

## RNeasy<sup>®</sup> Mini Handbook

RNeasy Mini Kit

RNeasy Plant Mini Kit

For total RNA minipreps from

animal cells

animal tissues

bacteria

yeast

plants

filamentous fungi

For RNA cleanup

May 1999



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<b>Germany</b>	<b>QIAGEN GmbH</b>	Max-Volmer-Straße 4 • 40724 Hilden Orders 02103-892-230 • Fax 02103-892-233 • Technical 02103-892-240
<b>USA</b>	<b>QIAGEN Inc.</b>	28159 Avenue Stanford • Valencia • CA 91355 Orders 800-426-8157 • Fax 800-718-2056 • Technical 800-DNA-PREP (800-362-7737)
<b>Australia</b>	<b>QIAGEN Pty Ltd</b> ACN 072 382 944	PO Box 25 • Clifton Hill • Victoria 3068 Orders 03-9489-3666 • Fax 03-9489-3888 • Technical 03-9489-3666
<b>Canada</b>	<b>QIAGEN Inc.</b>	2900 Argentia Road • Unit 23 • Mississauga • Ontario • L5N 7X9 Orders 800-572-9613 • Fax 800-713-5951 • Technical 800-DNA-PREP (800-362-7737)
<b>France</b>	<b>QIAGEN S. A.</b>	3 avenue du Canada • LP 809 • 91974 Courtaboeuf Cedex Orders 01-60-920-920 • Fax 01-60-920-925 • Technical 01-60-920-930
<b>Japan</b>	<b>QIAGEN K.K.</b>	Hakusan Takayanagi Bldg. 8F • 7-6, Hakusan 1 Chome • Bunkyo-ku, Tokyo 113-0001 Telephone 03-5805-7261 • Fax 03-5805-7263 • Technical 03-5805-7261
<b>Switzerland</b>	<b>QIAGEN AG</b>	Auf dem Wolf 39 • 4052 Basel Orders 061-319-30-30 • Fax 061-319-30-33 • Technical 061-319-30-31
<b>UK</b>	<b>QIAGEN Ltd.</b>	Boundary Court • Gatwick Road • Crawley • West Sussex, RH10 2AX Orders 01293-422-911 • Fax 01293-422-922 • Technical 01293-422-999

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# Kit Contents

<b>RNeasy Mini Kits*</b>	<b>(20)</b>	<b>(50)</b>	<b>(250)</b>
<b>Catalog No.</b>	<b>74103</b>	<b>74104</b>	<b>74106</b>
Preparations per Kit	20	50	250
RNeasy Mini Spin Columns (pink)	20	50	250
Collection Tubes (1.5-ml)	20	50	250
Collection Tubes (2-ml)	20	50	250
Buffer RLT <sup>†</sup>	18 ml	45 ml	220 ml
Buffer RW1 <sup>†</sup>	18 ml	45 ml	220 ml
Buffer RPE <sup>‡</sup>	5 ml	11 ml	55 ml
RNase-free Water	10 ml	10 ml	50 ml
Handbook	1	1	1

<b>RNeasy Plant Mini Kits*</b>	<b>(20)</b>	<b>(50)</b>
<b>Catalog No.</b>	<b>74903</b>	<b>74904</b>
Preparations per Kit	20	50
RNeasy Mini Spin Columns (pink)	20	50
QIAshredder Spin Columns (lilac)	20	50
Collection Tubes (1.5-ml)	20	50
Collection Tubes (2-ml)	20	50
Buffer RLT <sup>†</sup>	18 ml	45 ml
Buffer RLC <sup>†</sup>	18 ml	45 ml
Buffer RW1 <sup>†</sup>	18 ml	45 ml
Buffer RPE <sup>‡</sup>	5 ml	11 ml
RNase-free Water	10 ml	10 ml
Handbook	1	1

\* Buffers available separately as RNeasy Buffer Set. Buffer RLT available separately. See ordering information (page 61).

<sup>†</sup> Not compatible with disinfecting reagents containing bleach. Contains a guanidine salt which is an irritant. Take appropriate safety measures.

<sup>‡</sup> Buffer RPE is supplied as a concentrate. Before using for the first time add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.

## Storage Conditions

RNeasy® Kits should be stored dry at room temperature (15 to 25°C) and are stable for at least 9 months under these conditions.

## Product Use Limitations

RNeasy Kits are developed, designed, and sold for research purposes only. They are not to be used for human diagnostic or drug purposes or to be administered to humans unless expressly cleared for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of many of the materials described in this text.

## 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.

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 (listed on the last page).

## 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 RNeasy 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 listed on the last page.

## Introduction

RNeasy Mini Kits and RNeasy Plant Mini Kits are designed to isolate total RNA from small quantities of starting material. They provide a fast and simple method for the preparation of up to 100 µg of total RNA from animal cells and tissues, bacteria, and yeast (**RNeasy Mini Kits**) or plant cells and tissues and filamentous fungi (**RNeasy Plant Mini Kits**). In addition, all RNeasy Kits can be used to desalt or to purify RNA from enzymatic reactions such as DNase digestion, proteinase digestion, RNA ligation, and labeling reactions.

RNeasy Mini Kits and RNeasy Plant Mini Kits make multiple, simultaneous processing of a wide variety of biological samples possible in less than 30 min. 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 procedure. The purified RNA is ready for use in standard downstream applications such as:

- RT-PCR
- Northern, dot, and slot blotting
- Poly A<sup>+</sup> RNA selection
- Primer extension
- RNase/S1 nuclease protection
- cDNA synthesis
- Differential display

A list of references describing the use of RNeasy Kits in a variety of applications can be found on page 52.

## The RNeasy Principle and Procedure

The RNeasy procedure represents a novel technology for RNA isolation. This technology combines the selective binding properties of a silica-gel-based membrane with the speed of microspin technology. A specialized high-salt buffer system allows up to 100 µg of RNA longer than 200 bases to bind to the RNeasy silica-gel membrane. Biological samples are first lysed and homogenized in the presence of a highly denaturing guanidine isothiocyanate (GITC)-containing buffer which immediately inactivates RNases to ensure isolation of intact RNA. Ethanol is added to provide appropriate binding conditions and the sample is then applied to an RNeasy mini spin column where the 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.

The RNeasy Mini and RNeasy Plant Mini procedures isolate all RNA molecules longer than 200 nucleotides. Small RNAs such as 5.8S RNA, 5S RNA, and tRNAs, approximately 160, 120, and 70–90 nucleotides in length respectively, will not bind quantitatively under the conditions used. Since low-molecular-weight RNA species make up 15–20% of the total RNA, the RNeasy procedure enriches for larger RNA molecules. Therefore, the size distribution of RNA isolated with the RNeasy procedure is comparable to that obtained

by centrifugation through a CsCl cushion, because small RNAs do not sediment efficiently during centrifugation through CsCl.

In this handbook separate protocols are provided for the various starting materials. The protocols differ primarily in the lysis and homogenization of the sample, and in the adjustment of the conditions for binding RNA to the RNeasy membrane. Once the sample is bound to the membrane, the protocols are similar (see flowchart, page 9).

### **Isolation of Total RNA from Animal Cells and Tissues**

Samples (maximum  $1 \times 10^7$  cells or 30 mg tissue, see pages 12–13) are disrupted in lysis buffer containing GITC (Buffer RLT) and homogenized. An overview of disruption and homogenization methods is given on page 16. Ethanol is then added to the lysate, creating conditions which promote selective binding of RNA to the RNeasy membrane. The sample is then applied to the RNeasy mini spin column. Total RNA binds to the membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in water.

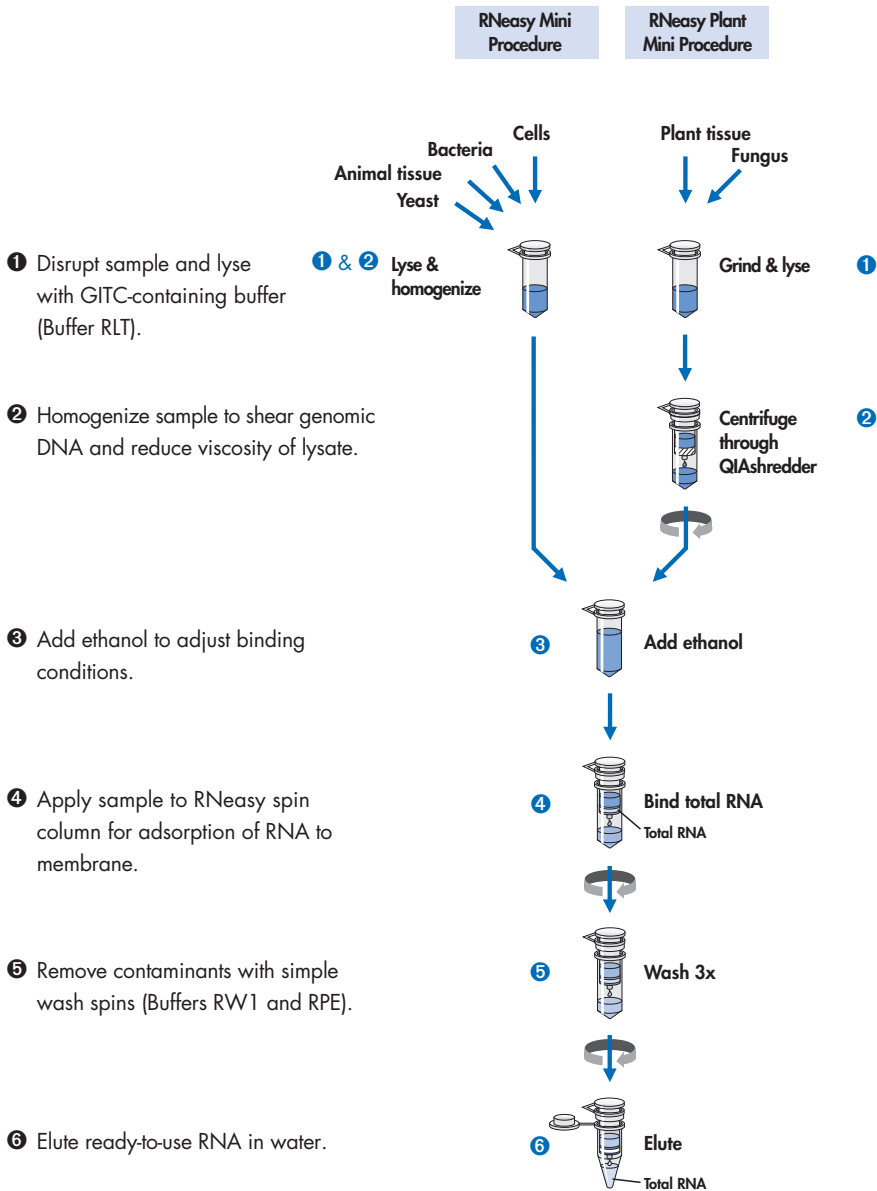
### **Isolation of Cytoplasmic RNA from Animal Cells**

Since the cytoplasm contains RNA in its mature form, this protocol is particularly advantageous in applications where unspliced or partially spliced RNA is not desirable. Cytoplasmic RNA accounts for approximately 85% of total cellular RNA. This protocol is also optimal in applications where the absence of DNA contamination is critical, since the nuclei are removed and no genomic DNA is released.

Cultured cells (maximum  $1 \times 10^7$ ) are lysed in a buffer (Buffer RLN) containing the non-ionic detergent Nonidet® P-40 which lyses the plasma membrane. Nuclei remain intact during the lysis procedure and are removed by centrifugation. Lysis buffer (Buffer RLT) and ethanol are added to the supernatant to provide optimal conditions for selectively binding RNA to the RNeasy membrane. The sample is then applied to the RNeasy mini spin column. Total RNA binds to the membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in water.

### **Isolation of Total RNA from Bacteria**

Bacteria (maximum  $1 \times 10^9$  cells) are incubated in a buffer containing lysozyme to digest the bacterial cell wall prior to lysis. After addition of GITC-containing lysis buffer (Buffer RLT) and ethanol the sample is loaded onto an RNeasy mini spin column. Total RNA binds to the membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in water.



## Isolation of Total RNA from Yeast

Two different protocols are provided for the isolation of total RNA from yeast (maximum  $5 \times 10^7$  cells) using RNeasy Mini Kits. The protocols differ primarily in the way the yeast cell walls are disrupted (enzymatically or mechanically). In general, the protocols function equally well. For some applications the Enzymatic Lysis Protocol may be preferable as no additional laboratory equipment is required. However, the Mechanical Disruption Protocol can be used in time-course experiments where enzymatic incubation steps cannot be tolerated.

### Enzymatic Lysis Protocol (Standard and Abbreviated Version)

This protocol uses zymolase or lyticase digestion of the cell walls to convert cells to spheroplasts which are processed using the RNeasy Mini Kit. In the standard protocol, spheroplasts are separated from the digestion mixture before lysis by centrifugation. In the abbreviated version of this protocol, for use with up to  $2 \times 10^7$  cells, the digestion mixture is used directly in the RNeasy procedure without prior separation of the spheroplasts. Spheroplasts are lysed in GITC-containing lysis buffer (Buffer RLT) and ethanol is added to the digestion mixture to provide optimal conditions for selectively binding RNA to the RNeasy membrane. The sample is then applied to the RNeasy mini spin column. Total RNA binds to the membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in water.

### Mechanical Disruption Protocol

Using this protocol, yeast cells are lysed and homogenized by mechanical disruption during high-speed agitation in a bead mill in the presence of glass beads and GITC-containing lysis buffer (Buffer RLT). Ethanol is added to the lysate creating conditions which promote selective binding of RNA to the RNeasy membrane. The sample is then applied to the RNeasy mini spin column. Total RNA binds to the membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in water.

## Isolation of Total RNA from Plant Cells and Tissues and Filamentous Fungi

In the RNeasy Plant and Fungi Protocol, samples (maximum 100 mg) are first ground in liquid nitrogen and then lysed under highly denaturing conditions. The RNeasy Plant Mini Kit includes a choice of lysis buffers, Buffer RLT and Buffer RLC, which contain GITC or guanidine hydrochloride (GuHCl) respectively. The higher cell disruption and denaturing properties of Buffer RLT frequently make it the buffer of choice. However some tissues, such as milky endosperm of maize or mycelia of filamentous fungi, solidify in this buffer making the extraction of RNA impossible. In these cases Buffer RLC should be used. After lysis with either buffer, samples are centrifuged through QIAshredder™, which is supplied with the RNeasy Plant Mini Kit. This simultaneously removes insoluble material and reduces the viscosity of the lysates by disrupting gelatinous material often formed in plant

and fungal lysates. Ethanol is added to the cleared lysate, creating conditions which promote selective binding of RNA to the RNeasy membrane. The sample is then applied to the RNeasy mini spin column. Total RNA binds to the membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in water.

RNeasy Plant Mini Kits can also be used for total RNA minipreparation from animal cells and tissues, bacteria, and yeast.

### **RNA Cleanup**

RNeasy Mini Kits and RNeasy Plant Mini Kits can be used to purify RNA from enzymatic reactions (e.g. DNase digestion or RNA labeling) or for desalting RNA samples (maximum 100 µg RNA). Lysis buffer and ethanol are added to the sample to provide conditions which promote selective binding of RNA to the RNeasy membrane. The sample is then applied to the RNeasy mini spin column. RNA binds to the membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in water.

# Important Points before Using RNeasy Kits

## How much starting material can I use?

It is essential to use the correct amount of starting material in order to obtain optimal results. The two main factors used to determine the amount of starting material are:

- The volume of Buffer RLT required for efficient lysis
- The RNA binding capacity of the RNeasy mini spin column (100 µg)

For samples containing very high amounts of RNA, this means that smaller amounts of starting material than listed in Table 1 should be used in order to avoid exceeding the RNA binding capacity of the column. Average RNA yields from various sources are provided in Table 2 and can be used as a guide for calculating amounts of starting material.

For samples containing average or low amounts of RNA, the maximum amount of starting material can be used. In these cases, even though the RNA binding capacity of the column may not be reached, no more starting material may be used or lysis will be incomplete, resulting in lower yields and purity.

**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 <sup>7</sup> *
Animal tissue	30 mg*
Bacteria	1 x 10 <sup>9</sup> *
Yeast	5 x 10 <sup>7</sup> *
Plant tissue	100 mg
Filamentous fungi	100 mg

\* For larger amounts RNeasy Midi or Maxi Kits are recommended. Please call our Technical Service Group or see ordering information on page 62.

**Note:** If the RNA binding capacity of the RNeasy mini spin column is exceeded, yields of total RNA will not be consistent and less than 100 µg of total RNA may be recovered. If lysis of the starting material is incomplete, the yield of total RNA will be lower than expected even if the binding capacity of the RNeasy column is not exceeded.

**Table 2. Yields of total RNA with RNeasy Mini or RNeasy Plant Mini Kits**

Source	Average yield of total RNA* (µg)	Source	Average yield of total RNA* (µg)
<b>Cell cultures (1 x 10<sup>6</sup> cells)</b>		<b>Bacteria (1 x 10<sup>9</sup> cells)</b>	
NIH/3T3	10	<i>E. coli</i>	55
HeLa	15	<i>B. subtilis</i>	33
COS-7	35	<b>Yeast (1 x 10<sup>7</sup> cells)</b>	
LMH	12	<i>S. cerevisiae</i>	25
Huh	15	<b>Plant (100 mg leaves)</b>	
<b>Mouse/rat tissues (10 mg)</b>		Arabidopsis	35
Embryo (day 13 p.c.)	25	Maize	25
Brain	8	Tomato	65
Heart	10	Tobacco	60
Kidney	35		
Liver	40		
Spleen	35		
Thymus	45		
Lung	10		

\* Amounts can vary due to developmental stage, growth conditions used, etc. Since RNeasy enriches for RNA >200 bases long, RNA yield does not include 5S RNA, tRNA, and other low-molecular-weight RNAs.

**Note:** If the starting material you are using is not shown in Table 2 and you have no information regarding RNA content, we recommend beginning with half the maximum amount of starting material indicated in Table 1. Depending on the yield obtained, the sample size may be increased in subsequent preparations.

### Examples of how to determine the correct amount of starting material

COS cells: High RNA content (approximately 35 µg RNA per 1 x 10<sup>6</sup> cells)

→ no more than 3 x 10<sup>6</sup> cells can be used, otherwise the RNA binding capacity of the RNeasy mini spin column will be exceeded

Rat lung: Low RNA content (approximately 10 µg RNA per 10 mg tissue)

→ no more than 30 mg of tissue can be used (maximum amount which can be efficiently lysed) even though the RNA binding capacity of the RNeasy mini spin column will not be reached

## How to quantitate my starting material

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 dishes after confluent growth is given in Table 3.

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

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

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

† Cell numbers given are for HeLa cells (approximate length = 15 µm) assuming confluent growth. Cell numbers vary since animal cells can vary in length from 10–100 µm.

### Animal tissue

A 3-mm cube (27 mm<sup>3</sup>) of most animal tissues weighs 30–35 mg.

### Bacteria and yeast

Bacterial and yeast growth are usually measured using a spectrophotometer. However, it is very difficult to give specific and reliable recommendations for the relations

between OD values and cell numbers in bacterial and yeast cultures. Cell density is influenced by a variety of factors (e.g. species, media, and shaker speed) and OD readings of cultures measure light scattering rather than absorption. Measurements of light scattering are highly dependent on the distance between the sample and the detector and therefore readings vary between different types of spectrophotometer. In addition, different species show different OD values at defined wavelengths (e.g. 600 or 436 nm).

We therefore recommend calibrating the spectrophotometer used by comparing OD measurements at appropriate wavelengths with viable cell densities determined by plating experiments (e.g. see Ausubel, F.M. et al., eds. (1991) *Current Protocols in Molecular Biology*, New York: Wiley Interscience). OD readings should be between 0.05 and 0.3 to ensure significance. Samples with readings above 0.3 should be diluted so that the readings fall within this range and the dilution factor used in calculating the number of cells per ml.

The following calculation can be considered as a rough guide which may be helpful. An *E. coli* culture of  $1 \times 10^9$  cells per ml, diluted 1 in 4, gives OD<sub>600</sub> values of 0.25 measured using a Beckman DU<sup>®</sup>-7400 or 0.125 using a Beckman DU-40 spectrophotometer. These correspond to calculated OD values of 1.0 or 0.5 respectively for  $1 \times 10^9$  cells per ml. The same OD<sub>600</sub> values of 1.0 or 0.5 respectively are obtained for a *S. cerevisiae* culture with a cell density of  $1-2 \times 10^7$  cells per ml.

### **Plant tissue**

A 1.5-cm-diameter leaf disc weighs 25–75 mg.

### **Handling and storage of starting material**

RNA in sample material is subject to degradation by intracellular RNases until it is flash frozen or disrupted and homogenized in the presence of RNase-inhibiting or denaturing agents. It is therefore imperative that samples are immediately flash frozen in liquid nitrogen and stored at  $-70^\circ\text{C}$  or are processed as soon as harvested. Frozen animal or plant tissue should not be allowed to thaw during handling (e.g., weighing) but cell pellets can thaw enough to allow cell pellets to be dislodged by flicking. The relevant procedures should be carried out as quickly as possible. Samples can also be stored at  $-70^\circ\text{C}$  in lysis buffer (Buffer RLT or RLC) after disruption and homogenization. Frozen samples are stable for months.

**Note:** Only freshly harvested samples can be used for enzymatic lysis of yeast cells or isolation of cytoplasmic RNA from animal cells.

## Disruption and homogenization of starting materials

Efficient disruption and homogenization of the starting material is essential for all intracellular RNA isolation procedures. Disruption and homogenization are two distinct steps.

**Disruption:** Complete disruption of cells walls and 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 yields.

**Homogenization:** Homogenization is necessary to reduce the viscosity of the cell lysates produced by disruption. Homogenization shears the high-molecular-weight genomic DNA and other high-molecular-weight cellular components to create a homogeneous lysate. Incomplete homogenization results in significantly reduced yields.

Some disruption methods simultaneously homogenize the sample, while others require an additional homogenization step. Table 4 (page 18) gives an overview of different disruption and homogenization methods suitable for various starting materials. It can be used as a guide to choose the appropriate method for the starting material with which you are working. The different disruption and homogenization methods listed in Table 4 are described in more detail below.

### Disruption and homogenization using rotor–stator homogenizers

Rotor–stator homogenizers thoroughly disrupt and simultaneously homogenize, in the presence of lysis buffer, animal tissues in 5–90 sec depending on the toughness 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, by 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 up to 300 µl and can be used for homogenization in microfuge tubes. Probes with a diameter of 10 mm or above require larger tubes. See page 60 for a list of suppliers of rotor–stator homogenizers.

### Disruption and homogenization using a bead mill

Bead-milling disrupts cells and tissues by rapid agitation in the presence of glass beads and lysis buffer. Disruption is caused by the shearing and crushing action of the glass beads as they collide with the cells. Disruption efficiency is influenced by:

- size of beads
- ratio of buffer to beads
- amount of starting material
- speed and configuration of agitator
- disintegration time

The optimal size of glass beads (mean diameter) is 0.1 mm for bacteria; 0.5 mm for yeast, mycelia, and unicellular animal cells; and 1.0–2.5 mm for tissues. It is essential that glass beads are pretreated by washing in concentrated nitric acid. All other disruption parameters must be determined empirically for each application.

A protocol for mechanical disruption of yeast cells is included in this handbook since this is the most widespread application for bead-milling. Please refer to suppliers guidelines for further details.

As an alternative to glass beads, plant tissue may also be disrupted in a bead mill using steel or tungsten carbide beads. In this case, plant material, beads, and disruption vessel must all be precooled in liquid nitrogen and disruption performed without lysis buffer.

### **Disruption using a mortar and pestle**

For disruption using a mortar and pestle, freeze the 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 methods below.

**Note:** Grinding the sample using a mortar and pestle will disrupt the sample but it will not homogenize it. Homogenization must be performed separately before proceeding with the RNeasy protocol.

### **Homogenization using QIAshredder**

QIAshredder is a fast and efficient way to homogenize cell and tissue lysates without cross contamination of the samples. The lysate (maximum volume 700 µl) is loaded onto the QIAshredder spin column sitting in a 2-ml collection tube, spun for 2 min at maximum speed in a microfuge and the homogenized lysate collected. QIAshredder spin columns are supplied in the RNeasy Plant Mini Kit and can be purchased separately for use with RNeasy Mini Kits. Call our Technical Service Group for further details or see ordering information on page 61.

### **Homogenization using a syringe and needle**

Cell and tissue lysates can be homogenized using a syringe and needle. High-molecular-weight DNA can be sheared by passing the lysate through a 20-G (Ø 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.

**Table 4. Guide to methods of disruption and homogenization of samples**

<b>Starting material</b>	<b>Disruption method</b>
Cultured animal cells	Addition of lysis buffer
Animal tissue	Rotor–stator homogenizer  Mortar and pestle
Bacteria	Enzymatic (lysozyme) digestion followed by addition of lysis buffer
Yeast	Enzymatic (lyticase/zymolase) digestion of cell wall followed by lysis of spheroplasts by addition of lysis buffer  Glass beads in a bead mill with lysis buffer
Plants and filamentous fungi	Mortar and pestle

\* QIAshredder is supplied with the RNeasy Plant Mini Kit and can be purchased separately for use with the RNeasy Mini Kit.

Homogenization method	Comments
Rotor–stator homogenizer or QIAshredder* or syringe and needle	If $\leq 1 \times 10^5$ cells are processed, lysate can be homogenized by vortexing. No homogenization needed for cytoplasmic RNA protocol.
Rotor–stator homogenizer  QIAshredder* or syringe and needle	Simultaneously disrupts and homogenizes.  Rotor-stator usually gives higher yields than mortar and pestle
Vortex	If more than $5 \times 10^8$ cells are being processed further homogenization using QIAshredder* or a syringe/needle may increase yield.
Vortex	
Glass beads in a bead mill with lysis buffer	Bead-milling simultaneously disrupts and homogenizes; bead-milling cannot be replaced by vortexing.
QIAshredder*	Mortar and pestle cannot be replaced by rotor–stator homogenizer.

## Reagents and equipment to be supplied by user

See page 60 for a list of suppliers of disruption and homogenization equipment and enzymes.

### For all protocols

14.5 M  $\beta$ -mercaptoethanol ( $\beta$ -ME)\* (commercially available solutions are usually 14.5 M)

Sterile, RNase-free tips

Microcentrifuge (with rotor for 2-ml tubes)

Equipment for disruption and homogenization (see pages 16–17)

Ethanol (96–100%)

### For animal cell and animal tissue protocols

Ethanol (70%)

### For cytoplasmic RNA protocol

Buffer RLN <sup>†</sup>	50 mM Tris-Cl, pH 8.0
	140 mM NaCl
	1.5 mM MgCl <sub>2</sub>
	0.5% Nonidet P-40

Just before use add:

1000 U/ml RNasin <sup>®</sup> (optional)
1 mM DTT (optional)

### For bacterial protocol

TE buffer<sup>†</sup> (10 mM Tris-Cl, 1 mM EDTA pH 8.0)

Lysozyme (20 mg/ml in water for Gram-negative or 50 mg/ml in water for Gram-positive bacteria respectively). Store lysozyme stock solution in single-use aliquots at  $-20^{\circ}\text{C}$ . Add lysozyme stock solution to TE buffer just before use.

For Gram-negative bacteria:

Add 2  $\mu\text{l}$  of 20 mg/ml lysozyme stock solution per 100  $\mu\text{l}$  of TE buffer.

For Gram-positive bacteria:

Add 6  $\mu\text{l}$  of 50 mg/ml lysozyme stock solution per 100  $\mu\text{l}$  of TE buffer.

\*  $\beta$ -ME must be added to Buffer RLT and Buffer RLC before use.  $\beta$ -ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 10  $\mu\text{l}$  of  $\beta$ -ME per 1 ml of Buffer RLT or RLC. The solution is stable for 1 month after the addition of  $\beta$ -ME.

<sup>†</sup> The use of molecular-biology-grade reagents is recommended.

## For yeast

### Enzymatic protocol

Ethanol (70%)

Buffer for enzymatic lysis:

Depending on the yeast strain and enzyme used, the amount of enzyme and composition of this buffer may vary. Please adhere to guidelines of enzyme supplier. However, in most cases Buffer Y1 (see below) can be used.

Buffer Y1<sup>†</sup>            1 M sorbitol  
                          0.1 M EDTA, pH 7.4

Just before use add:

0.1%  $\beta$ -ME  
50 U lyticase/zymolase per  $10^7$  cells

### Mechanical disruption protocol

Acid-washed glass beads, 0.5 mm diameter:

Prepare acid-washed glass beads (0.45–0.55 mm diameter) by soaking in concentrated nitric acid for 1 hour, washing extensively with deionized water, and drying in a baking oven.

Bead mill

### For plant and fungi protocol

Liquid nitrogen<sup>‡</sup>

Mortar and pestle (alternatively: bead mill)

<sup>†</sup> The use of molecular-biology-grade reagents is recommended.

<sup>‡</sup> Liquid nitrogen can cause severe burns. Take appropriate safety measures.

# RNeasy Mini Protocol for the Isolation of Total RNA from Animal Cells

## Important notes before starting

- Use an appropriate number of cells. Please read “How much starting material can I use?” (page 12).
- If using RNeasy for the first time, please read “Disruption and homogenization of starting materials” (page 16).
- If preparing RNA for the first time, please read appendix A (page 54).
- Cell pellets can be stored at  $-70^{\circ}\text{C}$  for later use or used directly in the procedure. Cell lysates in Buffer RLT (after step 2) can be stored at  $-70^{\circ}\text{C}$  for several months. To process frozen lysates, thaw and incubate at  $37^{\circ}\text{C}$  for 10 min to ensure that all the salt has dissolved. Continue with step 3.
- Buffer RLT may form a precipitate upon storage. If necessary, warm to redissolve.
- $\beta$ -Mercaptoethanol ( $\beta$ -ME) must be added to Buffer RLT before use (see page 20). Add 10  $\mu\text{l}$   $\beta$ -ME per 1 ml of Buffer RLT. The solution is stable for 1 month.
- 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.
- All steps of the RNeasy protocol should be performed at 20 to  $25^{\circ}\text{C}$ . During the procedure, work quickly.
- After cell harvesting, all centrifugation steps should be performed in a microcentrifuge at 20 to  $25^{\circ}\text{C}$ .

## 1. Harvest cells.

### a. Cells grown in suspension

Spin the appropriate number of cells (see pages 12–13) for 5 min at  $300 \times g$  in a centrifuge tube. Discard supernatant, completely removing all media. Continue with step 2.

**Note:** Incomplete removal of the supernatant will inhibit lysis and dilute the lysate which will affect the conditions for binding RNA to the RNeasy membrane.

**b. Cells grown in a monolayer**

Cells grown in a monolayer can either be lysed directly in the culture vessel (up to 10 cm diameter) or trypsinized and collected as a cell pellet prior to lysis.

**To trypsinize cells:** Aspirate medium and wash cells with PBS, aspirate and trypsinize. After cells detach from the dish or flask, add medium, transfer cells to a centrifuge tube, and pellet by centrifugation at 300 x g for 5 min. Completely aspirate supernatant and continue with step 2.

**To lyse directly in culture vessel (up to 10 cm diameter):** Completely aspirate supernatant and continue immediately with step 2.

**Note:** Incomplete removal of the supernatant will inhibit lysis and dilute the lysate which will affect the conditions for binding the RNA to the RNeasy membrane.

**2. Disrupt cells by addition of Buffer RLT.**

**Note:** Ensure  $\beta$ -ME is added to Buffer RLT before use (see "Important notes before starting").

**For pelleted cells:**

Loosen cell pellet by flicking the tube and add Buffer RLT (according to table below). Vortex or pipet to mix. No cell clumps should be visible before proceeding to step 3.

Buffer RLT ( $\mu$ l)	Number of pelleted cells
350	up to $5 \times 10^6$
600	$5 \times 10^6$ to $1 \times 10^7$

**For monolayer cells:**

Add Buffer RLT (according to table below) to monolayer cells. Collect cell lysate with a rubber policeman. Vortex or pipet to mix. No cell clumps should be visible before proceeding to step 3.

Buffer RLT ( $\mu$ l)	Dish diameter (cm)*
350	<6
600	6–10

\* Add the volumes indicated to completely cover the surface of the dish, regardless of the cell number.

### 3. Homogenize the sample.

Three alternative methods (a, b, or c) may be used to homogenize the sample. After homogenization proceed to step 4. See page 16 for a more detailed description of homogenization methods.

If  $\leq 1 \times 10^5$  cells are being processed, the cells can be homogenized by vortexing for 1 min.

**Note:** Incomplete homogenization will lead to significantly reduced yields and can cause clogging of the RNeasy mini spin column.

a. **Pipet lysate directly onto a QIAshredder column (see page 17 for details) sitting in the 2-ml collection tube, and centrifuge for 2 min at maximum speed to homogenize.**

or

b. **Homogenize for 30 seconds using a rotor–stator homogenizer.**

or

c. **Pass lysate at least 5 times through a 20-G ( $\varnothing$  0.9 mm) needle fitted to a syringe.**

### 4. Add 1 volume (usually 350 $\mu$ l or 600 $\mu$ l) of 70% ethanol to the homogenized lysate, and mix well by pipetting. Do not centrifuge.

If some lysate was lost during homogenization, adjust volume of ethanol accordingly. A precipitate may form after the addition of ethanol, but this will not affect the RNeasy procedure.

### 5. Apply up to 700 $\mu$ l of sample, including any precipitate which may have formed, to an RNeasy mini spin column sitting in a 2-ml collection tube, and centrifuge for 15 sec at $\geq 8000 \times g$ ( $\geq 10,000$ rpm).

If the volume of the mixture exceeds 700  $\mu$ l, successively load aliquots onto the RNeasy column and centrifuge as above. Reuse the collection tube but discard flow-through\* after each step. Reuse the collection tube in step 6.

### 6. Pipet 700 $\mu$ l Buffer RW1 onto the RNeasy column, and centrifuge for 15 sec at $\geq 8000 \times g$ ( $\geq 10,000$ rpm) to wash.

Discard flow-through\* and collection tube.

\* Flow-through contains Buffer RLT or RW1 and is therefore not compatible with bleach.

7. **Transfer RNeasy column into a new 2-ml collection tube (supplied). Pipet 500  $\mu$ l Buffer RPE onto the RNeasy column, and centrifuge for 15 sec at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to wash.**

Discard flow-through and reuse the collection tube in step 8.

**Note:** Ensure ethanol is added to Buffer RPE before use (see "Important notes before starting").

8. **Pipet 500  $\mu$ l Buffer RPE onto the RNeasy column. Centrifuge for 2 min at maximum speed to dry the RNeasy membrane. Continue directly with step 9, or to eliminate any chance of possible Buffer RPE carryover, continue first with step 8a.**

It is important to dry the RNeasy membrane since residual ethanol may interfere with subsequent reactions. This spin ensures that no ethanol is carried over during elution.

**Note:** Following the spin, remove the RNeasy column from the collection tube carefully so that the column does not contact the flow-through as this will result in carryover of ethanol.

- 8a. **(Optional): Place the RNeasy spin column in a new 2-ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.**

9. **Transfer RNeasy column into a new 1.5-ml collection tube (supplied), and pipet 30–50  $\mu$ l of RNase-free water directly onto the RNeasy membrane. Centrifuge for 1 min at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to elute. Repeat if the expected RNA yield is  $>30 \mu\text{g}$ .**

If a second elution step is performed, elute into the same collection tube using another 30–50  $\mu$ l RNase-free water.

# RNeasy Mini Protocol for the Isolation of RNA from the Cytoplasm of Animal Cells

## Important notes before starting

- Use an appropriate number of cells. Please read “How much starting material can I use?” (page 12).
- If preparing RNA for the first time, please read appendix A (page 54).
- Prepare Buffer RLN\* and precool to 4°C:

50 mM Tris-Cl, pH 8.0  
140 mM NaCl  
1.5 mM MgCl<sub>2</sub>  
0.5% Nonidet P-40

Just before use add:

1000 U/ml RNasin (optional)  
1 mM DTT (optional)

- Use only freshly harvested cells. Freezing and thawing cells results in ice crystal formation which destroys the nuclear membranes, releasing DNA and other nuclear molecules. After addition of Buffer RLT (step 4) samples may be stored at -70°C for several months. To process, frozen lysates should be thawed and incubated at 37°C for 10 min to ensure that all salt has been dissolved. Vortex vigorously. If any insoluble material remains, spin for 2 min and use supernatant. Continue with step 5.
- Plasma membrane lysis is performed on ice, while all subsequent steps of the RNeasy protocol should be performed at 20 to 25°C. With the exception of Buffer RLN, buffers should not be precooled.
- Buffer RLT may form a precipitate upon storage. If necessary, warm to redissolve.
- β-Mercaptoethanol (β-ME) must be added to Buffer RLT before use (see page 20). Add 10 μl β-ME per 1 ml of Buffer RLT. The solution is stable for 1 month.
- 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.
- After cell harvesting and pelleting of nuclei, all centrifugation steps should be performed in a microcentrifuge at 20 to 25°C.

\* The use of molecular-biology-grade reagents is recommended.

## 1. Harvest cells.

### a. Cells grown in suspension

Spin the appropriate number of cells (see pages 12–13) for 5 min at 300 x g in a centrifuge tube. Discard supernatant, ensuring all media is completely removed. Continue with step 2.

**Note:** Incomplete removal of the supernatant will inhibit lysis and dilute the lysate which will affect the conditions for binding the RNA to the RNeasy membrane.

### b. Cells grown in a monolayer

Cells grown in a monolayer can either be lysed directly in a culture vessel up to 3.5 cm diameter, or trypsinized and collected as a cell pellet prior to lysis.

**To trypsinize cells:** Aspirate medium and wash cells with PBS, aspirate, and trypsinize. After cells detach from the dish or flask, add medium, transfer cells to a centrifuge tube, and pellet by centrifugation at 300 x g for 5 min. Discard supernatant, ensuring complete removal of all liquid and continue with step 2.

**To lyse directly in culture vessel:** For culture dishes up to 3.5 cm diameter, cells can be lysed directly in the dish. Discard media ensuring that all the liquid is completely removed and continue immediately with step 2.

**Note:** Incomplete removal of the supernatant will inhibit lysis and dilute the lysate which will affect the conditions for binding the RNA to the RNeasy membrane.

## 2. Add Buffer RLN to lyse plasma membrane.

a. **For pelleted cells, loosen cell pellet by flicking the tube, carefully resuspend cells in 175 µl cold Buffer RLN (4°C), and incubate for 5 min on ice. Continue with step 3.**

b. **For direct lysis of cells in the culture vessel (up to 3.5 cm diameter), add 175 µl cold Buffer RLN (4°C) to the cells, detach gently using a rubber policeman, and transfer to a microcentrifuge tube prior to incubation on ice for 5 min. Continue with step 3.**

The suspension should clear rapidly, indicating lysis of the plasma membrane which occurs almost immediately.

## 3. Centrifuge lysate at 4°C for 2 min at 300 x g, transfer supernatant to a new tube (not supplied), and discard pellet.

The supernatant is the cytoplasmic extract and is generally slightly cloudy and yellow-white, depending on the cell type used. The pellet, containing nuclei and cell debris, is considerably smaller than the whole-cell pellet obtained during harvesting.

**4. Add 600  $\mu$ l Buffer RLT to the supernatant and vortex vigorously.**

No further homogenization is required since genomic DNA is not present.

**Note:** Ensure  $\beta$ -ME is added to Buffer RLT before use (see "Important notes before starting").

**5. Add 430  $\mu$ l ethanol (96–100%) to the sample, and mix well by pipetting. Do not centrifuge.**

A precipitate may form after the addition of ethanol, but this will not affect the RNeasy procedure.

**6. Apply 700  $\mu$ l of the sample, including any precipitate which may have formed, to an RNeasy mini spin column sitting in a 2-ml collection tube. Centrifuge for 15 sec at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm). Repeat with the remaining sample.**

The maximum loading volume is 700  $\mu$ l. Therefore, successively load aliquots onto the RNeasy column and centrifuge as above. Reuse the collection tube but discard flow-through\* after each step. Reuse the collection tube in step 7.

**7. Pipet 700  $\mu$ l Buffer RW1 onto the RNeasy column, and centrifuge for 15 sec at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to wash.**

Discard flow-through\* and collection tube

**8. Transfer RNeasy column into a new 2-ml collection tube (supplied). Pipet 500  $\mu$ l Buffer RPE onto the RNeasy column, and centrifuge for 15 sec at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to wash.**

Discard flow-through and reuse the collection tube in step 9.

**Note:** Ensure ethanol is added to Buffer RPE before use (see "Important notes before starting").

**9. Pipet 500  $\mu$ l Buffer RPE onto RNeasy column, and centrifuge for 2 min at maximum speed to dry the RNeasy membrane. Continue directly with step 10, or to eliminate any chance of possible Buffer RPE carryover, continue first with step 9a.**

It is important to dry the RNeasy membrane since residual ethanol may interfere with subsequent reactions. This spin ensures that no ethanol is carried over during elution.

**Note:** Following the spin, remove the RNeasy column from the collection tube carefully so that the column does not contact the flow-through as this will result in carryover of ethanol.

\* Flow-through contains Buffer RLT or RW1 and is therefore not compatible with bleach.

- 9a. (Optional): Place the RNeasy spin column in a new 2-ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
10. Transfer RNeasy column into a new 1.5-ml collection tube (supplied), and pipet 30–50  $\mu\text{l}$  of RNase-free water directly onto the RNeasy membrane. Centrifuge for 1 min at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to elute. Repeat if the expected RNA yield is  $>30 \mu\text{g}$ .

If a second elution step is performed, elute into the same collection tube using another 30–50  $\mu\text{l}$  RNase-free water.

# RNeasy Mini Protocol for Isolation of Total RNA from Animal Tissues

## Important notes before starting

- Use an appropriate amount of tissue. Please read “How much starting material can I use?” (page 12).
- If using RNeasy for the first time, please read “Disruption and homogenization of starting materials” (page 16).
- If preparing RNA for the first time, please read appendix A (page 54).
- Some tissues, including heart, spleen, and brain are difficult to homogenize. The volume of lysis buffer may need to be increased to facilitate complete homogenization and to avoid reduced yields. See protocol for amounts.
- Fresh or frozen tissue can be used. To freeze tissue for long-term storage, flash freeze in liquid nitrogen and transfer immediately to  $-70^{\circ}\text{C}$  for storage up to several months. To process, do not allow tissue to thaw (e.g. during weighing) prior to disruption in Buffer RLT. Tissue lysates (in Buffer RLT) can also be stored at  $-70^{\circ}\text{C}$  for several months. To process frozen lysates, thaw samples and incubate for 10 min at  $37^{\circ}\text{C}$  in a water bath to dissolve salt. Continue with step 2.
- Buffer RLT may form a precipitate upon storage. If necessary, warm to redissolve.
- $\beta$ -Mercaptoethanol ( $\beta$ -ME) must be added to Buffer RLT before use (see page 20). Add 10  $\mu\text{l}$   $\beta$ -ME per 1 ml of Buffer RLT. The solution is stable for 1 month.
- 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.
- All steps of the RNeasy protocol (including centrifugation) should be performed at 20 to  $25^{\circ}\text{C}$ . During the procedure, work quickly.

## 1. Disrupt tissue and homogenize lysate.

Two alternative methods (a or b) may be used. See page 16 for a more detailed description of disruption and homogenization methods.

**Note:** Incomplete disruption and homogenization will lead to significantly reduced yields, and can cause clogging of the RNeasy mini spin column.

- a. **Simultaneously disrupt and homogenize the sample using Buffer RLT and a rotor–stator homogenizer. Continue with step 2.**

Place fresh or frozen tissue in a suitably sized vessel for the homogenizer. Add the appropriate volume of Buffer RLT (see below), and homogenize immediately until a completely homogeneous lysate is obtained (typically 20–40 sec).

Starting material	Volume of Buffer RLT
up to 20 mg	350 $\mu$ l
20 to 30 mg or if tissue is difficult to lyse	600 $\mu$ l

**Note:** Ensure  $\beta$ -ME is added to Buffer RLT before use (see “Important notes before starting”).

- b. **Disrupt the sample with a mortar and pestle, and homogenize using either a needle and syringe or QIAshredder. Continue with step 2.**

Immediately place the weighed, fresh or frozen tissue in liquid nitrogen and grind to a fine powder with mortar and pestle under liquid nitrogen. Transfer tissue powder and liquid nitrogen into a liquid-nitrogen-cooled, 2-ml microfuge tube and allow the liquid nitrogen to evaporate. Do not allow the tissue to thaw. Add the appropriate amount of Buffer RLT (see below) and homogenize by passing the lysate at least 5 times through a 20-G ( $\varnothing$  0.9 mm) needle fitted to a syringe. Alternatively, pipet lysate onto a QIAshredder column sitting in a 2-ml collection tube and centrifuge for 2 min at maximum speed to homogenize.

Starting material	Volume of Buffer RLT
up to 20 mg	350 $\mu$ l
20 to 30 mg or if tissue is difficult to lyse	600 $\mu$ l

**Note:** Ensure  $\beta$ -ME is added to Buffer RLT before use (see “Important notes before starting”).

2. **Centrifuge lysate for 3 min at maximum speed in a microcentrifuge and use only the supernatant in subsequent steps.**

For some samples very small amounts of insoluble material will be present, making the pellet invisible.

3. **Add 1 volume (usually 350  $\mu$ l or 600  $\mu$ l) of 70% ethanol to the cleared lysate, and mix well by pipetting. Do not centrifuge.**

If some lysate is lost during homogenization, reduce volume of ethanol accordingly. A precipitate may form after the addition of ethanol, but this will not affect the RNeasy procedure.

4. **Apply 700  $\mu$ l of the sample, including any precipitate which may have formed, to an RNeasy mini spin column sitting in a 2-ml collection tube. Centrifuge for 15 sec at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm).**

If the volume of the mixture exceeds 700  $\mu$ l, load aliquots successively onto the RNeasy column and centrifuge as above. Reuse the collection tube but discard flow-through\* after each step. Reuse the collection tube in step 5.

5. **Pipet 700  $\mu$ l Buffer RW1 onto the RNeasy column, and centrifuge for 15 sec at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to wash.**

Discard flow-through\* and collection tube.

6. **Transfer RNeasy column to a new 2-ml collection tube (supplied). Pipet 500  $\mu$ l Buffer RPE onto the RNeasy column, and centrifuge for 15 sec at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to wash.**

Discard flow-through and reuse the collection tube in step 7.

**Note:** Ensure ethanol is added to Buffer RPE before use (see "Important notes before starting").

7. **Pipet 500  $\mu$ l Buffer RPE onto RNeasy column, and centrifuge for 2 min at maximum speed to dry the RNeasy membrane. Continue directly with step 8, or to eliminate any chance of possible Buffer RPE carryover, continue first with step 7a.**

It is important to dry the RNeasy membrane since residual ethanol may interfere with subsequent reactions. This spin ensures that no ethanol is carried over during elution.

**Note:** Following the spin, remove the RNeasy column from the collection tube carefully so that the column does not contact the flow-through as this will result in carry-over of ethanol.

- 7a. **(Optional): Place the RNeasy spin column in a new 2-ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.**

8. **Transfer RNeasy column into a new 1.5-ml collection tube (supplied) and pipet 30–50  $\mu$ l of RNase-free water directly onto the RNeasy membrane. Centrifuge for 1 min at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to elute. Repeat if the expected RNA yield is  $> 30 \mu$ g.**

If a second elution step is performed, elute into the same collection tube using another 30–50  $\mu$ l RNase-free water.

\* Flow-through contains Buffer RLT or RW1 and is therefore not compatible with bleach.

# RNeasy Mini Protocol for the Isolation of Total RNA from Bacteria

## Important notes before starting

- Use an appropriate number of cells. Please read “How much starting material can I use?” (page 12) and page 14 for information about cell densities and OD values of bacterial cultures.
  - For RNA isolation, bacteria should be harvested in log-phase growth.
  - If using RNeasy for the first time, please read “Disruption and homogenization of starting materials” (page 16).
  - If preparing RNA for the first time, please read appendix A (page 54).
  - Prepare TE buffer, pH 8.0 containing 400 µg/ml lysozyme if Gram-negative bacteria are to be processed and 3 mg/ml lysozyme if Gram-positive bacteria are to be processed. TE and lysozyme are not supplied with the kit. See page 20 for details.
  - Buffer RLT may form a precipitate upon storage. If necessary, warm to redissolve.
  - β-Mercaptoethanol (β-ME) must be added to Buffer RLT before use (see page 20). Add 10 µl β-ME per 1 ml of Buffer RLT. The solution is stable for 1 month.
  - 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.
  - All steps of the RNeasy protocol should be performed at 20 to 25°C. During the procedure, work quickly.
  - After cell harvesting, all centrifugation steps are performed in a microcentrifuge at 20 to 25°C.
1. **Harvest the appropriate number of cells (maximum  $1 \times 10^9$ ) by centrifuging at 5000 x g for 3–5 min at 4°C. Discard supernatant ensuring all media is completely removed.**

**Note:** Incomplete removal of the supernatant will inhibit cell wall digestion in step 2.

- Loosen cell pellet by flicking the bottom of the tube. Resuspend cells in 100  $\mu$ l lysozyme-containing TE buffer by vortexing. Different lysozyme concentrations and incubation times are required for Gram-positive and Gram-negative bacteria (see below).

	Lysozyme concentration in TE buffer	Incubation time at room temperature
<b>Gram-negative bacteria</b>	400 $\mu$ g/ml	3–5 min
<b>Gram-positive bacteria</b>	3 mg/ml	5–10 min

The amount of enzyme required and/or the incubation time may need to be modified depending on the bacterial strain used. Adhere to the guidelines of the lysozyme supplier. Complete digestion of the cell wall is essential for efficient lysis.

- Add 350  $\mu$ l Buffer RLT to the sample and vortex vigorously. If insoluble material is visible, centrifuge for 2 min at maximum speed in a microfuge and use only the supernatant in subsequent steps.**

If more than  $5 \times 10^8$  cells are being processed, further homogenization with QIAshredder or a syringe and needle may increase yield (see page 17).

**Note:** Ensure  $\beta$ -ME is added to Buffer RLT before use (see “Important notes before starting”).

- Add 250  $\mu$ l ethanol (96–100%) to the lysate, and mix well by pipetting. Do not centrifuge.**

A precipitate may form after the addition of ethanol, but this will not affect the RNeasy procedure.

- Apply sample (usually 700  $\mu$ l), including any precipitate which may have formed, to an RNeasy mini spin column sitting in a 2-ml collection tube. Centrifuge for 15 sec at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm).**

If the volume of the mixture exceeds 700  $\mu$ l, load aliquots successively onto the RNeasy column and centrifuge as above. Reuse the collection tube but discard flow-through\* after each step.

Reuse the collection tube in step 6.

\* Flow-through contains Buffer RLT or RW1 and is therefore not compatible with bleach.

- 6. Pipet 700  $\mu\text{l}$  Buffer RW1 onto the RNeasy column, and centrifuge for 15 sec at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to wash.**

Discard flow-through\* and collection tube.

- 7. Transfer RNeasy column into a new 2-ml collection tube (supplied). Pipet 500  $\mu\text{l}$  Buffer RPE onto the RNeasy column, and centrifuge for 15 sec at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to wash.**

Discard flow-through and reuse the collection tube in step 8.

**Note:** Ensure ethanol is added to Buffer RPE before use (see "Important notes before starting").

- 8. Pipet 500  $\mu\text{l}$  Buffer RPE onto RNeasy column, and centrifuge for 2 min at maximum speed to dry the RNeasy membrane. Continue directly with step 9, or to eliminate any chance of possible Buffer RPE carryover, continue first with step 8a.**

It is important to dry the RNeasy membrane since residual ethanol may interfere with subsequent reactions. This spin ensures that no ethanol is carried over during elution.

**Note:** Following the spin, remove the RNeasy column from the collection tube carefully so that the column does not contact the flow-through as this will result in carryover of ethanol.

- 8a. (Optional): Place the RNeasy spin column in a new 2-ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.**

- 9. Transfer RNeasy column into a new 1.5-ml collection tube (supplied), and pipet 30–50  $\mu\text{l}$  of RNase-free water directly onto the RNeasy membrane. Centrifuge for 1 min at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to elute. Repeat if the expected RNA yield is  $> 30 \mu\text{g}$ .**

If a second elution step is performed, elute into the same collection tube using another 30–50  $\mu\text{l}$  RNase-free water.

# RNeasy Mini Protocol for Isolation of Total RNA from Yeast

## I. Enzymatic Lysis Protocol — Standard Version

### Important notes before starting

- Use an appropriate number of cells. Please read “How much starting material can I use?” (page 12) and page 14 for information about cell densities and OD values of yeast cultures.
- For RNA isolation from yeast, cells should be harvested in log-phase growth.
- For preparation of spheroplasts, freshly harvested cells must be used.
- If preparing RNA for the first time, please read Appendix A (page 54).
- Prepare Buffer Y1 (see page 20 for details):

Buffer Y1 \*                      1 M sorbitol  
    0.1 M EDTA, pH 7.4

Just before use add:

0.1%  $\beta$ -ME  
50 U lyticase/zymolase per  $10^7$  cells

Depending on the yeast strain and enzyme used, the amount of enzyme and composition of Buffer Y1 may need to be modified. Please adhere to guidelines of enzyme supplier.

- 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 may form a precipitate upon storage. If necessary, warm to redissolve.
- $\beta$ -Mercaptoethanol ( $\beta$ -ME) must be added to Buffer RLT before use (see page 20). Add 10  $\mu$ l  $\beta$ -ME per 1 ml of Buffer RLT. The solution is stable for 1 month.
- After enzymatic lysis, all steps of the RNeasy protocol should be performed quickly at 20 to 25°C.
- After cell harvesting, all centrifugation steps are performed in a microcentrifuge at 20 to 25°C.

\* The use of molecular-biology-grade reagents is recommended.

1. **Harvest the appropriate number of cells (maximum  $5 \times 10^7$ ) in a 12- or 15-ml tube by centrifuging for 5 min at  $1000 \times g$  at  $4^\circ\text{C}$ . Discard supernatant, ensuring that all liquid is completely removed.**

**Note:** Incomplete removal of the supernatant will inhibit cell wall digestion in step 2.

2. **Resuspend cells in 2 ml enzyme-containing Buffer Y1, and incubate for 10–30 min at  $30^\circ\text{C}$  with gentle shaking to prepare spheroplasts. Spheroplasts must be handled gently.**

Depending on the yeast strain and enzyme used, the incubation time, amount of enzyme, and composition of Buffer Y1 may need to be modified. Complete spheroplasting is essential for efficient lysis.

**Note:** For preparation of spheroplasts, freshly harvested cells must be used.

3. **Centrifuge 5 min at  $300 \times g$  to pellet spheroplasts. Carefully remove and discard all supernatant.**

**Note:** Incomplete removal of all the supernatant will inhibit lysis of spheroplasts and dilute the lysate which will affect the conditions for binding RNA to the RNeasy membrane.

4. **Add 350  $\mu\text{l}$  Buffer RLT to lyse spheroplasts and vortex vigorously. If insoluble material is visible, centrifuge lysate for 2 min at maximum speed and transfer supernatant to a microcentrifuge tube (not supplied).**

**Note:** Ensure  $\beta$ -ME is added to Buffer RLT before use (see "Important notes before starting").

5. **Add 1 volume (usually 350  $\mu\text{l}$ ) of 70% ethanol, to the lysate, and mix well by pipetting. Do not centrifuge.**

A precipitate may form after the addition of ethanol, but this will not affect the RNeasy procedure.

6. **Apply sample (usually 700  $\mu\text{l}$ ), including any precipitate which might have formed, to an RNeasy mini spin column sitting in a 2-ml collection tube. Centrifuge for 15 sec at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm).**

If the volume of the mixture exceeds 700  $\mu\text{l}$ , load aliquots successively onto the RNeasy column and centrifuge as above. Reuse the collection tube but discard flow-through\* after each step.

Reuse the collection tube in step 7.

7. **Pipet 700  $\mu\text{l}$  Buffer RW1 onto the RNeasy column, and centrifuge for 15 sec at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to wash.**

Discard flow-through\* and collection tube.

\* Flow-through contains Buffer RLT and is therefore not compatible with bleach.

8. **Transfer RNeasy column into a new 2-ml collection tube (supplied). Pipet 500  $\mu$ l Buffer RPE onto the RNeasy column, and centrifuge for 15 sec at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm).**

Discard flow-through and reuse the collection tube in step 9.

**Note:** Ensure ethanol is added to Buffer RPE before use (see “Important notes before starting”).

9. **Pipet 500  $\mu$ l Buffer RPE onto the RNeasy column, and centrifuge for 2 min at maximum speed to dry the RNeasy membrane. Continue directly with step 10, or to eliminate any chance of possible Buffer RPE carryover, continue first with step 9a.**

It is important to dry the RNeasy membrane since residual ethanol may interfere with subsequent reactions. This spin ensures that no ethanol is carried over during elution.

**Note:** Following the spin, remove the RNeasy column from the collection tube carefully so that the column does not contact the flow-through as this will result in carryover of ethanol.

- 9a. **(Optional): Place the RNeasy spin column in a new 2-ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.**
10. **Transfer RNeasy column into a new 1.5-ml collection tube (supplied), and pipet 30–50  $\mu$ l of RNase-free water directly onto the RNeasy membrane. Centrifuge for 1 min at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to elute. Repeat if the expected RNA yield is  $> 30 \mu\text{g}$ .**

If a second elution step is performed, elute into the same collection tube using another 30–50  $\mu$ l RNase-free water.

\* Flow-through contains Buffer RW1 and is therefore not compatible with bleach.

# RNeasy Mini Protocol for Isolation of Total RNA from Yeast

## II. Enzymatic Lysis Protocol — Abbreviated Version (for up to $2 \times 10^7$ cells)

### Important notes before starting

- Please see page 14 for information about cell densities and OD values for yeast cultures.
- For RNA isolation from yeast, cells should be harvested in log-phase growth.
- If preparing RNA for the first time, please read Appendix A (page 54).
- Prepare Buffer Y1 \* (see page 20 for details):

Buffer Y1                      1 M sorbitol  
   0.1 M EDTA, pH 7.4  
Just before use add:

0.1%  $\beta$ -ME  
50 U lyticase/zymolase per  $10^7$  cells

- 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 may form a precipitate upon storage. If necessary, warm to redissolve.
- $\beta$ -mercaptoethanol ( $\beta$ -ME) must be added to Buffer RLT before use (see page 20). Add 10  $\mu$ l  $\beta$ -ME per 1 ml of Buffer RLT. The solution is stable for 1 month.
- After enzymatic lysis, all steps of the RNeasy protocol should be performed quickly at 20 to 25°C.
- After cell harvesting, all centrifugation steps should be performed in a microcentrifuge at 20 to 25°C.

**1. Harvest cells in a microcentrifuge tube by centrifuging for 5 min at 1000 x g at 4°C. Discard supernatant, ensuring that all liquid is completely removed.**

**Note:** Incomplete removal of the supernatant will inhibit cell wall digestion in step 2.

**2. Resuspend cells in 100  $\mu$ l enzyme-containing Buffer Y1, and incubate for 10–30 min at 30°C with gentle shaking to prepare spheroplasts. Spheroplasts must be handled gently.**

Depending on the yeast strain and enzyme used, the incubation time, amount of enzyme, and composition of Buffer Y1 may need to be modified. Complete spheroplasting is essential for efficient lysis.

3. **Add 350  $\mu$ l Buffer RLT to the spheroplasts and vortex vigorously. If insoluble material is visible, centrifuge lysate for 2 min at maximum speed and transfer lysate to a fresh tube.**

**Note:** Ensure  $\beta$ -ME is added to Buffer RLT before use (see “Important notes before starting”).

4. **Add 250  $\mu$ l ethanol (96–100%) to the lysate, and mix well by pipetting. Do not centrifuge.**

A precipitate may form after the addition of ethanol, but this will not affect the RNeasy procedure.

5. **Apply sample (usually 700  $\mu$ l), including any precipitate which might have formed, to an RNeasy mini spin column sitting in a 2-ml collection tube. Centrifuge for 15 sec at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) and discard flow-through\*.**

If the volume of the mixture exceeds 700  $\mu$ l, load aliquots successively onto the RNeasy column and centrifuge as above. Reuse the collection tube but discard flow-through\* after each step. Reuse the collection tube in step 6.

6. **Pipet 700  $\mu$ l Buffer RW1 onto the RNeasy column, and centrifuge for 15 sec at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to wash.**

Discard flow-through\* and collection tube.

7. **Transfer RNeasy column into a new 2-ml collection tube (supplied). Pipet 500  $\mu$ l Buffer RPE onto RNeasy column, and centrifuge for 15 sec at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm).**

Discard flow-through and reuse the collection tube in step 8.

**Note:** Ensure ethanol is added to Buffer RPE before use (see “Important notes before starting”).

8. **Pipet 500  $\mu$ l Buffer RPE onto RNeasy column, and centrifuge for 2 min at maximum speed to dry the RNeasy membrane. Continue directly with step 9, or to eliminate any chance of possible Buffer RPE carryover, continue first with step 8a.**

It is important to dry the RNeasy membrane since residual ethanol may interfere with subsequent reactions. This spin ensures that no ethanol is carried over during elution.

**Note:** Following the spin, remove the RNeasy column from the collection tube carefully so that the column does not contact the flow-through as this will result in carryover of ethanol.

\* Flow-through contains Buffer RLT or RW1 and is therefore not compatible with bleach.

- 8a. (Optional): Place the RNeasy spin column in a new 2-ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
9. Transfer RNeasy column into a new 1.5-ml collection tube (supplied), and pipet 30–50  $\mu\text{l}$  of RNase-free water directly onto the RNeasy membrane. Centrifuge for 1 min at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to elute. Repeat if the expected RNA yield is  $> 30 \mu\text{g}$ .

If a second elution step is performed, elute into the same collection tube using another 30–50  $\mu\text{l}$  RNase-free water.

## RNeasy Mini Protocol for Isolation of Total RNA from Yeast

### III. Mechanical Disruption Protocol

#### Important notes before starting

- Use an appropriate number of cells. Please read “How much starting material can I use?” (page 12) and page 14 for information about cell densities and OD values of yeast cultures.
- For RNA isolation from yeast, cells should be harvested in log-phase growth.
- If using RNeasy for the first time, please read “Disruption and homogenization of starting materials” (page 16).
- If preparing RNA for the first time, please read Appendix A (page 54).
- Cell pellets can be stored at  $-70^{\circ}\text{C}$  or used directly in the procedure.
- Prepare acid-washed glass beads (0.45–0.55 mm diameter) by soaking in concentrated nitric acid for 1 hour, washing extensively with deionized water, and drying in a baking oven.
- 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 may form a precipitate upon storage. If necessary, warm to redissolve.
- $\beta$ -Mercaptoethanol ( $\beta$ -ME) must be added to Buffer RLT before use (see page 20). Add 10  $\mu\text{l}$   $\beta$ -ME per 1 ml of Buffer RLT. The solution is stable for 1 month.
- After disruption, all steps of the RNeasy protocol should be performed quickly at 20 to  $25^{\circ}\text{C}$ .
- After cell harvesting, all centrifugation steps should be performed in a microcentrifuge at 20 to  $25^{\circ}\text{C}$ .

**1. Harvest cells by centrifuging for 5 min at 1000 x g at  $4^{\circ}\text{C}$ . Discard supernatant, ensuring that all liquid is completely removed.**

**Note:** Incomplete removal of the supernatant will inhibit lysis and dilute the lysate which will affect the conditions for binding the RNA to the RNeasy membrane.

**2. Add approximately 1 volume (600  $\mu\text{l}$ ) acid-washed glass beads to a tube that fits into the bead mill.**

See disruption and homogenization section on pages 16–17 for details.

**3. Loosen cell pellet (obtained in step 1) by flicking the bottom of the tube. Resuspend cells in 600  $\mu\text{l}$  Buffer RLT by vortexing. Add the cells resuspended in Buffer RLT to the glass beads prepared in step 2.**

**Note:** Ensure  $\beta$ -ME is added to Buffer RLT before use (see “Important notes before starting”).

- 4. Agitate sample at top speed in the bead mill while cooling until cells are completely disrupted. Allow beads to settle.**

Most small-capacity bead mills do not have a cooling mechanism and therefore require the user to stop the bead mill regularly and cool the sample on ice. The time required for disruption and the length and frequency of the cooling intervals will vary depending upon the type of bead mill used. Please refer to supplier's instructions.

**Note:** Do not replace bead-milling by vortexing as this significantly reduces yield.

- 5. Remove sample from the bead mill and allow beads to settle. Transfer lysate (usually 350  $\mu$ l) to new microcentrifuge tube, centrifuge for 2 min at maximum speed, and transfer supernatant to a new microfuge tube (tubes not supplied).**

This step removes cell debris.

- 6. Add 1 volume (usually 350  $\mu$ l) of 70% ethanol to the supernatant, and mix well by pipetting. Do not centrifuge.**

If some lysate is lost during disruption, adjust volume of ethanol accordingly. A precipitate may form after the addition of ethanol, but this will not affect the RNeasy procedure.

- 7. Apply 700  $\mu$ l of the sample, including any precipitate which may have formed, to an RNeasy mini spin column sitting in a 2-ml collection tube. Centrifuge for 15 sec at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm).**

If the volume of the mixture exceeds 700  $\mu$ l, load aliquots successively onto the RNeasy column and centrifuge as above. Reuse the same collection tube but discard flow-through\* after each step. Reuse the collection tube in step 8.

- 8. Pipet 700  $\mu$ l Buffer RW1 onto the RNeasy column, and centrifuge for 15 sec at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to wash.**

Discard flow-through\* and collection tube.

- 9. Transfer RNeasy column into a new 2-ml collection tube (supplied). Pipet 500  $\mu$ l Buffer RPE onto the RNeasy column, and centrifuge for 15 sec at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to wash.**

Discard flow-through and reuse the collection tube in step 10.

**Note:** Ensure ethanol is added to Buffer RPE before use (see "Important notes before starting").

\* Flow-through contains Buffer RLT or RW1 and is therefore not compatible with bleach.

10. Add 500  $\mu$ l Buffer RPE to RNeasy column, and centrifuge for 2 min at maximum speed to dry the RNeasy membrane. Continue directly with step 11, or to eliminate any chance of possible Buffer RPE carryover, continue first with step 10a.

It is important to dry the RNeasy membrane since residual ethanol may interfere with subsequent reactions. This spin ensures that no ethanol will be carried over during elution.

**Note:** Following the spin, remove the RNeasy column from the collection tube carefully so that the column does not contact the flow-through as this will result in carry-over of ethanol.

- 10a. (Optional): Place the RNeasy spin column in a new 2-ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
11. Transfer RNeasy column into a new 1.5-ml collection tube (supplied), and pipet 30–50  $\mu$ l of RNase-free water directly onto the RNeasy membrane. Centrifuge for 1 min at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to elute. Repeat if the expected RNA yield is  $>30 \mu\text{g}$ .

If a second elution step is performed, elute into the same collection tube using another 30–50  $\mu$ l RNase-free water.

# RNeasy Plant Mini Protocol for Isolation of Total RNA from Plant Cells and Tissues, and Filamentous Fungi

## Important notes before starting

- Total RNA isolation from plant cells and tissues and filamentous fungi requires the RNeasy Plant Mini Kit and cannot be performed with the RNeasy Mini Kit alone.
- A maximum of 100 mg plant material or  $1 \times 10^7$  cells can be used per preparation. See “How much starting material can I use?” (page 12) for more information.
- If using RNeasy for the first time, please read “Disruption and homogenization of starting materials” (page 16).
- If preparing RNA for the first time, please read Appendix A (page 54).
- The RNeasy Plant Mini Kit provides two different lysis buffers, Buffer RLT and Buffer RLC, which contain guanidine isothiocyanate (GITC) or guanidine hydrochloride (GuHCl), respectively. In most cases Buffer RLT is the lysis buffer of choice due to the greater cell disruption and denaturation properties of GITC. However, depending on the amount and type of secondary metabolites in some tissues (such as milky endosperm of maize or mycelia of filamentous fungi), GITC can cause solidification of the sample, making extraction of RNA impossible. In these cases Buffer RLC should be used.
- 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 may form a precipitate upon storage. If necessary, warm to redissolve.
- $\beta$ -Mercaptoethanol ( $\beta$ -ME) must be added to Buffer RLT or Buffer RLC before use (see page 20). Add 10  $\mu$ l  $\beta$ -ME per 1 ml of Buffer RLT or Buffer RLC. The solution is stable for 1 month.
- The time between harvesting of tissue and freezing should be minimized. Once tissue is frozen, do not allow to thaw.
- After disruption, all steps of the RNeasy protocol should be performed at 20 to 25°C. During the procedure, work quickly.
- All centrifugation steps should be performed in a microcentrifuge at 20 to 25°C.

1. **Grind sample under liquid nitrogen to a fine powder using a mortar and pestle. Transfer the tissue powder and liquid nitrogen to an appropriately sized tube, and allow the liquid nitrogen to evaporate. Do not allow the sample to thaw. Continue immediately with step 2.**

**Note:** Incomplete grinding of the starting material will lead to reduced RNA yields.

2. **Add 450  $\mu$ l of either Buffer RLT or Buffer RLC (see notes above) to a maximum of 100 mg of tissue powder. Vortex vigorously.**

A short (1–3 min) incubation at 56°C may help to disrupt tissue. However for samples with high starch content, incubation at elevated temperatures should be omitted to prevent swelling of the starting material.

**Note:** Ensure  $\beta$ -ME is added to Buffer RLT or Buffer RLC before use (see “Important notes before starting”).

3. **Apply lysate to the QIAshredder spin column (lilac) sitting in a 2-ml collection tube, and centrifuge for 2 min at maximum speed. Transfer flow-through fraction from QIAshredder to a new tube (not supplied) without disturbing the cell-debris pellet in the collection tube.**

It may be necessary to cut the end off the pipet tip to apply the lysate to the QIAshredder spin column. This centrifugation through QIAshredder removes cell debris and simultaneously homogenizes the lysate. While most of the cell debris is retained on the QIAshredder spin column, a very small amount of cell debris will pass through and form a pellet in the collection tube. Be careful not to disturb this pellet while transferring the lysate to a new tube (not supplied).

4. **Add 0.5 volumes (usually 225  $\mu$ l) ethanol (96–100%) to the cleared lysate and mix well by pipetting.**

If some lysate is lost during homogenization, reduce volume of ethanol proportionally. A precipitate may form after the addition of ethanol, but this will not affect the RNeasy procedure.

5. **Apply sample (usually 675  $\mu$ l), including any precipitate which may have formed, onto an RNeasy mini spin column (pink) sitting in a 2-ml collection tube. Centrifuge for 15 sec at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm).**

If the volume of the mixture exceeds 700  $\mu$ l, load aliquots successively onto the RNeasy column and centrifuge as above. Reuse the same collection tube but discard flow-through\* after each step. Reuse the collection tube in step 6.

\* Flow-through contains Buffer RLT and is therefore not compatible with bleach.

6. **Pipet 700  $\mu\text{l}$  Buffer RW1 onto the RNeasy column, and centrifuge for 15 sec at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to wash.**

Discard flow-through\* and collection tube.

7. **Transfer RNeasy column into a new 2-ml collection tube (supplied). Pipet 500  $\mu\text{l}$  Buffer RPE onto the RNeasy column, and centrifuge for 15 sec at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm).**

Discard flow-through and reuse the collection tube in step 8.

**Note:** Ensure ethanol is added to Buffer RPE before use (see "Important notes before starting").

8. **Add 500  $\mu\text{l}$  Buffer RPE to the RNeasy column, and centrifuge for 2 min at maximum speed to dry the RNeasy membrane. Continue directly with step 9, or to eliminate any chance of possible Buffer RPE carryover, continue first with step 8a.**

It is important to dry the RNeasy membrane since residual ethanol may interfere with subsequent reactions. This spin ensures that no residual ethanol will be carried over during elution. Discard flow-through and collection tube.

**Note:** Following the spin, remove the RNeasy column from the collection tube carefully so that the column does not contact the flow-through as this will result in carryover of ethanol.

- 8a. **(Optional): Place the RNeasy spin column in a new 2-ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.**

9. **Transfer RNeasy column into a new 1.5-ml collection tube (supplied), and pipet 30–50  $\mu\text{l}$  of RNase-free water directly onto the RNeasy membrane. Centrifuge for 1 min at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to elute. Repeat if the expected RNA yield is  $> 20 \mu\text{g}$ .**

If a second elution step is performed, elute into the same collection tube using another 30–50  $\mu\text{l}$  RNase-free water.

\* Flow-through contains Buffer RW1 and is therefore not compatible with bleach.

# RNeasy Mini Protocol for RNA Cleanup

RNeasy Kits can be used to clean up RNA previously isolated by different methods or after enzymatic reactions (e.g. DNase digestion).

## Important notes before starting

- Do not exceed the RNA binding capacity (100 µg) of the RNeasy mini spin column.
- Buffer RLT may form a precipitate upon storage. If necessary, warm to redissolve.
- β-Mercaptoethanol (β-ME) must be added to Buffer RLT before use (see page 20). Add 10 µl β-ME per 1 ml of Buffer RLT. The solution is stable for 1 month.
- 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.
- Before purification of in vitro transcripts, we recommend a DNase digestion of the in vitro transcription reaction to assure complete removal of DNA.
- All centrifugation steps should be performed in a microcentrifuge at 20 to 25°C.

1. **Adjust sample to a volume of 100 µl with RNase-free water, add 350 µl Buffer RLT to the sample, and mix thoroughly.**

**Note:** Ensure β-ME is added to Buffer RLT before use (see “Important notes before starting”).

2. **Add 250 µl ethanol (96–100%) to the lysate, and mix well by pipetting. Do not centrifuge.**

3. **Apply sample (700 µl) to an RNeasy mini spin column sitting in a collection tube. Centrifuge for 15 sec at ≥8000 x g (≥10,000 rpm).**

Discard flow-through\* and collection tube.

4. **Transfer the RNeasy column into a new 2-ml collection tube (supplied). Add 500 µl Buffer RPE and centrifuge for 15 sec at ≥8000 x g (≥10,000 rpm) to wash.**

Discard flow-through and reuse the collection tube in step 5.

**Note:** Ensure ethanol is added to Buffer RPE before use (see “Important notes before starting”).

\* Flow-through contains Buffer RLT and is therefore not compatible with bleach.

5. Pipet 500  $\mu$ l Buffer RPE onto the RNeasy column, and centrifuge for 2 min at maximum speed to dry the RNeasy membrane. Continue directly with step 6, or to eliminate any chance of possible Buffer RPE carryover, continue first with step 5a.

It is important to dry the RNeasy membrane since residual ethanol may interfere with subsequent reactions. This spin ensures that no residual ethanol will be carried over during elution.

**Note:** Following the spin, remove the RNeasy column from the collection tube carefully so that the column does not contact the flow-through as this will result in carryover of ethanol.

- 5a. (Optional): Place the RNeasy spin column in a new 2-ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
6. Transfer the RNeasy column into a new 1.5-ml collection tube (supplied), and pipet 30–50  $\mu$ l of RNase-free water directly onto the RNeasy membrane. Centrifuge for 1 min at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to elute. Repeat if the expected RNA yield is  $>30 \mu\text{g}$ .

If a second elution step is performed, elute into the same collection tube using another 30–50  $\mu$ l RNase-free water.

# Troubleshooting Guide

This troubleshooting guide, as well as the information provided in the appendices of this handbook, 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 last page for contact information).

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## Comments and Suggestions

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### Clogged RNeasy column

- |  |   |
|--|---|
| a) Insufficient disruption and/or homogenization | See “Disruption and homogenization methods” on pages 16–17. Increase <i>g</i> -force and centrifugation time. After lysis, spin lysate to pellet debris and continue with the protocol using the supernatant. |
| b) Too much starting material                    | In future preparations, reduce amount of starting material (see pages 12–15) and/or increase volume of lysis buffer.  |

### Little or no RNA eluted

- |  |  |
|--|--|
| a) Insufficient disruption and/or homogenization | See “Disruption and homogenization of starting material” (pages 16–17). Reduce the amount of starting material and/or increase the amount of lysis buffer. |
| b) Too much starting material                    | Overloading significantly reduces yield. Reduce amount of starting material (see pages 12–15).   |
| c) Incomplete removal of supernatant             | When processing cultured cells ensure complete removal of the supernatant after cell harvesting.   |
| d) RNA remains bound to the membrane             | Repeat elution. Incubate spin column for 10 min with RNase-free water.   |

### RNA degraded

- |                                       |  |
|---------------------------------------|--|
| a) RNA source inappropriately handled | Ensure that the starting material is frozen immediately in liquid nitrogen, that the RNA source has been properly stored, and that the protocol, especially the first few steps, has been performed quickly. See Appendix A (page 54) and “Handling and storage of starting material” (page 15). |
|---------------------------------------|--|

## Comments and Suggestions

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### b) 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 during handling. See Appendix A (page 54).

### DNA contamination

See also "DNA contamination", Appendix B, (page 56).

DNase digest the eluate containing the RNA. The RNA can either be used directly in the subsequent application or repurified using the RNeasy cleanup protocol (page 48).

The RNase-Free DNase Set can be used in future preparations for convenient on-column DNase digestion. The DNase is removed in the subsequent wash steps. See page 61 for ordering information.

In future preparations incubate RNeasy column for 5 minutes after addition of Buffer RW1.

If working with cultured animal cells, use the protocol for isolation of cytoplasmic RNA (see pages 8 and 26).

### Low $A_{260}/A_{280}$ ratio

Water with low pH used to dilute the sample. Use 10 mM Tris-Cl, pH 7.5 to dilute the sample before measuring purity (see Appendix B, page 56).

### RNA does not perform well in downstream experiments

#### a) Salt carryover during elution

Ensure that Buffer RPE is at room temperature.

#### b) Ethanol carryover

Ensure that during the second Buffer RPE wash, the column is spun at maximum speed for 2 minutes to dry the RNeasy membrane. Following the spin, remove the RNeasy column from the collection tube carefully so that the column does not contact the flow-through as this will result in carryover of ethanol. Continue with the optional second centrifugation step in the protocol.

## RNeasy References

This is only a partial list of references. Please contact our Technical Service Department for a complete list.

### Animal cells

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# Appendix A

## 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 only 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 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.

### 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 55). 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 detergent, thoroughly rinsed, and oven-baked at  $\geq 240^{\circ}\text{C}$  for at 4 h (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Oven-baking will both inactivate ribonucleases and ensure that no other nucleic acids (such as plasmid DNA) are left on the surface of the glassware. Alternatively, glassware can be treated with DEPC\* (diethyl pyrocarbonate). Fill glassware with 0.1% DEPC (0.1% in water), allow to stand overnight (12 h) at  $37^{\circ}\text{C}$ , and then autoclave or heat to  $100^{\circ}\text{C}$  for 15 min to remove residual DEPC.

**Note:** Corex® tubes should be rendered RNase-free by treatment with DEPC and not by baking. This will reduce the failure rate of this type of tube during centrifugation.

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

## Electrophoresis tanks

Electrophoresis tanks should be cleaned with detergent solution (e.g. 0.5% SDS), rinsed with water, dried with ethanol, and then filled with a solution of 3% H<sub>2</sub>O<sub>2</sub>. After 10 min at room temperature, the electrophoresis tanks should be rinsed thoroughly with RNase-free water.

## 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, shake vigorously to bring the DEPC into solution, and let the solution stand for 12 h at 37°C. Autoclave for 15 min to remove any trace of DEPC. DEPC will react with primary amines and cannot be used directly to treat Tris buffers. Therefore 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 removed from solutions or vessels by autoclaving or heating to 100°C for 15 min.

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

## Appendix B

### Storage of total RNA

Total RNA may be stored at -20°C or -70°C in water. Under these conditions, no degradation of RNA has been detected, even after 1 year.

### Quantitation of total RNA

The concentration and purity of RNA can be determined by measuring the absorbance at 260 nm ( $A_{260}$ ) and 280 nm ( $A_{280}$ ) in a spectrophotometer. Absorbance readings at 260 nm measure RNA concentration and should be greater than 0.15 to ensure significance. An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per ml ( $A_{260} = 1 = 40 \mu\text{g/ml}$ ). This relationship is valid for measurements in water. Therefore to quantify spectrophotometrically dilute RNA in water. The ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity (see "Purity", page 56).

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" above). Use the buffer in which the RNA is diluted to zero the spectrophotometer.

An example of the calculations involved in RNA quantitation is shown below:

Volume of RNA sample = 100  $\mu$ l

Dilution = 10  $\mu$ l of RNA sample + 490  $\mu$ l dH<sub>2</sub>O (1/50 dilution)

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

$$A_{260} = 0.23$$

When measured in water an  $A_{260}$  value of 1 is equal to 40  $\mu$ g/ml of RNA;

therefore, concentration of original RNA sample = 40  $\times$   $A_{260}$   $\times$  dilution factor

$$= 40 \times 0.23 \times 50$$

$$= 460 \mu\text{g/ml}$$

Total yield = concentration  $\times$  volume of sample (ml)

$$= 460 \mu\text{g/ml} \times 0.1 \text{ ml}$$

$$= 46 \mu\text{g}$$

## Purity

The ratio between the readings taken at 260 nm and 280 nm ( $A_{260}/A_{280}$ ) provides an estimate of the purity of RNA. The  $A_{260}/A_{280}$  ratio is influenced by pH. As water is unbuffered, the same RNA sample may show different  $A_{260}/A_{280}$  ratio in different types of water, ranging from 1.5–1.9. Also, the addition of the RNA itself influences the pH of the water. Therefore we recommend determining  $A_{260}/A_{280}$  ratios in 10 mM Tris-Cl, pH 7.5 if exact and reliable data are required. Pure RNA has an  $A_{260}/A_{280}$  ratio of 1.8–2.1 in 10 mM Tris-Cl, pH 7.5. RNA concentration should be determined in water (see "Quantitation of total RNA" page 55).

## DNA contamination

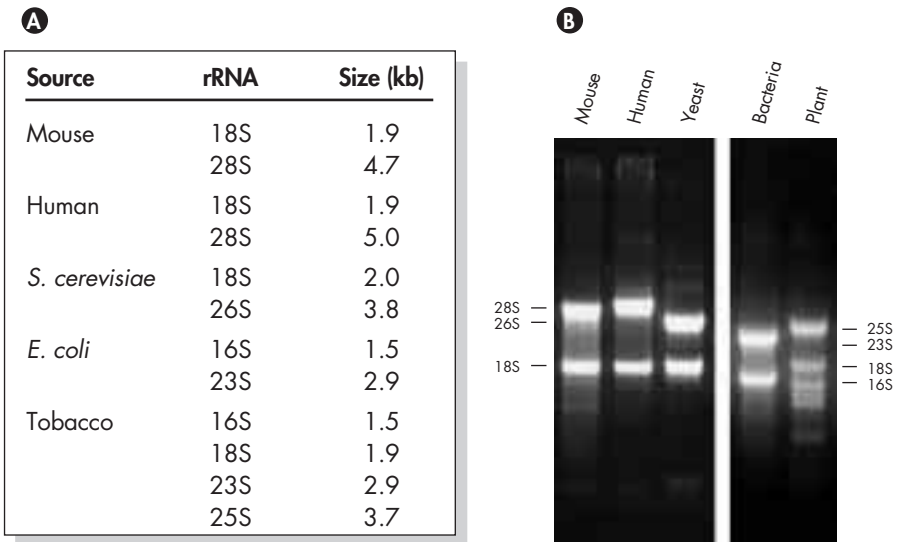
No currently available purification method can guarantee that RNA is completely free of DNA, even when it is not visible on an agarose gel. For very sensitive applications such as RT-PCR or differential display, on-column DNase digestion with the RNase-Free DNase Set is recommended (see page 61 for ordering information). Alternatively, the RNA can be digested with RNase-free DNase after purification. The RNA can then be repurified using the RNeasy cleanup protocol (page 48).

For RT-PCR applications it is preferable to work when possible with intron-spanning primers and to perform control experiments in which no reverse transcriptase is added prior to the PCR.

## Integrity

The integrity and size distribution of total RNA purified with RNeasy can be checked by denaturing-agarose gel electrophoresis and ethidium bromide staining. The relevant ribosomal species (Figure 1A) should appear as sharp bands on the stained gel. 28S and 23S ribosomal RNA bands should be present at approximately twice the amounts of the 18S and 16S RNA. 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 under analysis has suffered major degradation during its preparation.

RNA isolated from various sources using RNeasy and analyzed on a formaldehyde (FA) agarose gel is shown below. A protocol for FA gel electrophoresis is given in Appendix C (page 58).



**Figure 1.** **A** Sizes of ribosomal RNAs from various sources and **B** formaldehyde agarose gel of total RNA isolated from the indicated sources using RNeasy Kits. 10 µg RNA was loaded per lane.

## Appendix C

### Protocol for Formaldehyde Agarose (FA) Gel Electrophoresis

This protocol uses a more concentrated RNA loading buffer allowing a larger volume of RNA sample to be loaded onto the gel compared to 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).

#### 1.2% FA gel (100 ml)

1.2 g agarose

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

RNase-free water to 100 ml

Microwave to melt agarose. Cool to 65°C in a waterbath. Add 1.8 ml of 37% (12.3 M) formaldehyde\* and 1 µl of ethidium bromide\* (10 mg/ml). 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

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

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 per cm with 1x FA gel running buffer.

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

## 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

**Note:** Equilibrate gel in 1x FA gel running buffer for at least 30 min before starting electrophoresis.

### 5x RNA loading buffer

16  $\mu$ l saturated bromophenol blue solution<sup>†</sup>  
80  $\mu$ l 500 mM EDTA, pH 8.0  
720  $\mu$ l 37% (=12.3 M) formaldehyde\*  
2 ml 100% glycerol  
3084  $\mu$ l formamide  
4 ml 10x FA gel buffer

Add RNase-free water to 10 ml

Stability: Approximately 3 months at 4°C

Usage: For example, 40  $\mu$ l RNA and 10  $\mu$ l 5x RNA loading buffer, incubate for 3–5 min at 65°C, chill on ice, load onto gel.

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

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

## Appendix D

### Equipment and Reagent Suppliers\*

Rotor–stator homogenizers can be purchased from:

- OMNI International Inc., Warrenton, VA, USA (Omni Homogenizers) (OMNI also supply disposable tips for homogenizers)
- Bio-Spec Products, Bartlesville, OK, USA (Tissue-Tearor™)
- Charles Ross & Son Company, Hauppauge, NY, USA
- Craven Laboratories, Austin, TX, USA
- IKA Analysentechnik GmbH, Germany (Ultra Turrax®)
- IKA Works, Cincinnati, OH, USA
- Kinematica AG; sold by Brinkmann Instruments, Westbury, NY, USA (Polytron® Homogenizers)
- Silverson Machines, Bay Village, OH, USA
- Tekmar Inc., Cincinnati, OH, USA (Tissuemizer®)
- VirTis Company, Gardiner, NY, USA

Bead mills and glass beads can be purchased from:

- Retsch (Haan, Germany)

Lyticase/zymolase can be purchased from:

- Roche Molecular Biochemicals
- Sigma Chemicals
- Medac

Lysozyme can be purchased from:

- Roche Molecular Biochemicals
- Sigma Chemicals
- SERVA/Boehringer Ingelheim Bioproducts

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

## Ordering Information

Product	Contents	Cat. No.
<b>RNeasy Mini Kits — for up to 100 µg of total RNA from animal cells or tissues, yeast, and bacteria</b>		
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 Kit (250)	250 RNeasy Mini Spin Columns, Collection Tubes (1.5-ml and 2-ml), RNase-free Reagents and Buffers	74106
<b>RNeasy Plant Mini Kits — for up to 100 µg of total RNA from plants and fungi</b>		
RNeasy Plant Mini Kit (20)	20 RNeasy Mini Spin Columns, 20 QIAshredder Spin Columns, Collection Tubes (1.5 and 2 ml), RNase-free Reagents and Buffers	74903
RNeasy Plant Mini Kit (50)	50 RNeasy Mini Spin Columns, 50 QIAshredder Spin Columns, Collection Tubes (1.5 and 2 ml), RNase-free Reagents and Buffers	74904
<b>Accessories</b>		
RNase-Free DNase Set (50)	1500 units RNase-free DNase I, RNase-free Buffer, and RNase-free water for 50 RNA minipreps	79254
QIAshredder (50)	50 disposable cell-lysate homogenizers for use in nucleic acid minipreparation, caps	79654
QIAshredder (250)	250 disposable cell-lysate homogenizers for use in nucleic acid minipreparation, caps	79656
Buffer RLT	220 ml RNeasy Lysis Buffer	79216
Collection Tubes (2-ml)	1000 Collection Tubes (2-ml)	19201

# Ordering Information

Product	Contents	Cat. No.
<b>Related Products</b>		
<b>RNeasy Midi Kits — for up to 1 mg of total RNA from animal cells or tissues, yeast, and bacteria</b>		
RNeasy Midi Kit (10)*	10 RNeasy Midi Spin Columns, Collection Tubes (15-ml), RNase-free Reagents and Buffers	75142
<b>RNeasy Maxi Kits — for up to 6 mg of total RNA from animal cells or tissues, yeast, and bacteria</b>		
RNeasy Maxi Kit (6)*	6 RNeasy Maxi Spin Columns, Collection Tubes (50-ml), RNase-free Reagents and Buffers	75161
<b>QIAamp® RNA Blood Mini Kits — for isolation of total RNA from up to 1.5 ml whole blood</b>		
QIAamp RNA Blood Mini Kit (20)*	20 QIAamp Mini Spin Columns, 20 QIAshredder Spin Columns, Collection Tubes (1.5-ml and 2-ml), RNase-free Reagents and Buffers	52303
Buffer EL	1000 ml Erythrocyte Lysis Buffer	79217
<b>Oligotex mRNA Kits† — for isolation of mRNA from total RNA</b>		
Oligotex mRNA Mini Kit*	For 12 mRNA preps from up to 250 µg of total RNA each: 200 µl Oligotex Suspension, Small Spin Columns, Collection Tubes, RNase-free Reagents and Buffers	70022
<b>Oligotex Suspension†</b>		
Oligotex Suspension (0.5)*	0.5 ml (binds 300 µg mRNA)	79000
<b>QIAGEN RNA/DNA Kits — for simultaneous total RNA and genomic DNA isolation from animal cells or tissues, yeast, and bacteria</b>		
QIAGEN RNA/DNA Mini Kit*	25 QIAGEN-tip 20, RNase-free Reagents and Buffers	14123

\* Larger kit sizes available; please inquire.

† Not available in Japan.

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Syngen Biotech  
ul. Legnicka 61A  
54-204 Wrocław  
Tel: (071) 351 41 06  
or (0601 70 60 07  
Fax: (071) 351 04 88  
E-mail: [syngen@infonet.wroc.pl](mailto:syngen@infonet.wroc.pl)  
Web site: [www.syngen.com.pl](http://www.syngen.com.pl)

### Portugal

IZASA PORTUGAL, LDA  
Rua Cordeiro Ferreira, 9  
1700 Lisboa  
Tel: (1)-751 6000  
Fax: (1)-759 9529

### Singapore

Research Biolabs Pte Ltd.  
29 Lucky Crescent  
Singapore 467742  
Tel: 445 7927  
Fax: 448 3966  
E-mail:  
[BIOLABS@SINGNET.COM.SG](mailto:BIOLABS@SINGNET.COM.SG)

### Slovak Republic

BIO-CONSULT Slovakia spol. s.r.o.  
Ružová dolina 6  
SK-821 08 Bratislava  
Tel/Fax: (07) 50 221 336  
E-mail: [bio-cons@login.cz](mailto:bio-cons@login.cz)  
Web site: [www.bio-consult.cz](http://www.bio-consult.cz)

### South Africa

Southern Cross Biotechnology (Pty) Ltd  
P.O. Box 23681  
Claremont 7735  
Cape Town  
Tel: (021) 671 5166  
Fax: (021) 671 7734  
E-mail: [info@scb.co.za](mailto:info@scb.co.za)  
Web site: [www.scb.co.za](http://www.scb.co.za)

### Spain

IZASA, S.A.  
Aragón, 90  
08015 Barcelona  
Tel: (93) 902.20.30.90  
Fax: (93) 902.22.33.66

### Sweden

KEBO Lab  
Fagerstogatan 18A  
16394 Spånga  
Tel: (08) 621 34 00  
Fax: (08) 621 34 70  
E-mail: [cellmol@kebolab.se](mailto:cellmol@kebolab.se)  
Web site: [www.kebolab.se](http://www.kebolab.se)

### Taiwan

TAIGEN Bioscience Corporation  
3F. No. 306, Section 4  
Chen-Der Road  
Taipei  
Taiwan, R.O.C.  
Tel: (02) 2880 2913  
Fax: (02) 2880 2916  
E-mail: [taigen@ms10.hinet.net](mailto:taigen@ms10.hinet.net)

### Thailand

Theera Trading Co. Ltd.  
64 Charan Sanit Wong Road  
(Charan 13) Bangkokyay  
Bangkok 10600  
Tel: (02) 412-5672  
Fax: (02) 412-3244  
E-mail: [theetrad@samart.co.th](mailto:theetrad@samart.co.th)

### QIAGEN Importers

#### Saudi Arabia

Abdulla Fouad Co. Ltd.  
Medical Supplies Division  
Prince Mohammed Street  
P.O. Box 257, Dammam 31411  
Kingdom of Saudia Arabia  
Tel: (03) 8324400  
Fax: (03) 8346174  
E-mail:  
[sadiq.omar@abdulla-fouad.com](mailto:sadiq.omar@abdulla-fouad.com)

#### All other countries

QIAGEN GmbH, Germany

