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# RNeasy® Plus Mini Handbook

For purification of total RNA from animal cells and easy-to-lyse animal tissues using gDNA Eliminator columns


WWW.QIAGEN.COM

# Contents

## Kit Contents

| **RNeasy Plus Mini Kit** | **(50)** |
|---|---|
| **Catalog no.** | **74134** |
| **Number of preps** | **50** |
| gDNA Eliminator Mini Spin Columns (uncolored) (each in a 2 ml Collection Tube) | 50 |
| RNeasy Mini Spin Columns (pink) (each in a 2 ml Collection Tube) | 50 |
| Collection Tubes (1.5 ml) | 50 |
| Collection Tubes (2 ml) | 50 |
| Buffer RLT Plus* | 45 ml |
| Buffer RW1* | 45 ml |
| Buffer RPE† (concentrate) | 11 ml |
| RNase-Free Water | 10 ml |
| Handbook | 1 |

* Contains a guanidine salt. Not compatible with disinfectants containing bleach. See page 6 for safety information.

† Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.

## Storage

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

## Quality Control

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

## Product Use Limitations

The RNeasy Plus Mini Kit is intended for research use only. Prior to using it for other purposes, the user must validate the system in compliance with the applicable law, directives, and regulations.

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

## Product Warranty and Satisfaction Guarantee

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

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

## Technical Assistance

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

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

For technical assistance and more information please call one of the QIAGEN Technical Service Departments or local distributors (see back cover).

# Safety Information

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

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

Buffer RLT Plus contains guanidine thiocyanate and Buffer RW1 contains a small amount of guanidine thiocyanate. This chemical 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.

The following risk and safety phrases apply to the components of the RNeasy Plus Mini Kit.

**Buffer RLT Plus**

Contains guanidine thiocyanate: harmful. Risk and safety phrases:* R20/21/22-32, S13-26-36-46

**Buffer RW1**

Contains ethanol: flammable. Risk phrase:* R10

**24-hour emergency information**

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

* R10: Flammable; R20/21/22: Harmful by inhalation, in contact with skin and if swallowed; R32: Contact with acids liberates very toxic gas; S13: Keep away from food, drink and animal feedingstuffs; S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice; S36: Wear suitable protective clothing; S46: If swallowed, seek medical advice immediately and show the container or label.

# Introduction

The RNeasy Plus Mini Kit is designed to purify RNA from small amounts of animal cells or tissues. The kit is compatible with a wide range of cultured cells and with easy-to-lyse tissues. Genomic DNA contamination is effectively removed using a specially designed gDNA Eliminator spin column. The purified RNA is ready to use and is ideally suited for downstream applications that are sensitive to low amounts of DNA contamination, such as quantitative, real-time RT-PCR.* The purified RNA can also be used in other applications, including:

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

The RNeasy Plus Mini Kit allows the parallel processing of multiple samples in less than 25 minutes. Time-consuming and tedious methods such as CsCl step-gradient ultracentrifugation and alcohol precipitation steps, or methods involving the use of toxic substances such as phenol and/or chloroform, are replaced by the RNeasy Plus procedure.

## Principle and procedure

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

Biological samples are first lysed and homogenized in a highly denaturing guanidine-isothiocyanate–containing buffer, which immediately inactivates RNases to ensure isolation of intact RNA. The lysate is then passed through a gDNA Eliminator spin column. This column, in combination with the optimized high-salt buffer, allows efficient removal of genomic DNA.

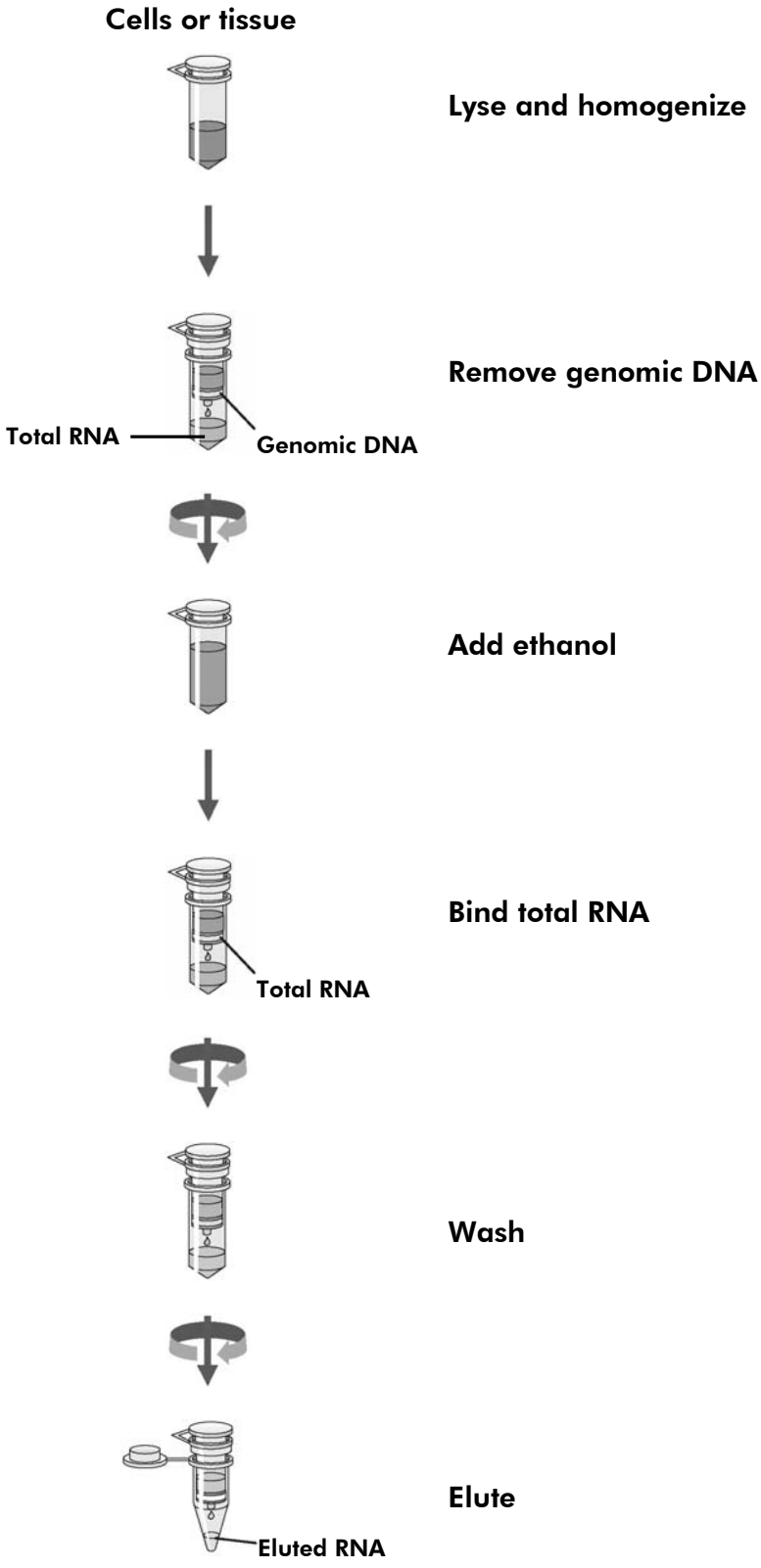
Ethanol is added to the flow-through to provide appropriate binding conditions for RNA, and the sample is then applied to an RNeasy spin column, where total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in 30 µl, or more, of water.

* Visit www.qiagen.com/geneXpression for information on standardized solutions for gene expression analysis, including QuantiTect® Kits and Assays for quantitative, real-time RT-PCR.

With the RNeasy Plus procedure, 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 the purified RNA is comparable to that obtained by centrifugation through a CsCl cushion, where small RNAs do not sediment efficiently. Protocols for purification of small RNA using RNeasy Kits are available at www.qiagen.com/goto/microRNAprotocols .

In this handbook, different protocols are provided for different starting materials. The protocols differ primarily in the lysis and homogenization of the sample. Once the sample is applied to the gDNA Eliminator spin column, the protocols are similar (see flowchart, next page).

## RNeasy Plus Procedure



# Equipment and Reagents to Be Supplied by User

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

### For all protocols

- 14.3 M β-mercaptoethanol (β-ME) (commercially available solutions are usually 14.3 M)
- Sterile, RNase-free pipet tips
- Microcentrifuge (with rotor for 2 ml tubes)
- 96–100% ethanol*
- 70% ethanol* in water
- Disposable gloves
- For tissue samples: RNA*later*™ RNA Stabilization Reagent (see ordering information, page 43) or liquid nitrogen
- Equipment for sample disruption and homogenization (see pages 15–17). Depending on the method chosen, one or more of the following are required:
  - Trypsin and PBS
  - QIAshredder homogenizer (see ordering information, page 43)
  - Blunt-ended needle and syringe
  - Mortar and pestle
  - TissueLyser (see ordering information, page 43)
  - Rotor–stator homogenizer

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

**Suppliers of rotor–stator homogenizers***

- BioSpec Products, Inc. ( www.biospec.com ): Tissue-Tearor™ homogenizer
- Charles Ross & Son Company ( www.mixers.com )
- IKA ( www.ika.de ): ULTRA-TURRAX® dispersers
- KINEMATICA AG ( www.kinematica.ch ) or Brinkmann Instruments, Inc. ( www.brinkmann.com ): POLYTRON® laboratory dispersing devices
- Omni International, Inc. ( www.omni-inc.com )
- Silverson ( www.silverson.com )
- VirTis ( www.virtis.com )

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

# Important Notes

## Determining the amount of starting material

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

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

When processing samples containing high amounts of DNA or RNA, less than the maximum amount of starting material shown in Table 1 should be used, so that the DNA removal capacity of the gDNA Eliminator spin column and the RNA binding capacity of the RNeasy spin column are not exceeded.

When processing samples containing average or low amounts of RNA, the maximum amount of starting material shown in Table 1 can be used. However, even though the RNA binding capacity of the RNeasy spin column is not reached, the maximum amount of starting material must not be exceeded. Otherwise, lysis will be incomplete and cellular debris may interfere with the binding of RNA to the RNeasy spin column membrane, resulting in lower RNA yield and purity.

More information on using the correct amount of starting material is given in each protocol. Table 2 shows expected RNA yields from various cells and tissues.

**Note:** If the DNA removal capacity of the gDNA Eliminator spin column is exceeded, the purified RNA will be contaminated with DNA. Although the gDNA Eliminator spin column can bind more than 100 µg DNA, we recommend using samples containing less than 20 µg DNA to ensure removal of virtually all genomic DNA. If the binding capacity of the RNeasy spin column is exceeded, RNA yields will not be consistent and may be reduced. If lysis of the starting material is incomplete, RNA yields will be lower than expected, even if the binding capacity of the RNeasy spin column is not exceeded.

**Table 1. RNeasy Mini Spin Column Specifications**

| | |
|---|---|
| Maximum binding capacity | 100 µg RNA |
| Maximum loading volume | 700 µl |
| RNA size distribution | RNA >200 nucleotides |
| Minimum elution volume | 30 µl |
| Maximum amount of starting material | |
| ■ Animal cells | 1 x $10^7$ cells |
| ■ Animal tissues | 30 mg |

**Table 2. Yields of Total RNA with the RNeasy Plus Mini Kit**

| Cell cultures (1 x $10^6$ cells) | Average yield of total RNA* (µg) | Mouse/rat tissues (10 mg) | Average yield of total RNA* (µg) |
|---|---|---|---|
| NIH/3T3 | 10 | Embryo (13 day) | 25 |
| HeLa | 15 | Brain | 5–10 |
| COS-7 | 35 | Heart | 4–8 |
| LMH | 12 | Kidney | 20–30 |
| Huh | 15 | Liver | 40–60 |
| | | Lung | 10–20 |

* Amounts can vary due to factors such as species, developmental stage, and growth conditions. Since the RNeasy procedure enriches for mRNA and other RNA species >200 nucleotides, the total RNA yield does not include 5S rRNA, tRNA, and other low-molecular-weight RNAs, which make up 15–20% of total cellular RNA.

Counting cells or weighing tissue is the most accurate way to quantitate the amount of starting material. However, the following may be used as a guide.

### Animal cells

The number of HeLa cells obtained in various culture vessels after confluent growth is given in Table 3.

**Table 3. Growth Area and Number of HeLa Cells in Various Culture Vessels**

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

* Per well, if multiwell plates are used; varies slightly depending on the supplier.

† Cell numbers are given for HeLa cells (approximate length = 15 µm), assuming confluent growth. Cell numbers will vary for different kinds of animal cells, which vary in length from 10 to 30 µm.

### Animal tissues

A 3 mm cube (27 $mm^3$) of most animal tissues weighs 30–35 mg.

## Handling and storing starting material

RNA in harvested tissue is not protected until the sample is treated with RNA*later* RNA Stabilization Reagent, flash-frozen, or disrupted and homogenized in the presence of RNase-inhibiting or denaturing reagents. Otherwise, unwanted changes in the gene expression profile will occur. It is therefore important that tissue samples are immediately frozen in liquid nitrogen and stored at –70°C, or immediately immersed in RNA*later* RNA Stabilization Reagent.

The procedures for tissue harvesting and RNA protection should be carried out as quickly as possible. Frozen tissue samples should not be allowed to thaw during handling or weighing. After disruption and homogenization in Buffer RLT Plus (lysis buffer), samples can be stored at –70°C for months.

## Disrupting and homogenizing starting material

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

- **Disruption:** Complete disruption of plasma membranes of cells and organelles is absolutely required to release all the RNA contained in the sample. Different samples require different methods to achieve complete disruption. Incomplete disruption results in significantly reduced RNA yields.
- **Homogenization:** Homogenization is necessary to reduce the viscosity of the lysates produced by disruption. Homogenization shears high-molecular-weight genomic DNA and other high-molecular-weight cellular components to create a homogeneous lysate. Incomplete homogenization results in inefficient binding of RNA to the RNeasy spin column membrane and therefore significantly reduced RNA yields.

Some disruption methods simultaneously homogenize the sample, while others require an additional homogenization step. Table 4 gives an overview of various disruption and homogenization methods, and is followed by a detailed description of each method.

**Table 4. Disruption and Homogenization Methods**

| Sample | Disruption method | Homogenization method |
|---|---|---|
| Animal cells | Addition of lysis buffer | Rotor–stator homogenizer or QIAshredder homogenizer or syringe and needle |
| Animal tissues | TissueLyser* | TissueLyser* |
| | Rotor–stator homogenizer† | Rotor–stator homogenizer† |
| | Mortar and pestle | QIAshredder homogenizer or syringe and needle |

* Simultaneously disrupts and homogenizes up to 192 samples in parallel. Results are comparable to those obtained using a rotor–stator homogenizer.

† Simultaneously disrupts and homogenizes individual samples.

### Disruption and homogenization using the TissueLyser system

In bead-milling, tissues can be disrupted by rapid agitation in the presence of beads and lysis buffer. Disruption and simultaneous homogenization occur by the shearing and crushing action of the beads as they collide with the cells. Disruption efficiency is influenced by:

- Size and composition of beads
- Ratio of buffer to beads
- Amount of starting material
- Speed and configuration of the TissueLyser
- Disintegration time

For animal tissues, the optimal beads are 3–7 mm diameter stainless steel beads. All other disruption parameters must be determined empirically for each application. For guidelines on disruption and homogenization of tissues using the TissueLyser system, refer to the *TissueLyser Handbook*. For other bead mills, please refer to suppliers' guidelines for further details.

**Note:** Do not use Buffer RLT Plus with tungsten carbide beads. Buffer RLT Plus reacts with tungsten carbide and can damage the surface of the beads.

### Disruption and homogenization using rotor–stator homogenizers

Rotor–stator homogenizers thoroughly disrupt and simultaneously homogenize, in the presence of lysis buffer, single samples of animal tissues in 15–90 seconds depending on the toughness and size of the sample. Rotor–stator homogenizers can also be used to homogenize cell lysates. The rotor turns at a very high speed, causing the sample to be disrupted and homogenized by a

combination of turbulence and mechanical shearing. Foaming of the sample should be kept to a minimum by using properly sized vessels, keeping the tip of the homogenizer submerged, and holding the immersed tip to one side of the tube. Rotor–stator homogenizers are available in different sizes and operate with differently sized probes. Probes with diameters of 5 mm and 7 mm are suitable for volumes of up to 300 µl and can be used for homogenization in microcentrifuge tubes. Probes with a diameter of 10 mm or above require larger tubes. In addition, round-bottomed tubes allow more efficient homogenization than conical-bottomed tubes.

### Disruption using a mortar and pestle

For disruption using a mortar and pestle, freeze the tissue sample immediately in liquid nitrogen and grind to a fine powder under liquid nitrogen. Transfer the suspension (tissue powder and liquid nitrogen) into a liquid-nitrogen–cooled, appropriately sized tube and allow the liquid nitrogen to evaporate without allowing the sample to thaw. Add lysis buffer and continue as quickly as possible with the homogenization according to one of the two methods below.

**Note:** Grinding the sample using a mortar and pestle will disrupt the sample, but will not homogenize it. Homogenization must be performed afterwards.

### Homogenization using QIAshredder homogenizers

Using QIAshredder homogenizers is a fast and efficient way to homogenize cell and tissue lysates without cross-contamination of samples. Up to 700 µl of lysate is loaded onto a QIAshredder spin column placed in a 2 ml collection tube, and spun for 2 minutes at maximum speed in a microcentrifuge. The lysate is homogenized as it passes through the spin column.

### Homogenization using a syringe and needle

Cell and tissue lysates can be homogenized using a syringe and needle. Lysate is passed through a 20-gauge (0.9 mm) needle attached to a sterile plastic syringe at least 5–10 times or until a homogeneous lysate is achieved. Increasing the volume of lysis buffer may be required to facilitate handling and minimize loss.

# Protocol: Purification of Total RNA from Animal Cells

### Determining the correct amount of starting material

It is essential to use the correct amount of starting material in order to obtain optimal RNA yield and purity. The minimum amount is generally 100 cells, while the maximum amount depends on:

- The RNA content of the cell type
- The DNA removal capacity of the gDNA Eliminator spin column
- The RNA binding capacity of the RNeasy spin column (100 µg RNA)
- The volume of Buffer RLT Plus required for efficient lysis (the maximum volume of Buffer RLT Plus that can be used limits the maximum amount of starting material to 1 x $10^7$ cells)

RNA content can vary greatly between cell types. The following examples illustrate how to determine the maximum amount of starting material:

- COS cells have high RNA content (approximately 35 µg RNA per $10^6$ cells). Do not use more than 3 x $10^6$ cells, otherwise the RNA binding capacity of the RNeasy spin column will be exceeded.
- HeLa cells have average RNA content (approximately 15 µg RNA per $10^6$ cells). Do not use more than 7 x $10^6$ cells, otherwise the RNA binding capacity of the RNeasy spin column will be exceeded.
- NIH/3T3 cells have low RNA content (approximately 10 µg RNA per $10^6$ cells). The maximum amount of starting material (1 x $10^7$ cells) can be used.

If processing a cell type not listed in Table 2 (page 13) and if there is no information about its RNA content, we recommend starting with no more than 3–4 x $10^6$ cells. Depending on RNA yield and purity, it may be possible to increase the cell number in subsequent preparations.

**Do not overload the gDNA Eliminator spin column, as this will lead to copurification of DNA with RNA. Do not overload the RNeasy spin column, as this will significantly reduce RNA yield and purity.**

As a guide, Table 3 (page 14) shows the expected numbers of HeLa cells in different cell-culture vessels.

### Important points before starting

- If using the RNeasy Plus Mini Kit for the first time, read "Important Notes" (page 12).
- If preparing RNA for the first time, read Appendix A (page 35).
- Cell pellets can be stored at –70°C for later use or used directly in the procedure. Determine the number of cells before freezing. Frozen cell pellets should be thawed slightly so that they can be dislodged by flicking the tube in step 2. Homogenized cell lysates from step 3 can be stored at –70°C for several months. Frozen lysates should be incubated at 37°C in a water bath until completely thawed and salts are dissolved. Avoid prolonged incubation, which may compromise RNA integrity. If any insoluble material is visible, centrifuge for 5 min at 3000–5000 x *g*. Transfer supernatant to a new RNase-free glass or polypropylene tube, and continue with step 4.
- β-mercaptoethanol (β-ME) must be added to Buffer RLT Plus before use. Add 10 µl β-ME per 1 ml Buffer RLT Plus. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT Plus is stable at room temperature (15–25°C) for 1 month after addition of β-ME.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.
- Buffer RLT Plus may form a precipitate during storage. If necessary, redissolve by warming, and then place at room temperature.
- Buffer RLT Plus and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 6 for safety information.
- Perform all steps of the procedure at room temperature. During the procedure, work quickly.
- Perform all centrifugation steps at 20–25°C in a standard microcentrifuge. Ensure that the centrifuge does not cool below 20°C.

### Procedure

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

**1a. Cells grown in suspension (do not use more than 1 x $10^7$ cells): Determine the number of cells. Pellet the appropriate number of cells by centrifuging for 5 min at 300 x *g* in a centrifuge tube (not supplied). Carefully remove all supernatant by aspiration, and proceed to step 2.**

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

**1b. Cells grown in a monolayer (do not use more than 1 x $10^7$ cells): Cells grown in a monolayer in cell-culture vessels can be either lysed directly in the vessel (up to 10 cm diameter) or trypsinized and collected as a cell pellet prior to lysis. Cells grown in a monolayer in cell-culture flasks should always be trypsinized.**

**To lyse cells directly:**
**Determine the number of cells. Completely aspirate the cell-culture medium, and proceed immediately to step 2.**

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

**To trypsinize and collect cells:**
**Determine the number of cells. Aspirate the medium, and wash the cells with PBS. Aspirate the PBS, and add 0.10–0.25% trypsin in PBS. After the cells detach from the dish or flask, add medium (containing serum to inactivate the trypsin), transfer the cells to an RNase-free glass or polypropylene centrifuge tube (not supplied), and centrifuge at 300 x *g* for 5 min. Completely aspirate the supernatant, and proceed to step 2.**

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

**2. Disrupt the cells by adding Buffer RLT Plus.**

**For pelleted cells, loosen the cell pellet thoroughly by flicking the tube. Add the appropriate volume of Buffer RLT Plus (see Table 5). Vortex or pipet to mix, and proceed to step 3.**

**Note:** Incomplete loosening of the cell pellet may lead to inefficient lysis and reduced RNA yields. Ensure that β-ME is added to Buffer RLT Plus before use (see "Important points before starting").

**Table 5. Volumes of Buffer RLT Plus for Lysing Pelleted Cells**

| Number of pelleted cells | Volume of Buffer RLT Plus |
|---|---|
| $<5 \times 10^6$ | 350 µl |
| $5 \times 10^6 – 1 \times 10^7$ | 600 µl |

**For direct lysis of cells grown in a monolayer, add the appropriate volume of Buffer RLT Plus (see Table 6) to the cell-culture dish. Collect the lysate with a rubber policeman. Pipet the lysate into a microcentrifuge tube (not supplied). Vortex or pipet to mix, and ensure that no cell clumps are visible before proceeding to step 3.**

**Note:** Ensure that β-ME is added to Buffer RLT Plus before use (see "Important points before starting").

**Table 6. Volumes of Buffer RLT Plus for Direct Cell Lysis**

| Dish diameter | Volume of Buffer RLT Plus* |
|---|---|
| <6 cm | 350 µl |
| 6–10 cm | 600 µl |

* Regardless of the cell number, use the buffer volumes indicated to completely cover the surface of the dish.

**3. Homogenize the lysate according to step 3a, 3b, or 3c.**

See "Disrupting and homogenizing starting material", page 15, for more details on homogenization. If processing ≤1 x $10^5$ cells, they can be homogenized by vortexing for 1 min. After homogenization, proceed to step 4.

**Note:** Incomplete homogenization leads to significantly reduced RNA yields and can cause clogging of the RNeasy spin column. Homogenization with a rotor–stator or QIAshredder homogenizer generally results in higher RNA yields than with a syringe and needle.

**3a. Pipet the lysate directly into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 min at maximum speed. Proceed to step 4.**

**3b. Homogenize the lysate for 30 s using a rotor–stator homogenizer. Proceed to step 4.**

**3c. Pass the lysate at least 5 times through a 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe. Proceed to step 4.**

**4. Transfer the homogenized lysate to a gDNA Eliminator spin column placed in a 2 ml collection tube (supplied). Centrifuge for 30 s at ≥8000 x *g* (≥10,000 rpm). Discard the column, and save the flow-through.**

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

5. **Add 1 volume (usually 350 µl or 600 µl) of 70% ethanol to the flow-through, and mix well by pipetting. Do not centrifuge.**

   If some lysate was lost during homogenization and DNA removal, adjust the volume of ethanol accordingly.

   **Note:** When purifying RNA from certain cell lines, precipitates may be visible after addition of ethanol. This does not affect the procedure.

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

   Reuse the collection tube in step 7.

   If the sample volume exceeds 700 µl, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.

7. **Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.***

   Reuse the collection tube in step 8.

   **Note:** After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.

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

   Reuse the collection tube in step 9.

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

9. **Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane.**

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

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

* Flow-through contains Buffer RLT Plus or Buffer RW1 and is therefore not compatible with bleach. See page 6 for safety information.

10. **Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Centrifuge at full speed for 1 min.**

    Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 9.

11. **Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm) to elute the RNA.**

12. **If the expected RNA yield is >30 µg, repeat step 11 using another 30–50 µl of RNase-free water, or using the eluate from step 11 (if high RNA concentration is required). Reuse the collection tube from step 11.**

    If using the eluate from step 11, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.

# Protocol: Purification of Total RNA from Animal Tissues

### Determining the correct amount of starting material

It is essential to use the correct amount of starting material in order to obtain optimal RNA yield and purity. A maximum amount of 30 mg fresh or frozen tissue or 15–20 mg RNA*later* stabilized tissue (which is partially dehydrated) can generally be processed. For most tissues, the DNA removal capacity of the gDNA Eliminator spin column, the RNA binding capacity of the RNeasy spin column, and the lysing capacity of Buffer RLT Plus will not be exceeded by these amounts. However, smaller amounts may allow more efficient DNA removal. Average RNA yields from various tissues are given in Table 2 (page 13).

For maximum RNA yields from liver, 50% ethanol (instead of 70% ethanol) should be used in step 5 of the procedure.

Some tissues, such as spleen and thymus, contain very high amounts of DNA, which will overload the gDNA Eliminator spin column. For these tissues, we recommend using the RNeasy Mini Kit in combination with the RNase-Free DNase Set (see page 43 for ordering information).

RNA yields from fibrous tissues, such as skeletal muscle, heart, and skin, may be low due to the abundance of contractile proteins, connective tissue, and collagen. Proteinase K digestion is not compatible with the RNeasy Plus Mini Kit. For maximum RNA yields from these tissues, we recommend using RNeasy Fibrous Tissue Kits (see page 43 for ordering information).

If there is no information about the nature of your starting material, we recommend starting with no more than 10 mg tissue. Depending on RNA yield and purity, it may be possible to use up to 30 mg tissue in subsequent preparations.

**Do not overload the gDNA Eliminator spin column, as this will lead to copurification of DNA with RNA. Do not overload the RNeasy spin column, as this will significantly reduce RNA yield and quality.**

## Important points before starting

- If using the RNeasy Plus Mini Kit for the first time, read "Important Notes" (page 12).
- If preparing RNA for the first time, read Appendix A (page 35).
- For optimal results, stabilize harvested tissues immediately in RNA*later* RNA Stabilization Reagent (see the *RNA*later *Handbook*). Tissues can be stored in the reagent for up to 1 day at 37°C, 7 days at 18–25°C, or 4 weeks at 2–8°C, or archived at –20°C or –80°C.
- Fresh, frozen, or RNA*later* stabilized tissues can be used. Tissues can be stored at –70°C for several months. Flash-freeze tissues in liquid nitrogen, and immediately transfer to –70°C. Do not allow tissues to thaw during weighing or handling prior to disruption in Buffer RLT Plus. Homogenized tissue lysates from step 3 can also be stored at –70°C for several months. Incubate frozen lysates at 37°C in a water bath until completely thawed and salts are dissolved before continuing with step 4. Avoid prolonged incubation, which may compromise RNA integrity.
- If desired, more than 30 mg tissue can be disrupted and homogenized at the start of the procedure (increase the volume of Buffer RLT Plus proportionately). Use a portion of the homogenate corresponding to no more than 30 mg tissue for RNA purification, and store the rest at –80°C.
- β-mercaptoethanol (β-ME) must be added to Buffer RLT Plus before use. Add 10 µl β-ME per 1 ml Buffer RLT Plus. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT Plus is stable at room temperature (15–25°C) for 1 month after addition of β-ME.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.
- Buffer RLT Plus may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature.
- Buffer RLT Plus and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 6 for safety information.
- Perform all steps of the procedure at room temperature. During the procedure, work quickly.
- Perform all centrifugation steps at 20–25°C in a standard microcentrifuge. Ensure that the centrifuge does not cool below 20°C.

**Procedure**

1. **Excise the tissue sample from the animal or remove it from storage. Remove RNA*later* stabilized tissues from the reagent using forceps. Determine the amount of tissue. Do not use more than 30 mg.**

   Weighing tissue is the most accurate way to determine the amount.

2. **Follow either step 2a or 2b.**

**2a. For RNA*later* stabilized tissues:**

**If using the entire tissue, place it directly into a suitably sized vessel for disruption and homogenization, and proceed to step 3.**

**If using only a portion of the tissue, cut it on a clean surface. Weigh the piece to be used, and place it into a suitably sized vessel for disruption and homogenization. Proceed to step 3.**

RNA in RNA*later* stabilized tissues is protected during cutting and weighing of tissues at ambient temperature (18–25°C). It is not necessary to cut the tissues on ice or dry ice or in a refrigerated room. Remaining tissues can be stored in RNA*later* Reagent. Previously stabilized tissues can be stored at –80°C without the reagent.

**2b. For unstabilized fresh or frozen tissues:**

**If using the entire tissue, place it directly into a suitably sized vessel for disruption and homogenization, and proceed immediately to step 3.**

**If using only a portion of the tissue, weigh the piece to be used, and place it into a suitably sized vessel for disruption and homogenization. Proceed immediately to step 3.**

RNA in harvested tissues is not protected until the tissues are treated with RNA*later* Reagent, flash-frozen, or disrupted and homogenized in step 3. Frozen tissues should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible.

**Note:** Remaining fresh tissues can be placed into RNA*later* Reagent to stabilize RNA (see the *RNA*later *Handbook*). However, previously frozen tissues thaw too slowly in the reagent, preventing the reagent from diffusing into the tissues quickly enough to prevent RNA degradation.

3. **Disrupt the tissue and homogenize the lysate in Buffer RLT Plus (do not use more than 30 mg tissue) according to step 3a, 3b, 3c, or 3d.**

   See "Disrupting and homogenizing starting material", page 15, for more details on disruption and homogenization.

   **Note:** Ensure that β-ME is added to Buffer RLT Plus before use (see "Important points before starting").

After storage in RNA*later* Reagent, tissues may become slightly harder than fresh or thawed tissues. Disruption and homogenization using standard methods is usually not a problem. For easier disruption and homogenization, we recommended using 600 µl Buffer RLT Plus.

**Note:** Incomplete homogenization leads to significantly reduced RNA yields and can cause clogging of the RNeasy spin column. Homogenization with the TissueLyser and rotor–stator homogenizers generally results in higher RNA yields than with other methods.

**Table 7. Volumes of Buffer RLT Plus for Tissue Disruption and Homogenization**

| Amount of starting material | Volume of Buffer RLT Plus |
|---|---|
| <20 mg | 350 µl or 600 µl* |
| 20–30 mg | 600 µl |

* Use 600 µl Buffer RLT Plus for tissues stabilized in RNA*later* Reagent or for difficult-to-lyse tissues.

**3a. Disruption and homogenization using a rotor–stator homogenizer: Place the weighed (fresh, frozen, or RNA*later* stabilized) tissue in a suitably sized vessel. Add the appropriate volume of Buffer RLT Plus (see Table 7). Immediately disrupt and homogenize the tissue using a conventional rotor–stator homogenizer until it is uniformly homogeneous (usually 20–40 s). Proceed to step 4.**

**3b. Disruption using a mortar and pestle followed by homogenization using a QIAshredder homogenizer:**
**Immediately place the weighed (fresh, frozen, or RNA*later* stabilized) tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled, 2 ml microcentrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw.**

**Add the appropriate volume of Buffer RLT Plus (see Table 7). Pipet the lysate directly into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 min at maximum speed. Proceed to step 4.**

**3c. Disruption using a mortar and pestle followed by homogenization using a needle and syringe:**
**Immediately place the weighed (fresh, frozen, or RNA*later* stabilized) tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled, 2 ml microcentrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw.**

**Add the appropriate volume of Buffer RLT Plus (see Table 7), and homogenize by passing lysate at least 5 times through a 20-gauge needle fitted to an RNase-free syringe. Proceed to step 4.**

**3d. Disruption and homogenization using the TissueLyser:**
**See the *TissueLyser Handbook*. Then proceed to step 4.**

**4. Centrifuge the lysate for 3 min at maximum speed. Carefully remove the supernatant by pipetting, and transfer it to a gDNA Eliminator spin column placed in a 2 ml collection tube (supplied). Centrifuge for 30 s at ≥8000 x *g* (≥10,000 rpm). Discard the column, and save the flow-through.**

This step is important, as it removes insoluble material that could clog the gDNA Eliminator spin column and interfere with DNA removal. In some preparations, very small amounts of insoluble material will be present after the 3 min centrifugation, making the pellet invisible.

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

**5. Add 1 volume (usually 350 µl or 600 µl) of 70% ethanol to the flow-through, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 6.**

If some lysate was lost during homogenization and DNA removal, adjust the volume of ethanol accordingly.

**Note:** Precipitates may be visible after addition of ethanol, but this does not affect the procedure.

**Note:** For maximum RNA yields from liver, use 50% ethanol instead of 70% ethanol.

6. **Transfer up to 700 µl of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flowthrough.***

   Reuse the collection tube in step 7.

   If the sample volume exceeds 700 µl, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.

7. **Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.***

   Reuse the collection tube in step 8.

   **Note:** After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.

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

   Reuse the collection tube in step 9.

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

9. **Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane.**

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

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

10. **Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Centrifuge at full speed for 1 min.**

    Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 9.

* Flow-through contains Buffer RLT Plus or Buffer RW1 and is therefore not compatible with bleach. See page 6 for safety information.

**11. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm) to elute the RNA.**

**12. If the expected RNA yield is >30 µg, repeat step 11 using another 30–50 µl of RNase-free water, or using the eluate from step 11 (if high RNA concentration is required). Reuse the collection tube from step 11.**

If using the eluate from step 11, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.

# Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or molecular biology applications (see back cover for contact information).

| | Comments and suggestions |
|---|---|
| **Clogged gDNA Eliminator spin column** | |
| a) Inefficient disruption and/or homogenization | See "Disrupting and homogenizing starting material" (page 15) for details on disruption and homogenization methods.<br><br>Increase *g*-force and centrifugation time if necessary.<br><br>In subsequent preparations, reduce the amount of starting material (see protocols, pages 18 and 24) and/or increase the homogenization time. |
| b) Too much starting material | Reduce the amount of starting material. It is essential to use the correct amount of starting material (see page 12). |
| c) Centrifugation temperature too low | The centrifugation temperature should be 20–25°C. Some centrifuges may cool to below 20°C even when set at 20°C. This can cause formation of precipitates that can clog the gDNA Eliminator spin column. If this happens, set the centrifugation temperature to 25°C. Warm the lysate to 37°C before transferring it to the gDNA Eliminator spin column. |
| **Low RNA yield** | |
| a) Insufficient disruption and homogenization | See "Disrupting and homogenizing starting material" (page 15) for details on disruption and homogenization methods.<br><br>In subsequent preparations, reduce the amount of starting material (see protocols, pages 18 and 24) and/or increase the volume of lysis buffer and the homogenization time. |
| b) Too much starting material | Overloading the RNeasy spin column significantly reduces RNA yield. Reduce the amount of starting material (see page 12). |

| | Comments and suggestions |
|---|---|
| c) Ethanol added to lysate before DNA removal | Pass the lysate through the gDNA Eliminator spin column before adding ethanol to it. |
| d) RNA still bound to RNeasy spin column membrane | Repeat RNA elution, but incubate the RNeasy spin column on the benchtop for 10 min with RNase-free water before centrifuging. |
| e) Ethanol carryover | During the second wash with Buffer RPE, be sure to centrifuge at ≥8000 x *g* (≥10,000 rpm) for 2 min at 20–25°C to dry the RNeasy spin column membrane.<br><br>Perform the optional centrifugation to dry the RNeasy spin column membrane if any flow-through is present on the outside of the column (step 10 of the protocols). |
| f) Incomplete removal of cell-culture medium (cell samples) | When processing cultured cells, ensure complete removal of cell-culture medium after harvesting cells (see protocol, page 18). |
| **Low $A_{260}/A_{280}$ value** | |
| Water used to dilute RNA for $A_{260}/A_{280}$ measurement | Use 10 mM Tris·Cl,* pH 7.5, not RNase-free water, to dilute the sample before measuring purity (see Appendix B, page 37). |
| **RNA degraded** | |
| a) Inappropriate handling of starting material | Ensure that tissue samples are properly stabilized and stored in RNA*later* RNA Stabilization Reagent.<br><br>For frozen cell pellets or frozen tissue samples, ensure that they were flash-frozen immediately in liquid nitrogen and properly stored at –70°C. Perform the RNeasy procedure quickly, especially the first few steps.<br><br>See Appendix A (page 35) and "Handling and storing starting material" (page 15). |

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

| | Comments and suggestions |
|---|---|
| b) RNase contamination | Although all RNeasy buffers have been tested and are guaranteed RNase-free, RNases can be introduced during use. Be certain not to introduce any RNases during the RNeasy procedure or later handling. See Appendix A (page 35) for general remarks on handling RNA. |

**DNA contamination in downstream experiments**

| | |
|---|---|
| a) Cell number too high | For some cell types, the efficiency of DNA removal may be reduced when processing very high cell numbers (containing more than 20 µg genomic DNA). If the eluted RNA contains substantial DNA contamination, try processing smaller cell numbers. |
| b) Incomplete removal of cell-culture medium or stabilization reagent | Be sure to remove any excess cell-culture medium or stabilization reagent to prevent significant dilution of the lysis buffer. The gDNA Eliminator spin column will not work effectively if the lysis buffer is diluted. |
| c) Tissue has high DNA content | For certain tissues with extremely high DNA content (e.g., thymus), DNA may not be completey removed. Try using smaller samples (containing less than 20 µg genomic DNA), or perform DNase digestion of the eluted RNA followed by RNA cleanup. |

**RNA concentration too low**

| | |
|---|---|
| Elution volume too high | Elute RNA with less than 2 x 50 µl of water. Do not use less than 1 x 30 µl of water. Although eluting with less than 2 x 50 µl of water results in increased RNA concentrations, RNA yields may be reduced. |

**RNA does not perform well in downstream experiments**

| | |
|---|---|
| a) Salt carryover during elution | Ensure that Buffer RPE is at 20–30°C.<br>When reusing collection tubes between washing steps, remove residual flow-through from the rim by blotting on clean paper towels. |

| | Comments and suggestions |
|---|---|
| b) Ethanol carryover | During the second wash with Buffer RPE, be sure to centrifuge at ≥8000 x *g* (≥10,000 rpm) for 2 min at 20–25°C to dry the RNeasy spin column membranes. After centrifugation, carefully remove the column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.<br><br>Perform the optional centrifugation to dry the RNeasy spin column membrane if any flow-through is present on the outside of the column (step 10 of the protocols). |

# Appendix A: General Remarks on Handling RNA

## Handling RNA

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

## General handling

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

## Disposable plasticware

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

## Nondisposable plasticware

Nondisposable plasticware should be treated before use to ensure that it is RNase-free. Plasticware should be thoroughly rinsed with 0.1 M NaOH,* 1 mM EDTA* followed by RNase-free water (see "Solutions", page 36). Alternatively, chloroform-resistant plasticware can be rinsed with chloroform* to inactivate RNases.

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

### Glassware

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

### Electrophoresis tanks

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

### Solutions

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

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

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

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

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

## Storage of RNA

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

## Quantification of RNA

The concentration of RNA should be determined by measuring the absorbance at 260 nm ($A_{260}$) in a spectrophotometer (see "Spectrophotometric quantification of RNA" below). For small amounts of RNA, however, it may be difficult to determine amounts photometrically. Small amounts of RNA can be accurately quantified using an Agilent 2100 bioanalyzer, quantitative RT-PCR, or fluorometric quantification.

## Spectrophotometric quantification of RNA

To ensure significance, $A_{260}$ readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 44 µg of RNA per ml ($A_{260}=1 \rightarrow 44$ µg/ml). This relation is valid only for measurements at a neutral pH. Therefore, if it is necessary to dilute the RNA sample, this should be done in a buffer with neutral pH.* As discussed below (see "Purity of RNA", page 38), the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity.

When measuring RNA samples, be certain that cuvettes are RNase-free, especially if the RNA is to be recovered after spectrophotometry. This can be accomplished by washing cuvettes with 0.1 M NaOH,* 1 mM EDTA,* followed by washing with RNase-free water (see "Solutions", page 36). Use the buffer in which the RNA is diluted to zero the spectrophotometer. An example of the calculation involved in RNA quantification is shown below:

Volume of RNA sample = 100 µl

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

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

$A_{260}$ = 0.2

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

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

### Purity of RNA

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

For determination of RNA concentration, however, we recommend dilution of the sample in a buffer with neutral pH since the relationship between absorbance and concentration ($A_{260}$ reading of 1 = 44 µg/ml RNA) is based on an extinction coefficient calculated for RNA at neutral pH (see "Spectrophotometric quantification of RNA", page 37).

### DNA contamination

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

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

To prevent any interference by DNA in real-time RT-PCR applications, such as with ABI PRISM® and LightCycler® instruments, we recommend designing primers that anneal at intron splice junctions so that genomic DNA will not be amplified. QuantiTect Assays from QIAGEN are designed for real-time RT-PCR analysis of RNA sequences (without detection of genomic DNA) where possible.

* 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.

## 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 or by using an Agilent 2100 bioanalyzer. The respective ribosomal RNAs should appear as sharp bands or peaks. The apparent ratio of 28S rRNA to 18S RNA should be approximately 2:1. If the ribosomal bands or peaks of a specific sample are not sharp, but appear as a smear towards smaller sized RNAs, it is likely that the sample suffered major degradation either before or during RNA purification.

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

# Appendix C: Protocol for Formaldehyde Agarose Gel Electrophoresis

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

### FA gel preparation

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

1.2 g agarose*

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

Add RNase-free water to 100 ml

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

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

### RNA sample preparation for FA gel electrophoresis

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

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

### Gel running conditions

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

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

## Composition of FA gel buffers

### 10x FA gel buffer

| | |
|---|---|
| 200 mM | 3-[N-morpholino]propanesulfonic acid (MOPS) (free acid)* |
| 50 mM | sodium acetate* |
| 10 mM | EDTA* |
| pH to 7.0 with NaOH* | |

### 1x FA gel running buffer

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

### 5x RNA loading buffer

| | |
|---|---|
| 16 µl | saturated aqueous bromophenol blue solution*† |
| 80 µl | 500 mM EDTA, pH 8.0 |
| 720 µl | 37% (12.3 M) formaldehyde |
| 2 ml | 100% glycerol* |
| 3.084 ml | formamide* |
| 4 ml | 10 x FA gel buffer |
| RNase-free water to 10 ml | |
| Stability: approximately 3 months at 4°C | |

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

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

# References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc.

For a complete list of references, visit the QIAGEN Reference Database online at www.qiagen.com/RefDB/search.asp or contact QIAGEN Technical Services or your local distributor.

# Ordering Information

| Product | Contents | Cat. no. |
|---|---|---|
| RNeasy Plus Mini Kit (50) | 50 RNeasy Mini Spin Columns, 50 gDNA Eliminator Mini Spin Columns, Collection Tubes, RNase-Free Reagents and Buffers | 74134 |
| **Accessories** | | |
| Collection Tubes (2 ml) | 1000 x 2 ml Collection Tubes | 19201 |
| RNA*later* RNA Stabilization Reagent (50 ml) | For stabilization of RNA in 25 x 200 mg tissue samples: 50 ml RNA*later* RNA Stabilization Reagent | 76104 |
| RNA*later* RNA Stabilization Reagent (250 ml) | For stabilization of RNA in 125 x 200 mg tissue samples: 250 ml RNA*later* RNA Stabilization Reagent | 76106 |
| RNA*later* TissueProtect Tubes (50 x 1.5 ml) | For stabilization of RNA in 50 x 150 mg tissue samples: 50 screw-top tubes containing 1.5 ml RNA*later* RNA Stabilization Reagent each | 76154 |
| RNA*later* TissueProtect Tubes (20 x 5 ml) | For stabilization of RNA in 20 x 500 mg tissue samples: 20 screw-top tubes containing 5 ml RNA*later* RNA Stabilization Reagent each | 76163 |
| QIAshredder (50)* | 50 disposable cell-lysate homogenizers | 79654 |
| TissueLyser† | Universal laboratory mixer-mill disruptor | Inquire |
| **Related products for RNA purification** | | |
| **RNeasy Kits — for purification of total RNA from animal cells or tissues, yeast, or bacteria** | | |
| RNeasy Mini Kit (50)* | 50 RNeasy Mini Spin Columns, Collection Tubes, RNase-Free Reagents and Buffers | 74104 |

* Larger kit size available; see www.qiagen.com .

† Visit www.qiagen.com/products/accessories for details about the TissueLyser and accessories.

| Product | Contents | Cat. no. |
|---|---|---|
| RNeasy Midi Kit (10)*† | 10 RNeasy Midi Spin Columns, Collection Tubes, RNase-Free Reagents and Buffers | 75142 |
| RNeasy Maxi Kit (12)† | 12 RNeasy Maxi Spin Columns, Collection Tubes, RNase-Free Reagents and Buffers | 75162 |
| **RNase-Free DNase Set — for DNase digestion during RNA purification** | | |
| RNase-Free DNase Set (50) | For 50 RNA minipreps: 1500 units RNase-Free DNase I, RNase-Free Buffer RDD, and RNase-Free Water | 79254 |
| **RNeasy MinElute® Cleanup Kit — for RNA cleanup and concentration with small elution volumes** | | |
| RNeasy MinElute Cleanup Kit (50) | 50 RNeasy MinElute Spin Columns, Collection Tubes, RNase-Free Reagents and Buffers | 74204 |
| **RNeasy Fibrous Tissue Kits — for purification of total RNA from fiber-rich tissues** | | |
| RNeasy Fibrous Tissue Mini Kit (50) | 50 RNeasy Mini Spin Columns, Collection Tubes, Proteinase K, RNase-Free DNase I, RNase-Free Reagents and Buffers | 74704 |
| RNeasy Fibrous Tissue Midi Kit (10)† | 10 RNeasy Midi Spin Columns, Collection Tubes, Proteinase K, RNase-Free DNase I, RNase-Free Reagents and Buffers | 75742 |
| **Related products for downstream applications** | | |
| **Omniscript® RT Kit — for reverse transcription using 50 ng to 2 µg RNA per reaction** | | |
| Omniscript RT Kit (50)* | For 50 x 20 µl reactions: Omniscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix, RNase-Free Water | 205111 |

* Larger kit size available; see www.qiagen.com .

† Requires use of a centrifuge capable of attaining 3000–5000 x *g* equipped with a swing-out rotor for 15 ml (Midi) or 50 ml (Maxi) centrifuge tubes.

| Product | Contents | Cat. no. |
|---|---|---|
| **Sensiscript® RT Kit — for reverse transcription using less than 50 ng RNA per reaction** | | |
| Sensiscript RT Kit (50)* | For 50 x 20 µl reactions: Sensiscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix, RNase-Free Water | 205211 |
| **QuantiTect Reverse Transcription Kit — for fast cDNA synthesis for sensitive real-time two-step RT-PCR** | | |
| QuantiTect Reverse Transcription Kit (50) | For 50 x 20 µl reactions: gDNA Wipeout Buffer, Quantiscript® Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, RNase-Free Water | 205311 |
| **QuantiTect SYBR® Green PCR Kit — for quantitative, real-time, two-step RT-PCR using SYBR Green I** | | |
| QuantiTect SYBR Green PCR Kit (200)*† | For 200 x 50 µl reactions: 3 x 1.7 ml 2x Master Mix, 2 x 2 ml RNase-Free Water | 204143 |
| **QuantiTect SYBR Green RT-PCR Kit — for quantitative, real-time, one-step RT-PCR using SYBR Green I** | | |
| QuantiTect SYBR Green RT-PCR Kit (200)*† | For 200 x 50 µl reactions: 3 x 1.7 ml 2x Master Mix, 100 µl RT Mix, 2 x 2 ml RNase-Free Water | 204243 |
| **QuantiTect Probe PCR Kit — for quantitative, real-time, two-step RT-PCR using sequence-specific probes** | | |
| QuantiTect Probe PCR Kit (200)*† | For 200 x 50 µl reactions: 3 x 1.7 ml 2x Master Mix, 2 x 2 ml RNase-Free Water | 204343 |
| **QuantiTect Probe RT-PCR Kit — for quantitative, real-time, one-step RT-PCR using sequence-specific probes** | | |
| QuantiTect Probe RT-PCR Kit (200)*† | For 200 x 50 µl reactions: 3 x 1.7 ml 2x Master Mix, 100 µl RT Mix, 2 x 2 ml RNase-Free Water | 204443 |

* Larger kit size available; see www.qiagen.com .

† Visit www.qiagen.com/GeneGlobe to search for and order primer sets or primer–probe sets.

| Product | Contents | Cat. no. |
|---|---|---|
| **QuantiTect Multiplex PCR Kits — for quantitative, multiplex, real-time, two-step RT-PCR using sequence-specific probes** | | |
| QuantiTect Multiplex PCR Kit (200)*†‡ | For 200 x 50 µl reactions: 3 x 1.7 ml 2x Master Mix (contains ROX dye), 2 x 2 ml RNase-Free Water | 204543 |
| QuantiTect Multiplex PCR NoROX Kit (200)*†§ | For 200 x 50 µl reactions: 3 x 1.7 ml 2x Master Mix (contains no ROX dye), 2 x 2 ml RNase-Free Water | 204743 |
| **QuantiTect Multiplex RT-PCR Kits — for quantitative, multiplex, real-time, one-step RT-PCR using sequence-specific probes** | | |
| QuantiTect Multiplex RT-PCR Kit (200)*†‡ | For 200 x 50 µl reactions: 3 x 1.7 ml 2x Master Mix (contains ROX dye), 100 µl RT Mix, 2 x 2 ml RNase-Free Water | 204643 |
| QuantiTect Multiplex RT-PCR NR Kit (200)*†§ | For 200 x 50 µl reactions: 3 x 1.7 ml 2x Master Mix (contains no ROX dye), 100 µl RT Mix, 2 x 2 ml RNase-Free Water | 204843 |

* Larger kit size available; see www.qiagen.com .

† Visit www.qiagen.com/GeneGlobe to search for and order primer–probe sets.

‡ Recommended for ABI PRISM and Applied Biosystems® cyclers.

§ Recommended for all other cyclers.

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