and RT-PCR Kits — for quantitative, real-time PCR and RT-PCR using SYBR Green		
QuantiTect SYBR® Green PCR Kit (200)	For 200 x 50 µL reactions: 3 x 1.7 mL QuantiTect SYBR® Green PCR Master Mix;‡ 2 x 2.0 mL RNase-free water	204143
QuantiTect SYBR® Green RT-PCR Kit (200)	For 200 x 50 µL reactions: 3 x 1.7 mL QuantiTect SYBR® Green RT-PCR Master Mix;† 1 x 100 µL QuantiTect RT Mix; 2 x 2.0 mL RNase-free water	204243
QuantiTect Probe PCR and RT-PCR Kits — for quantitative, real-time PCR and RT-PCR using sequence-specific probes		
QuantiTect Probe PCR Kit (200)	For 200 x 50 µL reactions: 3 x 1.7 mL QuantiTect Probe PCR Master Mix;§ 2 x 2.0 mL RNase-free water	204343
QuantiTect Probe RT-PCR Kit (200)	For 200 x 50 µL reactions: 3 x 1.7 mL QuantiTect Probe RT-PCR Master Mix;	204443

* Contains 15 mM $MgCl_2$
† Contains 15 mM $MgCl_2$
‡ Contains 5 mM $MgCl_2$
§ Contains 8 mM MgCl2

Product	Contents	Cat. no.
	1 x 100 µL QuantiTect RT Mix; 2 x 2.0 mL RNase- free water	

Contact Information

For technical assistance and more information, please see our Technical Support Center at www.qiagen.com/Support, call 00800-22-44-6000, or contact one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Document Revision History

Date	Description
10/2023	Removed discontinued products in Ordering Information and Text, Updated to latest template, Removal of protocols for isolation of cytoplasmic RNA

Limited License Agreement for RNeasy 96 Kit

Use of this product signifies the agreement of any purchaser or user of the product to the following terms:

1.The product may be used solely in accordance with the protocols provided with the product and this handbook and for use with components contained in the panel only. QIAGEN grants no license under any of its intellectual property to use or incorporate the enclosed components of this panel with any components not included within this panel except as described in the protocols provided with the product, this handbook, and additional protocols available at www.qiagen.com Some of these additional protocols have been provided by QIAGEN users for QIAGEN users. These protocols have not been thoroughly tested or optimized by QIAGEN. QIAGEN neither guarantees them nor warrants that they do not infringe the rights of third-parties.

2.Other than expressly stated licenses, QIAGEN makes no warranty that this panel and/or its use(s) do not infringe the rights of third-parties.

3.This panel and its components are licensed for one-time use and may not be reused, refurbished, or resold.

4.QIAGEN specifically disclaims any other licenses, expressed or implied other than those expressly stated.

5.The purchaser and user of the panel agree not to take or permit anyone else to take any steps that could lead to or facilitate any acts prohibited above. QIAGEN may enforce the prohibitions of this Limited License Agreement in any Court, and shall recover all its investigative and Court costs, including attorney fees, in any action to enforce this Limited License Agreement or any of its intellectual property rights relating to the panel and/or its components.

For updated license terms, see www.qiagen.com.