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September 2019

# RNeasy<sup>®</sup> Midi/Maxi Handbook

RNeasy Midi Kit

RNeasy Maxi Kit

For total RNA isolation from

animal cells

animal tissues

bacteria

yeast

whole blood

and for RNA cleanup

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# Kit Contents

<b>RNeasy Kits Catalog no.</b>	<b>Midi Kit (50) 75144</b>	<b>Maxi Kit (12) 75162</b>
Preparations per kit	50	12
RNeasy Midi columns in 15 ml tubes	50	–
RNeasy Maxi columns in 50 ml tubes	–	12
Collection tubes (15 ml) for elution	50	–
Collection tubes (50 ml) for elution	–	12
Buffer RLT*	220 ml	220 ml
Buffer RW1*	220 ml	220 ml
Buffer RPE†	55 ml	55 ml
RNase-free water	50 ml	50 ml
Quick-Start Protocol	1	1

\* Not compatible with disinfecting reagents containing bleach. Contains guanidine isothiocyanate (GITC), which is an irritant. Take appropriate safety measures and wear gloves when handling. Buffer RLT is also available separately. See "Ordering Information".

† Wash Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (90–100%) as indicated on the bottle, to obtain a working solution.

## Storage

RNeasy Kits should be stored dry at room temperature (15–25°C) and are stable for at least 9 months under these conditions, if not otherwise stated on the label.

## Safety Information

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

<p><b>CAUTION</b></p> 	<p>DO NOT add bleach or acidic solutions directly to the sample-preparation waste.</p>
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Buffers RLT and RW1 contain guanidine isothiocyanate, which can form highly reactive compounds when combined with bleach.

If liquid containing these buffers is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

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## Intended Use

RNeasy Midi and Maxi Kits are intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

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

## Quality Control

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

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# Introduction

## RNA isolation

RNeasy Midi and Maxi Kits are designed for rapid isolation of up to 1 mg or 6 mg of total RNA, respectively, from cells and tissues, and yeast. RNeasy Kits facilitate multiple, simultaneous processing of a wide variety of biological samples in 1 h or less. Current time-consuming and tedious methods such as CsCl step-gradient ultracentrifugation, 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 any downstream application such as:

- Ribonuclease (RNase) and S1 nuclease protection
- Primer extension
- Poly A<sup>+</sup> RNA selection
- cDNA synthesis
- RT-PCR
- Differential display
- Quantitative RT-PCR
- Expression-array and expression-chip analysis

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

## RNA stabilization in tissue

RNA stabilization is an absolute prerequisite for reliable gene-expression analysis. Immediate stabilization of RNA in biological materials is necessary because immediately after harvesting the biological sample, changes in the gene-expression pattern occur due to specific and

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nonspecific RNA degradation and transcriptional induction. Such changes in gene-expression pattern need to be avoided for all reliable quantitative gene-expression analyses, such as biochip and array analyses; quantitative RT-PCR, such as TaqMan® and LightCycler® technology.

The RNA/*ater*® RNA Stabilization Reagent represents a new technology enabling rapid and reliable preservation of gene-expression patterns in biological material so as to provide reliable gene-expression analysis. RNA/*ater* technology is designed for stabilization and protection of cellular RNA in animal tissues (in addition, RNA/*ater* technology can be used for cell-culture cells and white blood cells). The samples are harvested and immediately submerged in RNA/*ater* RNA Stabilization Reagent for storage for:

- 1 day at 37°C
- 7 days at 18–25°C
- 4 weeks at 2–8°C
- archival storage at –80°C or –20°C

During storage in RNA/*ater* RNA Stabilization Reagent, even at elevated temperatures (e.g., at room temperature or 37°C), the cellular RNA remains intact and undegraded. RNA/*ater* technology replaces current inconvenient, dangerous, and equipment-intensive methods such as snap-freezing of samples in liquid nitrogen, storage at –80°C, cutting and weighing on dry ice, or immediate processing of the harvested samples.

The RNA/*ater* RNA Stabilization Reagent cannot be used for stabilization of RNA in whole blood, plasma, or serum.

## Principle and procedure

RNeasy Kits represent a novel technology for RNA isolation. This technology combines the selective binding properties of a silica-gel–based membrane with the speed of spin technology. A specialized high-salt buffer system allows up to 1 mg (Midi) or 6 mg (Maxi) of RNA longer

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than 200 bases to adsorb 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 the RNeasy column where the total RNA binds, and contaminants are efficiently washed away. High-quality RNA is then eluted in RNase-free water, ready for use in any downstream application.

With the RNeasy procedure (Figure 1), all RNA molecules longer than 200 nucleotides are isolated. The procedure provides an enrichment for mRNA, because most RNAs <200 nucleotides (such as 5.8S rRNA, 5S rRNA, and tRNAs, which together comprise 15–20% of total RNA) are selectively excluded. The size distribution of purified RNA is comparable to that obtained by centrifugation through a CsCl cushion, where small RNAs do not sediment efficiently.

In this handbook, different protocols are provided for different 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 (Figure 1).

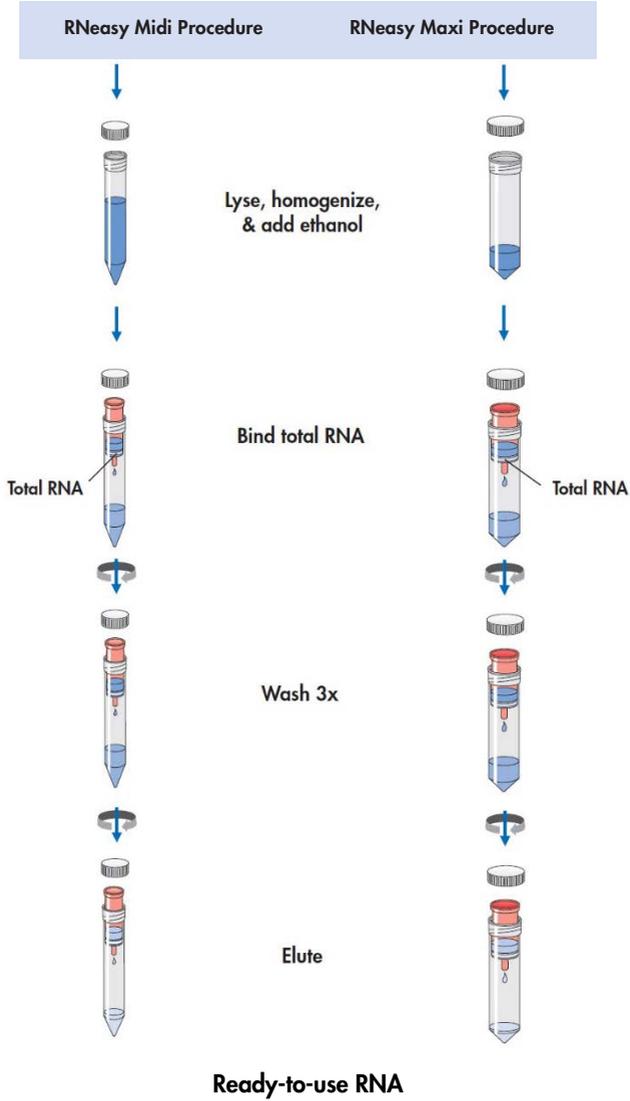


Figure 1. RNA isolation with RNeasy Midi and Maxi Kits.

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## Description of protocols

### Isolation of total RNA from animal cells

Samples (Midi:  $5 \times 10^6$  to  $1 \times 10^8$  cells; Maxi:  $5 \times 10^7$  to  $5 \times 10^8$  cells; amounts depend on cell line used) are disrupted in buffer containing guanidine isothiocyanate and homogenized. An overview of disruption and homogenization methods is given in “Disruption and homogenization of starting material”. Ethanol is then added to the lysate, creating conditions that promote selective binding of RNA to the RNeasy silica-gel membrane. The sample is then applied to the RNeasy column. Total RNA binds to the membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in RNase-free water.

### Isolation of total RNA from animal tissues

Fresh, frozen, or RNA/*ater*-stabilized tissue samples (Midi: 20–250 mg; Maxi: 0.15–1 g; amounts depend on tissue used) are disrupted in buffer containing guanidine isothiocyanate and homogenized. An overview of disruption and homogenization methods is given in “Disruption and homogenization of starting material”. Ethanol is then added to the lysate, creating conditions that promote selective binding of RNA to the RNeasy silica-gel membrane. The sample is then applied to the RNeasy column. Total RNA binds to the membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in RNase-free water.

### Isolation of total RNA from yeast

Two different protocols are provided for the isolation of total RNA from yeast (Midi:  $2 \times 10^7$  to  $5 \times 10^8$  yeast cells; Maxi:  $2.5 \times 10^8$  to  $2.5 \times 10^9$  yeast cells). The protocols differ primarily in the way the yeast cell walls are broken. In general, the protocols function equally well. For some applications the “Enzymatic lysis protocol” might be preferable, because no additional laboratory equipment is required. The “Mechanical disruption protocol”, however, is well-suited for time-course experiments where enzymatic digestion incubations are not practical.

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## **Enzymatic lysis protocol**

This protocol uses zymolase or lyticase digestion of the cell walls to convert cells to spheroplasts, which are processed using the RNeasy Midi or Maxi Kit. Spheroplasts are separated from the digestion mixture before lysis by centrifugation. After addition of GITC-containing lysis buffer and ethanol, samples are loaded onto the RNeasy column. Total RNA binds to the RNeasy silica-gel 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 homogenizer in the presence of glass beads and GITC-containing lysis buffer. Ethanol is added to the lysate creating conditions that promote selective binding of RNA to the RNeasy silica-gel membrane. The sample is then applied to the RNeasy column. Total RNA binds to the RNeasy silica-gel membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in water.

## **RNA cleanup**

RNeasy Kits can be used to purify RNA from enzymatic reactions (e.g., DNase digestion, RNA labeling) or for desalting RNA samples (Midi: maximum 1 mg RNA; Maxi: maximum 6 mg RNA). GITC-containing lysis buffer and ethanol are added to the sample to create conditions that promote selective binding of RNA to the RNeasy silica-gel membrane. The sample is then applied to the RNeasy column. RNA binds to the RNeasy silica-gel membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in water.

## **Specialized protocols**

In addition to the standard protocols in this handbook, 2 specialized protocols are included in the appendices. "Appendix C: RNeasy Midi/Maxi Protocol for Isolation of Total RNA from Heart, Muscle, and Skin Tissue" includes a proteinase digestion and optimized protocol for

these tissues, which have an abundance of contractile proteins, connective tissues, and collagen. "Appendix D: RNeasy Midi/Maxi Guidelines for Isolation of Total RNA from Plants" provides recommendations for isolation of RNA from up to 500 mg or 1 g of plant tissue using the RNeasy Midi or Maxi Kit, respectively. \*

The following supplementary protocols are also available:

- *RNeasy Midi/Maxi Protocol for Isolation of Cytoplasmic RNA from Animal Cells:*  
**[www.qiagen.com/HB-2683](http://www.qiagen.com/HB-2683)**
- *RNeasy Midi/Maxi Protocol for Isolation of Total RNA from Bacteria:*  
**[www.qiagen.com/HB-2684](http://www.qiagen.com/HB-2684)**
- *RNeasy Midi Protocol for Isolation of Total Cellular RNA from Whole Blood:*  
**[www.qiagen.com/HB-2685](http://www.qiagen.com/HB-2685)**

### Optional on-column DNase digestion with the RNase-Free DNase Set

Generally, DNase digestion is not required with RNeasy Kits, because the RNeasy silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). In these cases, residual DNA can be removed using the RNase-Free DNase Set (cat. no. 79254) for optional on-column DNase digestion (see Appendix E). The DNase is efficiently removed in subsequent wash steps. Alternatively, residual DNA can be removed by a DNase digestion after RNA isolation. The DNase digestion can then be cleaned up, if desired, using the RNeasy Midi/Maxi Protocol for RNA Cleanup.

\* For smaller amounts of plant material, up to 100 mg, the RNeasy Plant Mini Kit already comes complete with spin columns, RNase-free reagents, and detailed protocols (see "Ordering Information").

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## RNA stabilization in tissues with RNA/*later* RNA Stabilization Reagent

This handbook provides a detailed protocol for stabilization of RNA in animal tissues. Purification of total RNA from the stabilized tissues can subsequently be carried out using RNeasy Kits following the “Protocol for Isolation of Total RNA from Animal Tissues”. In addition, RNA/*later* technology can be used for RNA stabilization in other sample material, such as eukaryotic cell-culture cells or white blood cells (see “Appendix F: Guidelines for Other RNA/*later* Applications”).

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# Important Points Before Using RNeasy Kits

## Determining the amount of starting material

Using the correct amount of starting material is essential for obtaining high yield and pure RNA with RNeasy columns. The maximum amount that can be used is limited by:

- The volume of Buffer RLT required for efficient lysis and the maximum loading volume of the RNeasy column
- The RNA-binding capacity of the RNeasy column (Midi: 1 mg; Maxi: 6 mg)

For samples that contain high amounts of RNA, this means that less than the absolute maximum amounts of starting material listed in Table 1 should be used so as not to exceed the RNA-binding capacity of the column. For samples that contain 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, the maximum amount of starting material must not be exceeded, or lysis will be incomplete, resulting in lower yield and purity. The maximum amount of a specific biological sample that can be processed depends on the type of tissue or cells being processed and the corresponding RNA content. More information for calculating starting amounts of material is given in each protocol. Typical amounts of starting materials for use with RNeasy columns are shown in Table 1. Table 2 gives examples of expected RNA yields from various sources.

**Note:** If the binding capacity of the RNeasy column is exceeded, yields of total RNA will not be consistent and less than 1 mg (Midi) or 6 mg (Maxi) of total RNA may be recovered. If lysis of the starting material is incomplete, yields of total RNA will be lower than expected, even if the binding capacity of the RNeasy column is not exceeded.

**Table 1. RNeasy Midi and Maxi column specifications**

	RNeasy Midi column	RNeasy Maxi column
Maximum binding capacity	1 mg RNA	6 mg RNA
Maximum loading volume	4 ml	15 ml
RNA size distribution	All RNA >200 nt	All RNA >200 nt
Minimum elution volume	300 µl	800 µl
Amount of starting material:*		
Animal cells	$5 \times 10^6 - 1 \times 10^8$	$5 \times 10^7 - 5 \times 10^8$
Animal tissue	20–250 mg	0.15–1 g
Yeast	$2 \times 10^7 - 5 \times 10^8$	$2.5 \times 10^8 - 2.5 \times 10^9$

\* The upper value represents the absolute maximum for each type of sample; see Table 2 for expected amounts of RNA from specific samples.

**Table 2. Average yields of total RNA isolated from a variety of cells and tissues using RNeasy Midi and Maxi columns**

Source	RNeasy Midi column		RNeasy Maxi column	
	No. of cells or mg of tissue	Yield (µg)*	No. of cells or grams of tissue	Yield (mg)*
Mouse tissue				
Liver	200	700	1.0	3.6
Kidney	200	600	1.0	2.8
Spleen	200	600	1.0	3.2
Heart	200	200 <sup>†</sup>	0.8	0.8 <sup>†</sup>
Thymus	100	250	0.5	1.4
Lung	100	130	0.5	0.6
Brain	100	100	0.5	0.6
Embryo (13 days)	–	n.d.	0.5	2.1
Intestine	–	n.d.	0.4	0.5

n.d.: not determined

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

<sup>†</sup> Using the specialized “Appendix C: RNeasy Midi/Maxi Protocol for Isolation of Total RNA from Heart, Muscle, and Skin Tissue”.

Table continues on next page

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Source	RNeasy Midi column		RNeasy Maxi column	
	No. of cells or mg of tissue	Yield ( $\mu\text{g}$ )*	No. of cells or grams of tissue	Yield (mg)*
Rat tissue				
Heart	–	n.d.	0.8	0.5 <sup>†</sup>
Lung	–	n.d.	0.5	0.5
Pig kidney	–	n.d.	1.0	2.1
Trout kidney	–	n.d.	0.4	1.8
Cow kidney	–	n.d.	1.0	2.2
Cells				
LMH	$7 \times 10^7$	850	$5 \times 10^8$	5.7
HeLa	$7 \times 10^7$	1000	$4 \times 10^8$	6.0
COS-7	$3 \times 10^7$	950	$1.8 \times 10^8$	5.7
Yeast				
<i>S. cerevisiae</i>	$2 \times 10^8$	450	$1 \times 10^9$	2.4

n.d.: not determined

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

<sup>†</sup> Using the specialized "Appendix C: RNeasy Midi/Maxi Protocol for Isolation of Total RNA from Heart, Muscle, and Skin Tissue".

Counting cells and weighing tissue are the 2 most accurate ways to determine the amount of starting material. However, the following may be used as a guide:

- Animal tissue: A cube of rat kidney with a 5 mm edge length (125 mm<sup>3</sup>) weighs 150–175 mg.

Entire mouse organs\* may be used in the RNeasy Maxi procedure as long as they do not exceed 1 g. The following are average weights of various adult mouse organs:

- Kidney 180–250 mg
- Spleen 100–160 mg
- Lung 190–210 mg
- Heart 100–170 mg
- Liver 1–1.8 g (up to 1 g can be processed with the RNeasy Maxi Kit)

- Animal cells: Table 3 gives cell numbers for HeLa cells in various culture vessels.

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

Cell culture vessel	Growth area (cm <sup>2</sup> ) <sup>†</sup>	No. of cells <sup>‡</sup>
Dishes:		
100 mm	56	7 × 10 <sup>6</sup>
145–150 mm	145	2 × 10 <sup>7</sup>
Flasks:		
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>
900 ml	225	3 × 10 <sup>7</sup>

<sup>†</sup> Varies slightly depending on the supplier.

<sup>‡</sup> Cell numbers given are for HeLa cells (approximate length = 15 µm) assuming confluent growth. Cell numbers will vary for different kinds of animal cells, which vary in length from 10–30 µm.

\* Exception: Whole liver and intestine usually weigh more than 1 g. No more than 1 g can be processed with the RNeasy Maxi Kit.

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## Handling and storage of starting material

RNA in tissues is not protected after harvesting until the sample is treated with RNA<sup>later</sup> RNA Stabilization Reagent, flash frozen, or disrupted and homogenized in the presence of RNase-inhibiting or denaturing reagents.

Samples can be immediately flash frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  as soon as they are harvested or excised. Frozen animal or plant tissue should not be allowed to thaw during handling or weighing, but cell pellets can partially thaw enough to allow them 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) after disruption and homogenization. Frozen samples are stable for months.

**Note:** Only freshly harvested samples can be used for enzymatic lysis of yeast cells.

## Disruption and homogenization of starting material

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

**Disruption:** Complete disruption of cell walls and plasma membranes of cells and organelles is an absolute requirement to release all the RNA contained in the sample. Different samples require different methods to achieve complete disruption.

**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 inefficient binding of RNA to the RNeasy silica-gel membrane and therefore significantly reduced yields.

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Some disruption methods simultaneously homogenize the sample, while others require an additional homogenization step. An overview of different disruption and homogenization methods suitable for various starting materials is given in Table 4.

**Note:** After storage in RNA<sup>later</sup> RNA Stabilization Reagent, tissues become slightly harder than fresh or thawed tissues. Disruption and homogenization of this tissue, however, is usually not a problem.

### Disruption and homogenization using rotor–stator homogenizers

Rotor–stator homogenizers thoroughly disrupt and simultaneously homogenize most animal tissues in 45–90 s, 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 and by keeping the tip of the homogenizer submerged. Rotor–stator homogenizers are available in different sizes and operate with differently sized probes. See “Appendix I: Equipment and Reagent Suppliers” for a list of suppliers of rotor–stator homogenizers.

### Disruption and homogenization using the Mixer Mill MM 300 and other bead mills

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

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

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The optimal beads to use are 0.5 mm glass beads for yeast and unicellular animal cells, and 3–7 mm stainless steel beads for animal 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 with glass beads is included in this handbook, because this is the most widespread application for bead-milling. Please refer to suppliers' guidelines for further details.

### Disruption using a mortar and pestle

To disrupt tissue using a mortar and pestle, immediately freeze the sample in liquid nitrogen and grind under liquid nitrogen. Decant the suspension (tissue powder and liquid nitrogen) into an RNase-free, liquid-nitrogen-cooled 10–15 ml (Midi) or 50 ml (Maxi) polypropylene tube and allow the liquid nitrogen to evaporate but do not allow the sample to thaw. Add GITC-containing lysis buffer (Buffer RLT), and continue as quickly as possible with the homogenization according to the protocol.

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

### Homogenization using a syringe and needle

After disruption, cell and tissue lysates can be homogenized using a syringe and needle. High-molecular-weight DNA will be sheared by passing the lysate at least 5–10 times, or until a homogenous lysate is achieved, through an 18- to 21-gauge needle attached to a sterile plastic syringe. Adjusting the volume of lysis buffer may be required for ease of use and to minimize losses.

**Table 4. Disruption and homogenization methods**

Starting material	Disruption method	Homogenization	Comments
Cultured animal cells	Addition of lysis buffer	a) Rotor–stator homogenizer	
		b) Syringe and needle	
Animal tissue	Rotor–stator homogenizer	Rotor–stator homogenizer	Simultaneously disrupts and homogenizes
	Mortar and pestle	Syringe and needle	Rotor–stator homogenizer usually gives higher yields than mortar and pestle plus syringe and needle.
Yeast	Enzymatic (lyticase/zymolase) followed by lysis buffer	a) Rotor–stator homogenizer	Rotor–stator homogenizer usually gives higher yields than syringe and needle.
		b) Syringe and needle	
	Glass beads in a bead mill with lysis buffer	Glass beads in a bead mill	Bead-milling simultaneously disrupts and lysis buffer homogenizes; bead-milling cannot be replaced by vortexing.

# Equipment and Reagents to Be Supplied by User

## For all protocols

- 14.3 M  $\beta$ -mercaptoethanol ( $\beta$ -ME)\* (stock solutions are usually 14.3 M)
- Sterile, RNase-free pipette tips
- Laboratory centrifuge (capable of 3000–5000  $\times g$ )<sup>†</sup>
- Equipment for disruption and homogenization (see pages 19–22, 91)
- Vessels for homogenization (e.g., 10–15 ml centrifuge tubes for the RNeasy Midi Kit; 50 ml centrifuge tubes for the RNeasy Maxi Kit)
- Ethanol (96–100%)
- Disposable gloves

## For animal cell and animal tissue protocols

- Ethanol (70% in water)

\*  $\beta$ -ME must be added to Buffer RLT before use.  $\beta$ -ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 10  $\mu$ l of 14.3 M  $\beta$ -ME per 1 ml of Buffer RLT. The solution is stable for 1 month after the addition of  $\beta$ -ME.

<sup>†</sup> All centrifugation steps are carried out in a conventional laboratory centrifuge, e.g., QIAGEN Centrifuge 4-15C, Centrifuge 4K15C, Beckman CS-6KR, or equivalent, with a swinging bucket rotor for 15 ml (Midi) or 50 ml (Maxi) centrifuge tubes (the maximum speed of 3500–5000 rpm corresponds to 3000–5000  $\times g$  for most rotors). RNeasy Midi columns supplied with the kit fit into 15 ml centrifuge tubes. RNeasy Maxi columns supplied with the kit fit into 50 ml centrifuge tubes. These fit into the rotor of almost every standard laboratory centrifuge available. In the unlikely event that the tubes do not fit, the RNeasy columns can also be inserted into different 12–15 ml (Midi) or 50 ml (Maxi) RNase-free glass or polypropylene tubes. All centrifugation steps are carried out at 20–25°C.

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## For yeast protocols

### Enzymatic protocol

- Ethanol (70% in water)
- Buffer Y1 \* 1 M sorbitol

0.1 M EDTA, pH 7.4

Just before use, add: 0.1% (v/v)  $\beta$ -ME (add 1  $\mu$ l of 14.3 M  $\beta$ -ME per 1 ml of Buffer Y1)  
100 U lyticase/zymolase per 1 ml of Buffer Y1

Depending on the yeast strain and enzyme used, the incubation time, enzyme concentration, and composition of Buffer Y1 may vary. Please adhere to the guidelines of the enzyme supplier.

### Mechanical disruption protocol

- Acid-washed glass beads, 0.5 mm diameter
- Bead-mill homogenizer

**Note:** The yeast mechanical disruption protocol requires more Lysis Buffer RLT than do the other protocols in this handbook. Therefore, if  $>2 \times 10^8$  yeast cells are processed with the RNeasy Midi Kit, or  $>1 \times 10^9$  yeast cells with the RNeasy Maxi Kit, additional Buffer RLT is required. If necessary, additional Buffer RLT is available separately. See "Ordering Information".

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

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# RNeasy Midi/Maxi Protocol for Isolation of Total RNA from Animal Cells

## Determining the correct amount of starting material

It is essential to use the correct number of cells in order to obtain optimal RNA yield and purity with RNeasy columns. A minimum amount of  $5 \times 10^6$  or  $5 \times 10^7$  cells can generally be processed with RNeasy Midi or Maxi columns, respectively. The maximum number of cells that can be used depends on the specific RNA content of the cell line used, which varies greatly between cell types. Two main criteria limit the maximum number of cells to use:

- The binding capacity of the RNeasy Midi (1 mg RNA) and Maxi (6 mg RNA) spin columns.
- The volume of Buffer RLT required for efficient lysis. The maximum volume of Buffer RLT that can be used in the RNeasy procedure limits the amount of starting material to an absolute maximum of  $1 \times 10^8$  cells with the RNeasy Midi column and  $5 \times 10^8$  cells with the RNeasy Maxi column.

The following examples illustrate how to determine the correct maximum amount of starting material:

- COS cells:** High RNA content (approximately 3.1 mg RNA per  $10^8$  cells)  
No more than  $3 \times 10^7$  cells can be used with the RNeasy Midi column and no more than  $2 \times 10^8$  cells with the RNeasy Maxi column. Otherwise, the binding capacity of the spin column will be exceeded.
- HeLa cells:** Average RNA content (approximately 1.5 mg RNA per  $10^8$  cells)  
No more than  $7 \times 10^7$  cells can be used with the RNeasy Midi column and no more than  $4 \times 10^8$  cells with the RNeasy Maxi column. Otherwise, the binding capacity of the spin column will be exceeded.

**NIH/3T3 cells:** Low RNA content (approximately 0.9 mg RNA per  $10^8$  cells)

The maximum number of cells that can be processed with the RNeasy Midi and Maxi columns can be used:  $1 \times 10^8$  cells with the RNeasy Midi column or  $5 \times 10^8$  cells with the RNeasy Maxi column.

**Important:** If the cell type used is not shown in Table 2 (page 16) and you have no information about the RNA content of your starting material, we recommend starting with no more than  $3\text{--}4 \times 10^7$  cells per RNeasy Midi column or  $2 \times 10^8$  cells per RNeasy Maxi column. Depending on yield and purity, it may be possible to increase cell number in subsequent preparations.

**Important:** Do not overload the column. Overloading will significantly reduce yield and purity.

The numbers of HeLa cells expected in certain cell culture vessels are shown in Table 3.

### Important points before starting

- If using RNeasy Midi or Maxi Kits for the first time, read "Important Points Before Using RNeasy Kits".
- If working with RNA for the first time, read Appendix A.
- RNA in cells can be stabilized in RNA $/ater$  RNA Stabilization Reagent for later use. See Appendix F for details.
- RNA $/ater$  RNA Stabilization Reagent should be stored dry at room temperature and is stable for at least 9 months under these conditions, if not otherwise stated on the label. Storage of RNA $/ater$  RNA Stabilization Reagent at lower temperatures may cause precipitation. The precipitate can be redissolved by heating to  $37^\circ\text{C}$  with agitation. Redissolve any precipitate before using.
- Cell pellets can be stored at  $-70^\circ\text{C}$  for later use or used directly in the procedure. Determine the number of cells before freezing. Frozen cell pellets should be thawed slightly so that cell pellets can be dislodged by flicking in step 2. Homogenized cell lysates (in Buffer RLT, step 3) can be stored at  $-70^\circ\text{C}$  for several months. To process frozen lysates, thaw samples for 15–20 min at  $37^\circ\text{C}$  in a water bath to dissolve salts. If

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any insoluble material is visible, centrifuge for 5 min at 3000–5000  $\times g$ . Transfer supernatant to a new RNase-free glass or polypropylene tube, and continue with step 4.

- **Important:**  $\beta$ -ME must be added to Buffer RLT before use.  $\beta$ -ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 10  $\mu$ l  $\beta$ -ME per 1 ml Buffer RLT. Buffer RLT is stable for 1 month after addition of  $\beta$ -ME.
- **Important:** 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.
- Generally, DNase digestion is not required, because the RNeasy silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). In these cases, the small residual amounts of DNA remaining can be removed using the RNase-Free DNase Set for the optional on-column DNase digestion (see page 83) or by a DNase digestion after RNA isolation. For on-column DNase digestion with the RNase-Free DNase Set, prepare the DNase I stock solution as described in “Appendix E: Optional On-Column DNase Digestion with the RNase-Free DNase Set” before beginning the procedure.
- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature.
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. Guanidine is an irritant. Take appropriate safety measures and wear gloves when handling.
- All steps of the RNeasy protocol should be performed at room temperature. During the procedure, work quickly.
- All centrifugation steps are performed at 20–25°C in a standard laboratory centrifuge with a swinging bucket rotor capable of  $\geq 3000 \times g$  (see page 23). Ensure that the centrifuge does not cool below 20°C.

- Blue (marked with a ▲) denotes ▲ RNeasy Midi prep volumes (for  $5 \times 10^6$  to  $1 \times 10^8$  cells); red (marked with a ●) denotes ● RNeasy Maxi prep volumes (for  $5 \times 10^7$  to  $5 \times 10^8$  cells).

## Procedure

### 1. Harvest cells.

#### 1a. Cells grown in suspension (Do not use more than ▲ $1 \times 10^8$ or ● $5 \times 10^8$ cells.)

Determine the number of cells. Pellet the appropriate number of cells for 5 min at  $300 \times g$  in an RNase-free glass or polypropylene centrifuge tube (not supplied). Carefully remove all supernatant by aspiration, and continue with step 2 of the protocol.

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

#### 1b. Cells grown in a monolayer (Do not use more than ▲ $1 \times 10^8$ or ● $5 \times 10^8$ cells.)

Cells grown in a monolayer in cell-culture vessels can either be lysed directly in the culture dish or trypsinized and collected as a cell pellet prior to lysis. Cells grown in a monolayer in cell-culture flasks should always be trypsinized.

**To lyse cells directly in culture dish:** Determine the number of cells. (Table 3 gives approximate cell numbers for various cell-culture vessels.) Completely aspirate cell-culture medium, and continue immediately with step 2 of the protocol.

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

**To trypsinize cells:** Determine the number of cells. (Table 3 gives approximate cell numbers for various cell-culture vessels.) Aspirate medium and wash cells with PBS. Aspirate PBS and add 0.10–0.25% trypsin in PBS to trypsinize the cells. After cells detach from the dish or flask, add medium (containing serum to inactivate the trypsin), transfer cells to an RNase-free glass or polypropylene centrifuge tube (not

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supplied), and pellet by centrifugation at 300 x *g* for 5 min. Completely aspirate supernatant, and continue with step 2 of the protocol.

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

2. Disrupt cells by addition of Buffer RLT.

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

**Note:** Incomplete loosening of the cell pellet may lead to inefficient lysis and reduced yields. Ensure that  $\beta$ -ME is added to Buffer RLT before use (see “Important points before starting”).

For direct lysis of cells grown in monolayer, add the appropriate volume of Buffer RLT (see Table 5) to cell-culture dish. Collect cell lysate with a rubber policeman. Pipet lysate into an RNase-free glass or polypropylene tube (not supplied), and proceed to step 3.

If total sample is grown on several dishes, the volume of Buffer RLT must be divided proportionately.

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

**Table 5. Buffer RLT volumes for RNeasy Midi/Maxi isolation of total RNA from animal cells**

RNeasy column	Number of cells	Buffer RLT (ml)
Midi ▲	$5 \times 10^6 - 3 \times 10^7$	2.0
Midi ▲	$3 \times 10^7 - 5 \times 10^7$	2.0 (4.0)*
Midi ▲	$5 \times 10^7 - 1 \times 10^8$	4.0
Maxi ●	$5 \times 10^7 - 1.5 \times 10^8$	7.5
Maxi ●	$1.5 \times 10^8 - 2.5 \times 10^8$	7.5 (15.0)†
Maxi ●	$2.5 \times 10^8 - 5 \times 10^8$	15.0

\* If using the RNeasy Midi Kit to prepare RNA from cells containing more than 150 µg of total RNA per  $10^7$  cells, it may be necessary to use ▲ 4.0 ml Buffer RLT for  $>3 \times 10^7$  cells.

† If using the RNeasy Maxi Kit to prepare RNA from cells containing more than 2 mg of total RNA per  $1 \times 10^8$  cells, it may be necessary to use ● 15.0 ml Buffer RLT for  $>1.5 \times 10^8$  cells.

3. Homogenize cells using a conventional rotor–stator homogenizer for at least 45 s at maximum speed until the sample is uniformly homogeneous. Alternatively, vortex the sample for 10 s, and pass the lysate at least 5–10 times through an 18- to 20-gauge needle fitted to an RNase-free syringe.

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

4. Add 1 volume (▲ 2.0 ml or 4.0 ml; ● 7.5 ml or 15.0 ml) of 70% ethanol to the homogenized lysate, and mix thoroughly by shaking vigorously. Do *not* centrifuge. If some lysate is lost during homogenization, adjust volume of ethanol accordingly.

**Note:** Visible precipitates may form after the addition of ethanol when preparing RNA from certain cell lines. Resuspend precipitates completely by vigorous shaking, and proceed immediately to step 5. Insufficient resuspension of precipitates will cause DNA contamination and can lead to impure total RNA.

5. Apply the sample, including any precipitate that may have formed, to an ▲ RNeasy Midi column or ● RNeasy Maxi column placed in a ▲ 15 ml or ● 50 ml centrifuge tube

(supplied). Maximum loading volume is ▲ 4.0 ml or ● 15 ml. Close the tube gently, and centrifuge for 5 min at 3000–5000  $\times g$ . Discard the flow-through.\*

Reuse the centrifuge tube in step 6.

If the maximum amount of starting material is used, it may be necessary to increase centrifugation time to 10 min to allow the lysate to completely pass through the column.

If the volume exceeds ▲ 4.0 ml or ● 15 ml, load aliquots successively onto the RNeasy column, and centrifuge as above. Discard the flow-through after each centrifugation step.\*

**Optional:** QIAGEN offers the RNase-Free DNase Set for convenient on-column DNase digestion during RNA purification. Generally, DNase digestion is not required, because the RNeasy silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). If using the RNase-Free DNase Set, follow the procedure in Appendix E, after performing this step.

6. Add ▲ 4.0 ml or ● 15 ml Buffer RW1 to the RNeasy column. Close the centrifuge tube gently, and centrifuge for 5 min at 3000–5000  $\times g$  to wash the column. Discard the flow-through.\*

Skip this step if performing the optional on-column DNase digestion (Appendix E). Reuse the centrifuge tube in step 7.

7. Add ▲ 2.5 ml or ● 10 ml Buffer RPE to the RNeasy column. Close the centrifuge tube gently, and centrifuge for 2 min at 3000–5000  $\times g$  to wash the column. Discard the flow-through.

Reuse the centrifuge tube in step 8. In the RNeasy Midi procedure, it is not necessary to discard the flow-through.

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

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

8. Add another ▲ 2.5 ml or ● 10 ml Buffer RPE to the RNeasy column. Close the centrifuge tube gently, and centrifuge for ▲ 5 min or ● 10 min at 3000–5000 × *g* to dry the RNeasy silica-gel membrane.

It is important to dry the RNeasy membrane, because residual ethanol may interfere with downstream reactions. This centrifugation ensures that no ethanol is carried over during elution.

**Note:** After centrifugation, remove the RNeasy column from the centrifuge tube carefully so that the column does not come in contact with the flow-through, because this will result in carryover of ethanol.

9. To elute, transfer the RNeasy column to a new ▲ 15 ml or ● 50 ml collection tube (supplied). Pipet the appropriate volume of RNase-free water (see Table 6) directly onto the RNeasy silica-gel membrane. Close the tube gently. Let it stand for 1 min, and then centrifuge for 3 min at 3000–5000 × *g*.

**Table 6. RNase-free water volumes for RNeasy Midi/Maxi elution**

RNeasy column	Expected total RNA yield	RNase-free water
Midi ▲	≤150 µg	150 µl
Midi ▲	150 µg – 1 mg	250 µl
Maxi ●	≤1 mg	0.8 ml
Maxi ●	1–6 mg	1.2 ml

10. Repeat the elution step (step 9) as described with a second volume of RNase-free water.

To obtain a higher total RNA concentration, this second elution step may be performed by using the first eluate (from step 9). The yield will be 15–30% less than the yield obtained using a second volume of RNase-free water, but the final concentration will be higher.

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# RNeasy Midi/Maxi Protocol for Isolation of Total RNA from Animal Tissues

## Determining the correct amount of starting material

It is essential to begin with the correct amount of tissue in order to obtain optimal RNA yield and purity with RNeasy columns. A minimum amount of 20 mg or 150 mg tissue can generally be processed with RNeasy Midi or Maxi columns, respectively, and a maximum of 250 mg or 1 g. For most tissues, the binding capacity of the column (1 mg or 6 mg of RNA) and the lysing capacity of Buffer RLT will not be exceeded by these amounts. Depending on the type of tissue, 0.1–1.0 mg of total RNA can be expected from 250 mg tissue and 0.4–4 mg of total RNA from 1 g of tissue. Average RNA yields from various sources are given in Table 2.

Some tissues such as spleen, parts of brain, lung, and thymus are more difficult to lyse or tend to form precipitates during the procedure. The maximum amount of these tissues used in the RNeasy Midi or Maxi protocol may need to be decreased to 100–150 mg or 0.4–0.6 g, respectively. This will facilitate complete homogenization and avoid significantly reduced yields, DNA contamination, or clogging of the RNeasy column.

Total RNA isolation from skeletal muscle, heart, and skin tissue can be difficult due to the abundance of contractile proteins, connective tissue, and collagen. The specialized protocol in Appendix C includes a proteinase digestion and optimized RNA isolation procedure for these tissues.

**Important:** If you have no information about the nature of your starting material, we recommend starting with no more than 100 mg of tissue with the RNeasy Midi procedure or 0.6 g with the RNeasy Maxi procedure. Depending on the yield and purity obtained, it may be possible to increase the amount of tissue to 250 mg in the RNeasy Midi procedure or 1 g in the RNeasy Maxi procedure.

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**Important:** Do not overload the column. Overloading will significantly reduce yield and quality.

### Important points before starting

- If using RNeasy Midi or Maxi Kits for the first time, read “Important Points Before Using RNeasy Kits”.
- If working with RNA for the first time, read Appendix A.
- **Important:** For best results, stabilize animal tissues immediately in RNA/*later* RNA Stabilization Reagent. Tissues can be stored in RNA/*later* RNA Stabilization Reagent for up to 1 day at 37°C, 7 days at 18–25°C, 4 weeks at 2–8°C, or for archival storage at –20°C or –80°C.
- Fresh, frozen, or RNA/*later*-stabilized tissue can be used. To freeze unstabilized tissue for long-term storage, flash-freeze in liquid nitrogen, and immediately transfer to –70°C. Tissue can be stored for several months at –70°C. To process, do not allow tissue to thaw during weighing or handling prior to disruption in Buffer RLT. Homogenized tissue lysates (in Buffer RLT, step 4) can also be stored at –70°C for several months. To process frozen lysates, thaw samples and incubate for 15–20 min at 37°C in a water bath to dissolve salts. Continue with step 4.
- RNA/*later* RNA Stabilization Reagent should be stored dry at room temperature and is stable for at least 9 months under these conditions, if not otherwise stated on the label. Storage of RNA/*later* RNA Stabilization Reagent at lower temperatures may cause precipitation. The precipitate can be redissolved by heating to 37°C with agitation. Redissolve any precipitate before using.
- **Important:** β-ME must be added to Buffer RLT before use. β-ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 10 μl β-ME per 1 ml Buffer RLT. Buffer RLT is stable for 1 month after addition of β-ME.
- **Important:** 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.
- Generally, DNase digestion is not required, because the RNeasy silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to

very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). In these cases, the small residual amounts of DNA remaining can be removed using the RNase-Free DNase Set for the optional on-column DNase digestion (see page 83) or by a DNase digestion after RNA isolation. For on-column DNase digestion with the RNase-Free DNase Set, prepare the DNase I stock solution as described in “Appendix E: Optional On-Column DNase Digestion with the RNase-Free DNase Set” before beginning the procedure.

- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature.
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. Guanidine is an irritant. Take appropriate safety measures and wear gloves when handling.
- All steps of the RNeasy protocol should be performed at room temperature. During the procedure, work quickly.
- All centrifugation steps are performed at 20–25°C in a standard laboratory centrifuge with a swinging bucket rotor capable of  $\geq 3000 \times g$  (see page 23). Ensure that the centrifuge does not cool below 20°C.
- Blue (marked with a ▲) denotes ▲ RNeasy Midi prep volumes (for 20–250 mg tissue); red (marked with a ●) denotes ● RNeasy Maxi prep volumes (for 150 mg – 1 g tissue).

## Procedure

1. Excise the tissue sample from the animal or remove it from storage. Remove RNA/*ater*-stabilized tissues from the reagent using forceps.
2. Determine the amount of tissue. Do not use more than ▲ 250 mg or ● 1 g tissue. Weighing tissue is the most accurate way to determine the amount. See page 33 for guidelines to determine the amount of starting material.

### 3. For RNA/*ater*-stabilized tissues:

If the entire piece of RNA/*ater*-stabilized tissue can be used for RNA isolation, place it directly into a suitably sized vessel for disruption and homogenization, and proceed with step 4.

If only a portion of the RNA/*ater*-stabilized tissue is to be used, place the tissue on a clean surface for cutting, and cut it. Determine the weight of the piece to be used, and place it into a suitably sized vessel for homogenization. Proceed with step 4.

RNA in the RNA/*ater*-treated tissue is still protected while the tissue is processed at 18–25°C. This allows cutting and weighing of tissues at ambient temperatures. It is not necessary to cut the tissue on ice or dry ice or in a refrigerated room. The remaining tissue can be placed into RNA/*ater* RNA Stabilization Reagent for further storage. Previously stabilized tissues can be stored at –80°C without the reagent.

#### **For unstabilized fresh or frozen tissues:**

If the entire piece of tissue can be used for RNA isolation, place it directly into a suitably sized vessel for disruption and homogenization, and proceed immediately with step 4.

If only a portion of the tissue is to be used, determine the weight of the piece to be used, and place it into a suitably sized vessel for homogenization. Proceed immediately with step 4.

RNA in tissues is not protected after harvesting until the sample is treated with RNA/*ater* RNA Stabilization Reagent, flash frozen, or disrupted and homogenized in protocol step 4. Frozen animal tissue should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible.

**Note:** The remaining fresh tissue can be placed into RNA/*ater* RNA Stabilization Reagent for stabilization. However, previously frozen tissue samples thaw too slowly in the reagent, thus preventing it from diffusing into the tissue quickly enough before the RNA begins to degrade.

4. Disrupt tissue and homogenize lysate in Buffer RLT. (Do not use more than ▲ 250 mg or ● 1 g tissue.)

Disruption and homogenization of animal tissue for the RNeasy Midi or Maxi procedure can be performed by 2 alternative methods (4a or 4b). See in “Disruption and homogenization of starting material” for a more detailed description of disruption and homogenization methods.

After storage in RNA<sup>later</sup> RNA Stabilization Reagent, tissues may become slightly harder than fresh or thawed tissues. Disruption and homogenization of tissue samples using standard methods is usually not a problem.

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

- 4a. Rotor–stator homogenization

Place the weighed (fresh, frozen, or RNA<sup>later</sup>-stabilized) tissue in a suitably sized vessel for the homogenizer (e.g., in a ▲ 10–15 ml or ● 50 ml centrifuge tube, not supplied). Add the appropriate volume of Buffer RLT (see Table 7). Homogenize immediately using a conventional rotor–stator homogenizer for ▲ 45 s or ● 60 s until the sample is uniformly homogeneous. Continue the protocol with step 5. Rotor–stator homogenization simultaneously disrupts and homogenizes the sample.

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

- 4b. Mortar and pestle with needle and syringe homogenization

Immediately place the weighed (fresh, frozen, or RNA<sup>later</sup>-stabilized) tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen-cooled 2 ml microcentrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw.

Add the appropriate volume of Buffer RLT (see Table 7). Vortex for 5 s, and homogenize by passing lysate at least 5–10 times through an 18- to 20-gauge needle fitted to an RNase-free syringe. Continue the protocol with step 5. Grinding the sample using a mortar and pestle will disrupt the sample, but it will not homogenize it. Homogenization is carried out with the needle and syringe.

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

**Table 7. Buffer RLT volumes for RNeasy Midi/Maxi isolation of total RNA from animal tissues**

RNeasy column	Amount of tissue (mg)	Buffer RLT (ml)
Midi ▲	20–75	2.0
Midi ▲	75–130	2.0 (4.0)*
Midi ▲	130–250	4.0
Maxi ●	150–300	7.5
Maxi ●	300–500	7.5 (15.0)†
Maxi ●	500–1000	15.0

\* If using the RNeasy Midi Kit to prepare RNA from tissues that are difficult to lyse, it may be necessary to use ▲ 4.0 ml Buffer RLT for >75 mg tissue.

† If using the RNeasy Maxi Kit to prepare RNA from tissues that are difficult to lyse, it may be necessary to use ● 15.0 ml Buffer RLT for >300 mg tissue.

5. Centrifuge the tissue lysate for 10 min at 3000–5000  $\times$  *g*. Carefully transfer the supernatant to a new ▲ 10–15 ml or ● 50 ml tube (not supplied) by pipetting. Use only this supernatant (lysate) in subsequent steps.

In most preparations, a small pellet will form, which is sometimes accompanied by a fatty upper layer. Transferring the pellet or the fatty layer may reduce the amount of RNA that binds to the membrane and cause the spin column to clog.

**Note:** To avoid transferring contaminants, hold the pipette tip underneath the fatty upper layer, and do not disturb the pellet.

6. Add 1 volume (▲ 2.0 ml or 4.0 ml; ● 7.5 or 15.0 ml) of 70% ethanol to the homogenized lysate and mix immediately by shaking vigorously. Ensure that any precipitates are resuspended. Do *not* centrifuge. Continue without delay with step 7. If some lysate is lost during steps 4 and 5, adjust volume of ethanol accordingly.

**Note:** Visible precipitates may form after the addition of ethanol when preparing RNA from certain tissues (thymus, spleen, etc.). Resuspend precipitates completely by vigorous shaking, and proceed immediately to step 7. Insufficient resuspension of precipitates will cause DNA contamination and can lead to impure total RNA.

7. Apply the sample to an ▲ RNeasy Midi column or ● RNeasy Maxi column placed in a ▲ 15 ml or ● 50 ml centrifuge tube (supplied), and close the tube gently. Maximum loading volume is ▲ 4.0 ml or ● 15 ml. Centrifuge for 5 min at 3000–5000 x *g*. Discard the flow-through.\*

Reuse the centrifuge tube in step 8.

If the maximum amount of starting material is used, it may be necessary to increase centrifugation time to 10 min to allow the lysate to completely pass through the column. If volume exceeds 4.0 ml or 15 ml, load aliquots successively onto the RNeasy column, and centrifuge as above. Discard the flow-through after each centrifugation step.\*

**Optional:** QIAGEN offers the RNase-Free DNase Set for convenient on-column DNase digestion during RNA purification. Generally, DNase digestion is not required, because the RNeasy silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). If using the RNase-Free DNase Set, follow the steps shown on page 83 after performing this step.

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

8. Add ▲ 4.0 ml or ● 15 ml Buffer RW1 to the RNeasy column. Close the centrifuge tube gently, and centrifuge for 5 min at 3000–5000  $\times g$  to wash the column. Discard the flow-through.\*

Skip this step if performing the optional on-column DNase digestion (Appendix E). Reuse the centrifuge tube in step 9.

9. Add ▲ 2.5 ml or ● 10 ml Buffer RPE to the RNeasy column. Close the centrifuge tube gently, and centrifuge for 2 min at 3000–5000  $\times g$  to wash the column. Discard the flow-through.

Reuse the centrifuge tube in step 10. In the RNeasy Midi procedure, the flow-through does not need to be discarded.

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

10. Add another ▲ 2.5 ml or ● 10 ml Buffer RPE to the RNeasy column. Close the centrifuge tube gently, and centrifuge for ▲ 5 min or ● 10 min at 3000–5000  $\times g$  to dry the RNeasy silica-gel membrane.

It is important to dry the RNeasy membrane, because residual ethanol may interfere with downstream reactions. This centrifugation ensures no ethanol is carried over during elution.

**Note:** After centrifugation, remove the RNeasy column from the centrifuge tube carefully so that the column does not come in contact with the flow-through, because this will result in carryover of ethanol.

11. To elute, transfer the RNeasy column to a new ▲ 15 ml or ● 50 ml collection tube (supplied). Pipet the appropriate volume of RNase-free water (see Table 8) directly onto the RNeasy silica-gel membrane. Close the tube gently. Let it stand for 1 min, and then centrifuge for 3 min at 3000–5000  $\times g$ .

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

**Table 8. RNase-free water volumes for RNeasy Midi/Maxi elution**

RNeasy column	Expected total RNA yield	RNase-free water
Midi ▲	≤150 µg	150 µl
Midi ▲	150 µg – 1 mg	250 µl
Maxi ●	≤1 mg	0.8 ml
Maxi ●	1–6 mg	1.2 ml

12. Repeat the elution step (step 11) as described with a second volume of RNase-free water.

To obtain a higher total RNA concentration, this second elution step may be performed using the first eluate (from step 11). The yield will be 15–30% less than the yield obtained using a second volume of RNase-free water, but the final concentration will be higher.

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# RNeasy Midi/Maxi Protocols for Isolation of Total RNA from Yeast

There are two different protocols for the isolation of total RNA from yeast. The two protocols differ primarily in the way the yeast cell walls are disrupted. In general, the protocols function equally well. For some applications, the “Enzymatic lysis protocol for isolation of total RNA from yeast” may be preferable as no additional laboratory equipment is required. However, the “Mechanical disruption protocol for isolation of total RNA from yeast” can be used in time-course experiments where enzymatic incubation steps cannot be tolerated.

The **enzymatic lysis protocol** requires digestion of the cell wall with zymolase or lyticase to convert cells to spheroplasts. Spheroplasts are separated from the digestion mixture by centrifugation and are subsequently lysed and processed in the RNeasy procedure.

The **mechanical disruption protocol** uses high-speed agitation in a bead mill, in the presence of glass beads and lysis buffer, to lyse the cells and release the RNA.

## Determining the correct amount of starting material

It is essential to use the correct number of yeast cells to obtain optimal RNA yield and purity with RNeasy columns. A minimum amount of  $2 \times 10^7$  or  $2.5 \times 10^8$  yeast cells can generally be processed with RNeasy Midi or Maxi columns, respectively. Two main criteria limit the maximum number of yeast cells to use:

- The binding capacity of the RNeasy Midi (1 mg RNA) and Maxi (6 mg RNA) columns
- The volume of Buffer RLT required for efficient lysis. The maximum volume of Buffer RLT that can be used in the RNeasy procedure limits the amount of starting material to an absolute maximum of  $5 \times 10^8$  yeast cells with the RNeasy Midi column and  $2.5 \times 10^9$  yeast cells with the RNeasy Maxi column.

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For yeast cultures containing high levels of RNA, fewer yeast cells should be used, so as not to exceed the RNA-binding capacity of the spin column. For yeast cultures containing lower levels of RNA, the maximum number of yeast cells can be used. In these cases, even though the RNA-binding capacity of the column may not be reached, the use of more cells would lead to incomplete lysis, resulting in lower RNA yield and purity.

Usually,  $2 \times 10^7$  –  $5 \times 10^8$  yeast cells can be processed with the RNeasy Midi column and  $2.5 \times 10^8$  –  $2.5 \times 10^9$  yeast cells with the RNeasy Maxi column. Depending on the strain and growth conditions used, 0.2–1.2 mg RNA can be expected from  $4.0 \times 10^8$  yeast cells and 1.2–6.0 mg RNA from  $2.0 \times 10^9$  yeast cells.

**Important:** If you have no information about the RNA content of your starting material, we recommend starting with no more than  $2 \times 10^8$  yeast cells per RNeasy Midi column or  $1 \times 10^9$  yeast cells per RNeasy Maxi column. Depending on yield and purity, it may be possible to increase the cell number in subsequent preparations.

**Important:** Do not overload the column. Overloading will significantly reduce yield and purity.

Yeast growth is usually measured using a spectrophotometer. However, it is very difficult to give specific and reliable recommendations for the relations between OD values and cell number in cultures. Cell density is influenced by a variety of factors (e.g., species, media, incubation time, 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. Therefore, readings vary between different types of spectrophotometers. In addition, different species show different OD values at the same wavelength.

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. The dilution factor should then be used in calculating the number of cells per milliliter.

The following values may be used as a rough guide. An *S. cerevisiae* culture containing  $1-2 \times 10^7$  cells per milliliter, diluted 1 in 4, gives an OD<sub>600</sub> value of approximately 0.25 with a Beckman DU®-7400 spectrophotometer or 0.125 with a Beckman DU-40 spectrophotometer.

## Enzymatic lysis protocol for isolation of total RNA from yeast

Use an appropriate number of yeast cells (see page 42).

### Important points before starting

- If using RNeasy Midi or Maxi Kits for the first time, read "Important Points Before Using RNeasy Kits".
- If working with RNA for the first time, read Appendix A.
- For RNA isolation from yeast, cells should be harvested in log-phase growth. Use only freshly harvested cells for the enzymatic lysis protocol.
- Prepare Buffer Y1

Buffer Y1 \* 1 M sorbitol

1 M EDTA, pH 7.4

Just before use, add:

0.1% (v/v)  $\beta$ -ME (add 1  $\mu$ l of 14.3 M  $\beta$ -ME per 1 ml of Buffer Y1)

100 U lyticase/zymolase per 1 ml of Buffer Y1

Depending on the yeast strain and enzyme used, the incubation time, enzyme concentration, and composition of Buffer Y1 may vary. Please adhere to the guidelines of the enzyme supplier.

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

- **Important:**  $\beta$ -ME must be added to Buffer RLT before use.  $\beta$ -ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 10  $\mu$ l  $\beta$ -ME per 1 ml Buffer RLT. Buffer RLT is stable for 1 month after addition of  $\beta$ -ME.
- **Important:** 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.
- Generally, DNase digestion is not required, because the RNeasy silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). In these cases, the small residual amounts of DNA remaining can be removed using the RNase-Free DNase Set for the optional on-column DNase digestion (see page 83) or by a DNase digestion after RNA isolation. For on-column DNase digestion with the RNase-Free DNase Set, prepare the DNase I stock solution as described in “Appendix E: Optional On-Column DNase Digestion with the RNase-Free DNase Set” before beginning the procedure.
- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature.
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. Guanidine is an irritant. Take appropriate safety measures and wear gloves when handling.
- After enzymatic lysis, all steps of the RNeasy protocol should be performed at room temperature. During the procedure, work quickly.
- After harvesting the cells, all centrifugation steps should be performed at 20–25°C in a standard laboratory centrifuge with a swinging bucket rotor capable of  $\geq 3000 \times g$  (see page 23). Ensure that the centrifuge does not cool below 20°C.
- **Blue** (marked with a ▲) denotes ▲ RNeasy Midi prep volumes (for  $2 \times 10^7$  to  $5 \times 10^8$  yeast cells); **red** (marked with a ●) denotes ● RNeasy Maxi prep volumes (for  $2.5 \times 10^8$  to  $2.5 \times 10^9$  yeast cells).

## Procedure

1. Harvest cells by centrifuge at  $500 \times g$  for 5 min at  $4^{\circ}\text{C}$ . (Do not use more than  $\blacktriangle 5 \times 10^8$  or  $\bullet 2.5 \times 10^9$  yeast cells.) Decant supernatant, and carefully remove remaining media by aspiration. After centrifuging, heat the centrifuge to  $20\text{--}25^{\circ}\text{C}$  if the same centrifuge is to be used in the following centrifugation steps of the protocol.

Incomplete removal of the supernatant will affect digestion of the cell wall in step 2.

**Note:** Freshly harvested cells must be used.

2. Resuspend cells in freshly prepared Buffer Y1 containing lyticase or zymolase. Use 1 ml Buffer Y1 per  $10^8$  yeast cells. Incubate for 30 min at  $30^{\circ}\text{C}$  with gentle shaking to generate spheroplasts. Spheroplasts must be handled gently.

Incubation time, amount of enzyme, and composition of Buffer Y1 may vary, depending on the yeast strain used. For best results, follow the guidelines of lyticase/zymolase supplier. Complete spheroplasting is essential for efficient lysis.

3. Centrifuge for 5 min at  $500 \times g$  to pellet the spheroplasts. Carefully remove and discard the supernatant.

**Note:** Incomplete removal of the supernatant will inhibit lysis and dilute the lysate, affecting the conditions for binding of RNA to the RNeasy membrane. Both effects may reduce RNA yield.

4. Disrupt spheroplasts by adding the appropriate volume of Buffer RLT (see Table 9).

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

**Table 9. Buffer RLT volumes for RNeasy Midi/Maxi isolation of total RNA from yeast**

RNeasy column	Number of yeast cells	Buffer RLT (ml)
Midi $\blacktriangle$	$2 \times 10^7 - 2 \times 10^8$	2.0
Midi $\blacktriangle$	$2 \times 10^8 - 5 \times 10^8$	4.0
Maxi $\bullet$	$2.5 \times 10^8 - 1 \times 10^9$	7.5
Maxi $\bullet$	$1 \times 10^9 - 2.5 \times 10^9$	15.0

5. Homogenize yeast cells using a conventional rotor–stator homogenizer for at least 45 s at maximum speed until the sample is uniformly homogeneous. Alternatively, vortex the sample for 10 s, and pass the lysate at least 5–10 times through an 18- to 20-gauge needle fitted to an RNase-free syringe.

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

6. Centrifuge the yeast for ▲ 5 min or ● 10 min at 3000–5000 x *g*. Carefully transfer supernatant to a new ▲ 10–15 ml or ● 50 ml tube (not supplied) by pipetting. Use only this supernatant (lysate) in subsequent steps.

In most preparations, a small pellet will form. Avoid transferring the components of the pellet, because this may cause the column to clog later on in the procedure.

7. Add 1 volume (▲ 2.0 ml or 4.0 ml; ● 7.5 ml or 15.0 ml) of 70% ethanol to the homogenized lysate, and mix thoroughly by shaking vigorously. Do *not* centrifuge. Continue immediately with step 8.

If some lysate is 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.

8. Apply the sample to an ▲ RNeasy Midi column or ● RNeasy Maxi column placed in a ▲ 15 ml or ● 50 ml centrifuge tube (supplied). Maximum loading volume is ▲ 4.0 ml or ● 15 ml. Close the tube gently, and centrifuge for 5 min at 3000–5000 x *g*. Discard the flow-through.\*

Reuse the centrifuge tube in step 9.

If the volume exceeds ▲ 4.0 ml or ● 15 ml, load aliquots successively onto the RNeasy column, and centrifuge as above. Discard the flow-through after each centrifugation step.\*

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

**Optional:** QIAGEN offers the RNase-Free DNase Set for convenient on-column DNase digestion during RNA purification. Generally, DNase digestion is not required, because the RNeasy silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). If using the RNase-Free DNase Set, follow the steps shown on page 106 after performing this step.

9. Add ▲ 4.0 ml or ● 15 ml Buffer RW1 to the RNeasy column. Close the centrifuge tube gently, and centrifuge for 5 min at 3000–5000  $\times g$  to wash the column. Discard the flow-through.\*

Skip this step if performing the optional on-column DNase digestion (Appendix E). Reuse the centrifuge tube in step 10.

10. Add ▲ 2.5 ml or ● 10 ml Buffer RPE to the RNeasy column. Close the centrifuge tube gently, and centrifuge for 2 min at 3000–5000  $\times g$  to wash the column. Discard the flow-through.

Reuse the centrifuge tube in step 11. In the RNeasy Midi procedure, the flow-through does not need to be discarded.

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

11. Add another ▲ 2.5 ml or ● 10 ml Buffer RPE to the RNeasy column. Close the centrifuge tube gently, and centrifuge for ▲ 5 min or ● 10 min at 3000–5000  $\times g$  to dry the RNeasy silica-gel membrane.

It is important to dry the RNeasy membrane, because residual ethanol may interfere with downstream reactions. This centrifugation ensures that no ethanol is carried over during elution.

**Note:** After centrifugation, remove the RNeasy column from the centrifuge tube carefully so that the column does not come in contact with the flow-through, because this will result in carryover of ethanol.

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

12. To elute, transfer RNeasy column to a new ▲ 15 ml or ● 50 ml collection tube (supplied). Pipet the appropriate volume of RNase-free water (see Table 10) directly onto the RNeasy silica-gel membrane. Close the tube gently. Let it stand for 1 min, and then centrifuge for 3 min at 3000–5000  $\times g$ .

**Table 10. RNase-free water volumes for RNeasy Midi/Maxi elution**

RNeasy column	Expected total RNA yield	RNase-free water
Midi ▲	≤150 µg	150 µl
Midi ▲	150 µg – 1 mg	250 µl
Maxi ●	≤1 mg	0.8 ml
Maxi ●	1–6 mg	1.2 ml

13. Repeat the elution step (step 12) as described with a second volume of RNase-free water. To obtain a higher total RNA concentration, this second elution step may be performed using the first eluate (from step 12). The yield will be 15–30% less than the yield obtained using a second volume of RNase-free water, but the final concentration will be higher.

## Mechanical disruption protocol for isolation of total RNA from yeast

### Determining the correct amount of starting material

Use an appropriate number of cells (see page 42 for detailed information).

### Important points before starting

- If using RNeasy Midi or Maxi Kits for the first time, read "Important Points Before Using RNeasy Kits".
- If working with RNA for the first time, read Appendix A.
- For RNA isolation from yeast, cells should be harvested in log-phase growth. Cell pellets can be stored at –70°C for later use or used directly in the procedure. Cell lysates (in

Buffer RLT, step 5) can be stored at  $-70^{\circ}\text{C}$  for several months. To process frozen lysates, thaw samples for 15–20 min at  $37^{\circ}\text{C}$  in a water bath to dissolve salts. Continue with step 6.

- **Important:** Prepare the appropriate amount (see Table 11) of acid-washed glass beads (0.45–0.55 mm in diameter) by soaking in concentrated nitric acid for 1 h, washing extensively with deionized water, and drying in a baking oven.
- **Important:**  $\beta$ -ME must be added to Buffer RLT before use.  $\beta$ -ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 10  $\mu\text{l}$   $\beta$ -ME per 1 ml Buffer RLT. Buffer RLT is stable for 1 month after addition of  $\beta$ -ME.
- The yeast mechanical disruption protocol requires more Lysis Buffer RLT than do the other protocols in this handbook. Therefore, if  $>2 \times 10^8$  yeast cells are processed with the RNeasy Midi procedure or  $>1 \times 10^9$  yeast cells with the RNeasy Maxi procedure, additional Buffer RLT is required. If necessary, additional Buffer RLT is available separately. See “Ordering Information”.
- 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.
- Generally, DNase digestion is not required, because the RNeasy silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). In these cases, the small residual amounts of DNA remaining can be removed using the RNase-Free DNase Set for the optional on-column DNase digestion (see page 83) or by a DNase digestion after RNA isolation. For on-column DNase digestion with the RNase-Free DNase Set, prepare the DNase I stock solution as described in “Appendix E: Optional On-Column DNase Digestion with the RNase-Free DNase Set” before beginning the procedure.
- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature.
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. Guanidine is an irritant. Take appropriate safety measures and wear gloves when handling.

- After disruption, all steps of the RNeasy protocol should be performed at room temperature. During the procedure, work quickly.
- After harvesting the cells, all centrifugation steps should be performed at 20–25°C in a standard laboratory centrifuge with a swinging bucket rotor capable of  $\geq 3000 \times g$  (see page 23). Ensure that the centrifuge does not cool below 20°C.
- Blue (marked with a ▲) denotes ▲ RNeasy Midi prep volumes (for  $2 \times 10^7$  to  $5 \times 10^8$  yeast cells); red (marked with a ●) denotes ● RNeasy Maxi prep volumes (for  $2.5 \times 10^8$  to  $2.5 \times 10^9$  yeast cells).

## Procedure

1. Add the appropriate amount of acid-washed glass beads (see Table 1 1) to a tube that fits the bead mill (see page 24 for details).
2. Harvest yeast cells by centrifuging at  $500 \times g$  for 5 min at 4°C. (Do not use more than ▲  $5 \times 10^8$  or ●  $2.5 \times 10^9$  yeast cells.) Decant supernatant, and carefully remove remaining media by aspiration. After centrifuging, heat the centrifuge to 20–25°C if the same centrifuge is to be used in the following centrifugation steps of the protocol.

**Note:** Incomplete removal of the supernatant will inhibit lysis and dilute the lysate, affecting the conditions for binding of RNA to the RNeasy membrane. Both effects may reduce RNA yield.

3. Loosen the cell pellet thoroughly by flicking the tube. Add the appropriate volume of Buffer RLT (see Table 1 1). Vortex to resuspend the cell pellet. Add the sample to the glass beads prepared in step 1. Vortex and agitate at top speed in a bead-mill homogenizer with cooling until cells are completely disrupted.

Some 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 cell disruption may vary depending on the type of bead mill used. Please refer to the supplier's instructions.

**Note:** Do not replace bead-milling with vortexing, because this significantly reduces RNA yield. Ensure that  $\beta$ -ME is added to Buffer RLT before use (see “Important points before starting”).

**Table 11. Buffer and reagent volumes for RNeasy Midi/Maxi isolation of total RNA from yeast (Mechanical Disruption Protocol)**

RNeasy column	Number of yeast cells	Acid-washed glass beads (ml)	Buffer RLT (ml)
Midi ▲	$2 \times 10^7 - 2 \times 10^8$	Approx. 2.0	2.0
Midi ▲	$2 \times 10^8 - 5 \times 10^8$	Approx. 4.0	4.0
Maxi ●	$2.5 \times 10^8 - 1 \times 10^9$	Approx. 7.5	7.5
Maxi ●	$1 \times 10^9 - 2.5 \times 10^9$	Approx. 15.0	15.0

- Remove the sample from the bead mill and allow beads to settle. Remove 0.5 volume of the supernatant (▲ 1.0 ml or 2.0 ml; ● 3.75 ml or 7.5 ml) by aspirating, and transfer it to a new ▲ 10–15 ml or ● 50 ml centrifuge tube (not supplied).
- Replace the aspirated supernatant with an equivalent volume (▲ 1.0 ml or 2.0 ml; ● 3.75 ml or 7.5 ml) of Buffer RLT, added to the glass beads. Shake and allow the beads to settle again. Remove 0.5 volume of the supernatant (▲ 1.0 ml or 2.0 ml; ● 3.75 ml or 7.5 ml) from the glass beads by aspiration, and combine it with the supernatant from step 4. The total volume of the removed supernatant (lysate) is now ▲ 2.0 ml or 4.0 ml or ● 7.5 ml or 15 ml.

This step is necessary for maximum recovery, because some of the lysis buffer – and therefore part of the sample – remains trapped in the bed volume of the glass beads.

**Note:** The yeast mechanical disruption protocol requires more Lysis Buffer RLT than do the other protocols in this handbook. Therefore, if ▲  $>2 \times 10^8$  yeast cells or ●  $>1 \times 10^9$  yeast cells are processed, additional Buffer RLT is required. If necessary, additional Buffer RLT is available separately. See “Ordering Information”.

6. Centrifuge the yeast lysate for ▲ 5 min or ● 10 min at 3000–5000 x *g*. Carefully transfer the supernatant into a new ▲ 10–15 ml or ● 50 ml centrifuge tube (not supplied) by pipetting. Use only this supernatant (lysate) in subsequent steps.  
This centrifugation step pellets the cell debris. Avoid transferring any of the pellet.
7. Add 1 volume (▲ 2.0 ml or 4.0 ml; ● 7.5 ml or 15 ml) of 70% ethanol to the lysate, and mix thoroughly by shaking vigorously. Do not centrifuge. Continue immediately with step 8.
8. Apply the sample to an ▲ RNeasy Midi column or ● RNeasy Maxi column placed in a ▲ 15 ml or ● 50 ml centrifuge tube (supplied). Maximum loading volume is ▲ 4.0 ml or ● 15 ml. Close the tube gently, and centrifuge for 5 min at 3000–5000 x *g*. Discard the flow-through.\*

Reuse the centrifuge tube in step 9.

If the maximum amount of starting material is used, it may be necessary to increase centrifugation time to 10 min to allow the lysate to completely pass through the column.

If the volume exceeds ▲ 4.0 ml or ● 15 ml, load aliquots successively onto the RNeasy column and centrifuge as above. Discard the flow-through after each centrifugation step.\*

**Optional:** QIAGEN offers the RNase-Free DNase Set for convenient on-column DNase digestion during RNA purification. Generally, DNase digestion is not required, because the RNeasy silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). If using the RNase-Free DNase Set, follow the steps shown in “Appendix E: Optional On-Column DNase Digestion with the RNase-Free DNase Set” after performing this step.

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

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9. Add ▲ 4.0 ml or ● 15 ml Buffer RW1 to the RNeasy column. Close the centrifuge tube gently, and centrifuge for 5 min at 3000–5000  $\times g$  to wash the column. Discard the flow-through.

Skip this step if performing the optional on-column DNase digestion (Appendix E). Reuse the centrifuge tube in step 10.

10. Add ▲ 2.5 ml or ● 10 ml Buffer RPE to the RNeasy column. Close the centrifuge tube gently, and centrifuge for 2 min at 3000–5000  $\times g$  to wash the column. Discard the flow-through.

Reuse the centrifuge tube in step 11. In the RNeasy Midi procedure, the flow-through does not need to be discarded.

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

11. Add another ▲ 2.5 ml or ● 10 ml Buffer RPE to the RNeasy column. Close the centrifuge tube gently, and centrifuge for ▲ 5 min or ● 10 min at 3000–5000  $\times g$  to dry the RNeasy silica-gel membrane.

It is important to dry the RNeasy membrane, because residual ethanol may interfere with downstream reactions. This centrifugation ensures that no ethanol is carried over during elution.

**Note:** After centrifugation, remove the RNeasy column from the centrifuge tube carefully so that the column does not come in contact with the flow-through, because this will result in carryover of ethanol.

12. To elute, transfer the RNeasy column to a new ▲ 15 ml or ● 50 ml collection tube (supplied). Pipet the appropriate volume of RNase-free water (see Table 12) directly onto the RNeasy silica-gel membrane. Close the tube gently. Let it stand for 1 min, and then centrifuge for 3 min at 3000–5000  $\times g$ .

**Table 12. RNase-free water volumes for RNeasy Midi/Maxi elution**

RNeasy column	Expected total RNA yield	RNase-free water
Midi ▲	≤150 µg	150 µl
Midi ▲	150 µg – 1 mg	250 µl
Maxi ●	≤1 mg	0.8 ml
Maxi ●	1–6 mg	1.2 ml

13. Repeat the elution step (step 12) as described with a second volume of RNase-free water.

To obtain a higher total RNA concentration, this second elution step may be performed using the first eluate (from step 12). The yield will be 15–30% less than the yield obtained using a second volume of RNase-free water, but the final concentration will be higher.

# RNeasy Midi/Maxi Protocol for RNA Cleanup

## Determining the correct amount of starting material

A maximum of 1 mg or 6 mg of RNA can be used in the RNA cleanup protocol using RNeasy Midi or Maxi columns, respectively. These amounts correspond to the binding capacity of the RNeasy columns.

## Important points before starting

- If using RNeasy Midi or Maxi Kits for the first time, read “Important Points Before Using RNeasy Kits”.
- If working with RNA for the first time, read Appendix A.
- $\beta$ -ME must be added to Buffer RLT before use.  $\beta$ -ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 10  $\mu$ l  $\beta$ -ME per 1 ml Buffer RLT. Buffer RLT is stable for 1 month after addition of  $\beta$ -ME.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.
- Generally, DNase digestion is not required, because the RNeasy silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). We recommend a DNase digestion of the reaction mixture before starting the procedure. The DNase is then removed during the cleanup procedure. Alternatively, the RNase-Free DNase Set can be used for the optional on-column DNase digestion (see page 83). For on-column DNase digestion with the RNase-Free DNase Set, prepare the DNase I stock solution as described in “Appendix E: Optional On-Column DNase Digestion with the RNase-Free DNase Set” before beginning the procedure.
- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature.

- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. Guanidine is an irritant. Take appropriate safety measures and wear gloves when handling.
- All steps of the RNeasy protocol should be performed at room temperature. During the procedure, work quickly.
- All centrifugation steps are performed at 20–25°C in a standard laboratory centrifuge with a swinging bucket rotor capable of  $\geq 3000 \times g$  (see page 23). Ensure that the centrifuge does not cool below 20°C.
- **Blue** (marked with a ▲) denotes ▲ RNeasy Midi prep volumes (for  $\leq 1$  mg starting RNA); **red** (marked with a ●) denotes ● RNeasy Maxi prep volumes (for 1–6 mg starting RNA).

## Procedure

1. Adjust sample to the appropriate volume (see Table 13) with RNase-free water. Add the appropriate volume of Buffer RLT and mix thoroughly.
2. Add the appropriate volume of ethanol (96–100%) to the diluted RNA (see Table 13). Mix thoroughly by shaking vigorously. Continue immediately with step 3.

**Table 13. Buffer volumes for RNeasy Midi/Maxi RNA cleanup**

RNeasy column	Amount of RNA (mg)	Add RNase-free water to (ml)	Buffer RLT (ml)	Ethanol (96–100%) (ml)
Midi ▲	$\leq 0.5$	0.5	2.0	1.4
Midi ▲	0.5–1.0	1.0	4.0	2.8
Maxi ●	1.0–3.0	2.0	7.5	5.5
Maxi ●	3.0–6.0	4.0	15.0	11.0

3. Apply the sample to an ▲ RNeasy Midi column or ● RNeasy Maxi column placed in a ▲ 15 ml or ● 50 ml centrifuge tube (supplied). Maximum loading volume is ▲ 4.0 ml or

● **15 ml.** Close tube gently, and centrifuge for 5 min at 3000–5000 x *g*. Discard the flow-through.\*

Reuse centrifuge tube in step 4.

If the volume exceeds ▲ **4.0 ml** or ● **15 ml**, load aliquots successively onto the RNeasy column, and centrifuge as above. Discard the flow-through after each centrifugation step.\*

**Optional:** QIAGEN offers the RNase-Free DNase Set for convenient on-column DNase digestion during RNA purification. Generally, DNase digestion is not required, because the RNeasy silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). If using the RNase-Free DNase Set, follow the steps shown on page 83 after performing this step.

4. Add ▲ **2.5 ml** or ● **10 ml** Buffer RPE to the RNeasy column. Close the centrifuge tube gently, and centrifuge for 2 min at 3000–5000 x *g* to wash the column. Discard the flow-through.

Reuse centrifuge tube in step 5. In the RNeasy Midi procedure, the flow-through does not need to be discarded.

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

5. Add another ▲ **2.5 ml** or ● **10 ml** Buffer RPE to the RNeasy column. Close the centrifuge tube gently, and centrifuge for ▲ **5 min** or ● **10 min** at 3000–5000 x *g* to dry the RNeasy silica-gel membrane.

It is important to dry the RNeasy membrane, because residual ethanol may interfere with downstream reactions. This centrifugation ensures that no ethanol is carried over during elution.

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

**Note:** After centrifugation, remove the RNeasy column from the centrifuge tube carefully so that the column does not come in contact with the flow-through, because this will result in carryover of ethanol.

- To elute, transfer the RNeasy column to a new ▲ 15 ml or ● 50 ml collection tube (supplied). Pipet the appropriate volume of RNase-free water (see Table 14) directly onto the RNeasy silica-gel membrane. Close the tube gently. Let it stand for 1 min, and then centrifuge for 3 min at 3000–5000  $\times g$ .

**Table 14. RNase-free water volumes for RNeasy Midi/Maxi elution**

RNeasy column	Expected total RNA yield	RNase-free water
Midi ▲	$\leq 150 \mu\text{g}$	150 $\mu\text{l}$
Midi ▲	150 $\mu\text{g}$ – 1 mg	250 $\mu\text{l}$
Maxi ●	$\leq 1 \text{ mg}$	0.8 ml
Maxi ●	1–6 mg	1.2 ml

- Repeat the elution step (step 6) as described with a second volume of RNase-free water. To obtain a higher total RNA concentration, this second elution step may be performed using the first eluate (from step 6). The yield will be 15–30% less than the yield obtained using a second volume of RNase-free water, but the final concentration will be higher.

# 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. For more information, see also the Frequently Asked Questions page at our Technical Support Center: [www.qiagen.com/FAQ/FAQList.aspx](http://www.qiagen.com/FAQ/FAQList.aspx). The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and/or protocols in this handbook or sample and assay technologies (for contact information, visit [www.qiagen.com](http://www.qiagen.com)).

## Comments and suggestions

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### Clogged RNeasy column

- |   |   |
|---|---|
| a) Inefficient disruption and/or homogenization   | See "Disruption and homogenization of starting material" for a detailed description of homogenization methods.<br>Increase $g$ -force and centrifugation time if necessary.<br>In subsequent preparations, reduce the amount of starting material (see "Determining the amount of starting material" and protocols) and/or increase the volume of lysis buffer and the homogenization time.<br>If working with tissues rich in proteins, the "RNeasy Midi/Maxi Protocol for Isolation of Total RNA from Heart, Muscle, and Skin Tissue" (Appendix C) may provide better results than with the standard tissue protocol. |
| b) Too much starting material   | Reduce amounts of starting material. It is essential to use the correct amount of starting material (see "Determining the amount of starting material" and protocols).  |
| c) Tissues and yeast (mechanical disruption protocol): centrifugation before adding ethanol not performed | Centrifuge lysate before adding ethanol, and use only this supernatant in subsequent steps (see protocols).<br>Pellets contain cell debris that can clog the RNeasy column.   |
| d) Centrifugation temperature too low   | The centrifugation temperature should be 20–25°C.<br>Some centrifuges may cool to below 20°C even when set at 20°C. This can cause precipitates to form that can clog the RNeasy column. If this happens, set the centrifugation temperature to 25°C. Warm the ethanol-containing lysate to 37°C before transferring to the RNeasy column.  |

## Comments and suggestions

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### Low RNA yield

- a) Insufficient disruption and homogenization
- See "Disruption and homogenization of starting material" for a detailed description of homogenization methods.
- Increase  $g$ -force and centrifugation time if necessary.
- In subsequent preparations, reduce the amount of starting material (see "Determining the amount of starting material" and protocols) and/or increase the volume of lysis buffer and the homogenization time.
- If working with tissues rich in proteins, the "RNeasy Midi/Maxi Protocol for Isolation of Total RNA from Heart, Muscle, and Skin Tissue" (Appendix C) may provide better results than with the standard tissue protocol.
- b) Too much starting material
- Reduce amounts of starting material. It is essential to use the correct amount of starting material (see "Determining the amount of starting material" and protocols).
- c) RNA still bound to the membrane
- Repeat elution, but incubate the RNeasy column on the benchtop for 10 min with RNase-free water before centrifuging.
- d) Cells: incomplete removal of cell-culture medium
- When processing cultured cells, ensure complete removal of the cell-culture medium after harvesting cells (see protocols).
- e) RNA/*ater*-stabilized cells: not pelleted completely or efficiently
- Because RNA/*ater* RNA Stabilization Reagent has a higher density than most cell-culture media, higher centrifugal forces may be necessary to pellet the cells. Initially, try to pellet cells by centrifugation at 3000  $\times g$ . If necessary, increase the  $g$ -force in increments of 2000  $\times g$  until a cell pellet is obtained that is similar to the size of the cell pellet before stabilization. See Appendix F.

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

Use 10 mM Tris-Cl, pH 7.5 (not RNase-free water) to dilute the sample before measuring purity (see Appendix B).

### RNA degraded

- a) Starting material not immediately stabilized
- Submerge the sample in the appropriate volume of the RNA/*ater* RNA Stabilization Reagent immediately after harvesting the material.
- b) Too much starting material for proper stabilization
- Reduce the amount of starting material or increase the amount of RNA/*ater* RNA Stabilization Reagent used for stabilization.
- c) Sample material too thick for stabilization
- Cut large samples into slices less than 0.5 cm thick for stabilization in RNA/*ater* RNA Stabilization Reagent.
- d) Frozen samples used for stabilization
- Use only fresh, unfrozen material for stabilization.

### Comments and suggestions

- 
- |                                   |   |
|-----------------------------------|---|
| e) Storage duration exceeded      | Storage of RNA/ <i>later</i> -stabilized material is possible for up to 1 day at 37°C, up to 7 days at 18–25°C, and up to 4 weeks at 2–8°C. Store at –80°C or –20°C for archival storage.   |
| f) Sample inappropriately handled | RNA/ <i>later</i> -stabilized samples: Ensure that samples are properly stabilized and stored in RNA/ <i>later</i> RNA Stabilization Reagent.<br><br>Unstabilized samples: For frozen cell pellets or tissue samples, ensure that they were flash-frozen immediately in liquid nitrogen and properly stored at –70°C. Perform the protocol quickly, especially the first few steps. See Appendix A and “Handling and storage of starting material”. |
| g) RNase contamination            | Although all buffers have been tested and guaranteed RNase-free, RNases can be introduced during use. Be certain not to introduce any RNases during the procedure or later handling. See Appendix A.<br><br>Do not put RNA samples into a vacuum dryer that has been used in DNA preparation where RNases may have been used.   |

### DNA contamination in downstream experiments

- |                                      |  |
|--------------------------------------|--|
| a) Cells: optimal procedure not used | For animal cells, the cytoplasmic RNA protocol is recommended for applicants where the absence of DNA contamination is critical, because the intact nuclei are removed at the start of the procedure. Follow the <i>RNeasy Midi/Maxi Protocol for Isolation of Cytoplasmic RNA from Animal Cells</i> supplementary protocol.   |
| b) No incubation with Buffer RW1     | In subsequent preparations, incubate the RNeasy column for 5 min at room temperature with Buffer RW1 before centrifuging.  |
| c) No DNase treatment                | Follow the optional on-column DNA digestion using the RNase-Free DNase Set (Appendix E) at the point indicated in the individual protocols.<br><br>Alternatively after the RNeasy procedure, a DNA digestion of the eluate containing the RNA can be performed. After inactivating DNase by heat treatment, the RNA can be either used directly in the subsequent application without further treatment or repurified using the “RNeasy Midi/Maxi Protocol for RNA Cleanup”. |

### RNA does not perform well in downstream experiments

- |                                  |   |
|----------------------------------|---|
| d) Salt carryover during elution | Ensure that Buffer RPE is at room temperature.  |
| e) Ethanol carryover             | During the second Buffer RPE wash, be sure to dry the RNeasy membrane by centrifugation at 3000–5000 × <i>g</i> for 10 min at 20–25°C. After centrifugation, remove the RNeasy column from the centrifuge tube carefully so that the column does not come in contact with the flow-through, because this will result in carryover of ethanol. |

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# Appendix A: General Remarks on Handling RNA

## Handling RNA

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

## General handling

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

## Disposable plasticware

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

## 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

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RNase-free water (see “Solutions,” page 64). Alternatively, chloroform-resistant plasticware can be rinsed with chloroform to inactivate RNases.

## Glassware

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

## Electrophoresis tanks

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

## Solutions

Solutions (water and other solutions) should be treated with 0.1% DEPC.\* DEPC is a strong, but not absolute, inhibitor of RNases. It is commonly used at a concentration of 0.1% to inactivate RNases on glass or plasticware or to create RNase-free solutions and water. DEPC inactivates RNases by covalent modification.

Add 0.1 ml DEPC to 100 ml of the solution to be treated, and then shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 hours at 37°C. Autoclave for 15 min to remove any trace of DEPC.

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

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

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DEPC will react with primary amines and cannot be used directly to treat Tris buffers. DEPC is highly unstable in the presence of Tris buffers and decomposes rapidly into ethanol and CO<sub>2</sub>. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris to make the appropriate buffer.

Trace amounts of DEPC will modify purine residues in RNA by carboxymethylation. Carboxymethylated RNA is translated with very low efficiency in cell-free systems. However, its ability to form DNA:RNA or RNA:RNA hybrids is not seriously affected unless a large fraction of the purine residues have been modified. Residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 min.

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

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## Appendix B: Storage, Quantitation, and Determination of Quality of Total RNA

### Storage of RNA

Purified RNA may be stored at  $-70^{\circ}\text{C}$  or  $-20^{\circ}\text{C}$  in water. Under these conditions, no degradation of RNA is detectable after 1 year.

### Quantitation of RNA

The concentration of RNA should be determined by measuring the absorbance at 260 nm ( $A_{260}$ ) in a spectrophotometer. To ensure significance, readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 40  $\mu\text{g}$  of RNA per ml ( $A_{260} = 1 \rightarrow 40 \mu\text{g}/\text{ml}$ ). This relation is valid only for measurements in water. Therefore, if it is necessary to dilute the RNA sample, this should be done in water. As discussed below (see "Purity of RNA", page 67), the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity.

When measuring RNA samples, be certain that cuvettes are RNase-free, especially if the RNA is to be recovered after spectrophotometry. This can be accomplished by washing cuvettes with 0.1 M NaOH, 1 mM EDTA followed by washing with RNase-free water (see "Solutions," page 64). Use the buffer in which the RNA is diluted to zero the spectrophotometer.

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

Volume of RNA sample = 2.2 ml

Dilution = 10  $\mu$ l of RNA sample + 490  $\mu$ l distilled water (1/50 dilution)

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

$$A_{260} = 0.75$$

Concentration of RNA sample =  $40 \times A_{260} \times \text{dilution factor}$

$$= 40 \times 0.75 \times 50$$

$$= 1500 \mu\text{g/ml}$$

Total yield = concentration  $\times$  volume of sample in milliliters

$$= 1500 \mu\text{g/ml} \times 2.2 \text{ ml}$$

$$= 3300 \mu\text{g} = 3.3 \text{ mg RNA}$$

## Purity of RNA

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

For determination of RNA concentration, however, we still recommend dilution of the sample in water, because the relationship between absorbance and concentration ( $A_{260}$  reading of 1 = 40  $\mu\text{g/ml}$  RNA) is based on an extinction coefficient calculated for RNA in water (see "Quantitation of RNA", page 66).

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

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

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## DNA contamination

No currently available purification method can guarantee that RNA is completely free of DNA, even when DNA is not visible on an agarose gel. To prevent any interference by DNA in RT-PCR applications, we recommend designing primers that anneal at intron splice junctions so that genomic DNA will not be amplified. Alternatively, DNA contamination can be detected on agarose gels following RT-PCR by performing control experiments in which no reverse transcriptase is added prior to the PCR step or by using intron-spanning primers. For sensitive applications, such as differential display, or if it is not practical to use splice-junction primers, DNase digestion of the purified RNA with RNase-free DNase is recommended.

A protocol for optional on-column DNase digestion using the RNase-Free DNase Set is provided in Appendix E. The DNase is efficiently washed away in the subsequent wash steps. Alternatively, after the RNeasy procedure, the eluate containing the RNA can be treated with DNase. The RNA can then be repurified with the “RNeasy Midi/Maxi Protocol for RNA Cleanup”, or after heat inactivation of the DNase, the RNA can be used directly in downstream applications.

## Integrity of RNA

The integrity and size distribution of total RNA purified with RNeasy Kits can be checked by denaturing agarose gel electrophoresis and ethidium bromide staining (see Appendix H). The respective ribosomal bands (Table 15) should appear as sharp bands on the stained gel. 28S ribosomal RNA bands should be present with an intensity approximately twice that of the 18S RNA band. If the ribosomal bands in a given lane are not sharp but appear as a smear of smaller-sized RNAs, it is likely that the RNA sample suffered major degradation during preparation.

**Table 15. Size of ribosomal RNAs from various sources**

<b>Source</b>	<b>rRNA</b>	<b>Size (kb)</b>
<i>E. coli</i>	16S	1.5
	23S	2.9
<i>S. cerevisiae</i>	18S	2.0
	26S	3.8
Mouse	18S	1.9
	28S	4.7
Human	18S	1.9
	28S	5.0

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## Appendix C: RNeasy Midi/Maxi Protocol for Isolation of Total RNA from Heart, Muscle, and Skin Tissue

Total RNA isolation from skeletal muscle, heart, and skin tissue can be difficult due to the abundance of contractile proteins, connective tissue, and collagen. To remove these proteins, which can interfere with the procedure, the standard “RNeasy Midi/Maxi Protocols for Isolation of Total RNA from Animal Tissues” has been adapted to include a proteinase K digest. Samples are lysed in a GITC-containing lysis buffer (Buffer RLT). After dilution of the lysate, the sample is treated with proteinase K. Debris is pelleted by centrifugation. Ethanol is then added to the cleared lysate, and RNA is bound to the RNeasy membrane. Contaminants are washed away, and total RNA is eluted in RNase-free water.

The procedure has been used successfully for isolation of total RNA from heart, muscle, and skin tissue. For other tissues, the standard “RNeasy Midi/Maxi Protocol for Isolation of Total RNA from Animal Tissues” is generally the method of choice. If working with other protein-rich tissues where a proteinase K digestion might be desired, we recommend performing a comparison of the 2 protocols. Because the RNase-inactivating Buffer RLT must be diluted to permit proteinase K digestion, this protocol should not be used for RNase-rich tissues such as spleen or intestine.

### Additional reagents to be supplied by user

- QIAGEN Proteinase K, >600 mAU/ml (cat. no. 19131 or 19133). Proteinase K must be used in the procedure. If using proteinase K from another supplier, use a 20 mg/ml solution in water.
- Double-distilled water

## Determining the correct amount of starting material

It is essential to begin with the correct amount of tissue in order to obtain optimal RNA yield and purity with RNeasy columns. A minimum amount of 20 mg and a maximum of 250 mg tissue can generally be processed with RNeasy Midi columns. A minimum amount of 150 mg and a maximum of 1 g tissue can generally be processed with RNeasy Maxi columns. For most tissues, the binding capacity of the column (1 mg or 6 mg of RNA) and the lysing capacity of Buffer RLT will not be exceeded by these amounts. Depending on the type of tissue, 0.1–1.0 mg of total RNA can be expected from 250 mg tissue, and 0.4–4 mg of total RNA from 1 g of tissue. Average RNA yields from various sources are given in Table 2.

Some tissues such as spleen, parts of brain, lung, and thymus are more difficult to lyse or tend to form precipitates during the procedure. The maximum amount of these tissues used in the RNeasy Midi or Maxi protocol may need to be decreased to 100–150 mg or 0.4–0.6 g, respectively, to facilitate complete homogenization and to avoid significantly reduced yields, DNA contamination, or clogging of the RNeasy column.

**Note:** If you have no information about the nature of your starting material, we recommend starting with no more than 100 mg of tissue with the RNeasy Midi procedure or 0.6 g with the RNeasy Maxi procedure. Depending on the yield and purity obtained, it may be possible to increase the amount of tissue to 250 mg in the RNeasy Midi procedure or 1 g in the RNeasy Maxi procedure.

**Important:** Do not overload the column. Overloading will significantly reduce yield and quality.

### Important points before starting

- If using RNeasy Midi or Maxi Kits for the first time, read "Important Points Before Using RNeasy Kits".
- If working with RNA for the first time, read Appendix A .

- **Important:** For best results, stabilize animal tissues immediately in RNA/*later* RNA Stabilization Reagent. Tissues can be stored in RNA/*later* RNA Stabilization Reagent for up to 1 day at 37°C, 7 days at 18–25°C, 4 weeks at 2–8°C, or for archival storage at –20°C or –80°C.
- Fresh, frozen, and RNA/*later*-stabilized tissue can be used. To freeze unstabilized tissue for long-term storage, flash-freeze in liquid nitrogen, and immediately transfer to –70°C. Tissue can be stored for several months at –70°C. To process, do not allow tissue to thaw during weighing or handling prior to disruption in Buffer RLT. Homogenized tissue lysates (in Buffer RLT, step 4) can also be stored at –70°C for several months. To process frozen lysates, thaw samples and incubate for 15–20 min at 37°C in a water bath to dissolve salts. Continue with step 5.
- RNA/*later* RNA Stabilization Reagent should be stored dry at room temperature and is stable for at least 9 months under these conditions, if not otherwise stated on the label. Storage of RNA/*later* RNA Stabilization Reagent at lower temperatures may cause precipitation. The precipitate can be redissolved by heating to 37°C with agitation. Redissolve any precipitate before using.
- Heat a water bath or heating block to 55°C for proteinase K digestion in step 3.
- **Important:** β-ME must be added to Buffer RLT before use. β-ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 10 μl β-ME per 1 ml Buffer RLT. Buffer RLT is stable for 1 month after addition of β-ME.
- **Important:** 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.
- Generally, DNase digestion is not required, because the RNeasy silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). In these cases, the small residual amounts of DNA remaining can be removed using the RNase-Free DNase Set for the optional on-column DNase digestion (see page 83) or by a DNase digestion after RNA isolation. For on-column DNase digestion with the RNase-Free DNase Set, prepare the DNase I stock solution as described in “Appendix E:

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Optional On-Column DNase Digestion with the RNase-Free DNase Set” before beginning the procedure.

- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature.
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. Guanidine is an irritant. Take appropriate safety measures and wear gloves when handling.
- Unless otherwise indicated, all steps of the RNeasy protocol should be performed at room temperature. During the procedure, work quickly.
- All centrifugation steps are performed at 20–25°C in a standard laboratory centrifuge with a swinging bucket rotor capable of  $\geq 3000 \times g$  (see page 23). Ensure that the centrifuge does not cool below 20°C.
- **Blue** (marked with a ▲) denotes ▲ RNeasy Midi prep volumes (for 100–150 mg tissue); **red** (marked with a ●) denotes ● RNeasy Maxi prep volumes (for 200–800 mg tissue).

## Procedure

1. Excise the tissue sample from the animal or remove it from storage. Remove RNA/*later*-stabilized tissues from the reagent using forceps.
2. Determine the amount of tissue. Do not use more than ▲ 150 mg or ● 800 mg tissue.

Weighing tissue is the most accurate way to determine the amount. See page 71 for guidelines to determine the amount of starting material.

### 3. For RNA/*later*-stabilized tissues:

If the entire piece of RNA/*later*-stabilized tissue can be used for RNA isolation, place it directly into a suitably sized vessel for disruption and homogenization, and proceed with step 4.

If only a portion of the RNA/*later*-stabilized tissue is to be used, place the tissue on a clean surface for cutting, and cut it. Determine the weight of the piece to be used, and place it into a suitably sized vessel for homogenization. Proceed with step 4.

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RNA in the RNA/*ater*-treated tissue is still protected while the tissue is processed at 18–25°C. This allows cutting and weighing of tissues at ambient temperatures. It is not necessary to cut the tissue on ice or dry ice or in a refrigerated room. The remaining tissue can be placed into RNA/*ater* RNA Stabilization Reagent for further storage. Previously stabilized tissues can be stored at –80°C without the reagent.

**For unstabilized fresh or frozen tissues:**

If the entire piece of tissue can be used for RNA isolation, place it directly into a suitably sized vessel for disruption and homogenization, and proceed immediately with step 4.

If only a portion of the tissue is to be used, determine the weight of the piece to be used, and place it into a suitably sized vessel for homogenization. Proceed immediately with step 4.

RNA in tissues is not protected after harvesting until the sample is treated with RNA/*ater* RNA Stabilization Reagent, flash frozen, or disrupted and homogenized in protocol step 4. Frozen animal tissue should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible.

**Note:** The remaining fresh tissue can be placed into RNA/*ater* RNA Stabilization Reagent for stabilization. However, previously frozen tissue samples thaw too slowly in the reagent, thus preventing it from diffusing into the tissue quickly enough before the RNA begins to degrade.

4. Disrupt tissue and homogenize lysate.

Place the weighed (fresh, frozen, or RNA/*ater*-stabilized) tissue in a suitably sized vessel for the homogenizer. Add ▲ 2.0 ml or ● 7.5 ml Buffer RLT, and homogenize immediately using a conventional rotor–stator homogenizer until the sample is uniformly homogeneous (usually 45–60 s at maximum speed). Continue the protocol with step 5.

Rotor–stator homogenization simultaneously disrupts and homogenizes the sample.

After storage in RNA/*ater* RNA Stabilization Reagent, tissues may become slightly harder than fresh or thawed tissues. Disruption and homogenization of tissue samples using standard methods is usually not a problem.

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

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

**Alternative method for disruption and homogenization:** The sample can alternatively be disrupted using a mortar and pestle and homogenized using a needle and syringe. This method, however, generally results in lower RNA yields. Homogenization with rotor–stator homogenizers is the method of choice for heart, muscle, or skin tissue. Immediately place the weighed fresh or frozen tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into a liquid-nitrogen-cooled tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw. Add ▲ 2.0 ml or ● 7.5 ml Buffer RLT. Homogenize by passing the lysate at least 5–10 times through a 20-gauge needle (0.9 mm diameter) fitted to a syringe. Continue the protocol with step 5.

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

5. Add ▲ 4.0 ml or ● 15.0 ml double-distilled water to the homogenate. Then add ▲ 65  $\mu$ l or ● 250  $\mu$ l QIAGEN Proteinase K solution, and mix thoroughly by pipetting. Due to the rigorous denaturing conditions of the homogenate, the water added in this step does not need to be RNase-free. Do not use the RNase-free water provided in the RNeasy Midi or Maxi Kit for this step, because it will be needed for elution.
6. Incubate at 55°C for 20 min.
7. Centrifuge for 5 min at 3000–5000  $\times g$ .  
A small pellet of tissue debris will form, sometimes accompanied by a thin layer or film on top of the supernatant.

- Pipet the supernatant (approximately ▲ 6.0 ml or ● 22.5 ml) into a new ▲ 10–15 ml or ● 50 ml tube (not supplied).

Avoid transferring any of the pellet. If unavoidable, however, a small amount of pelleted debris may be carried over without affecting the RNeasy procedure. Hold the pipette tip under the thin layer or film on top of the supernatant, if present. This layer will usually adhere to the outside of the pipette tip and should not be transferred.

- Add 0.5 volumes (usually ▲ 3.0 ml or ● 11.0 ml) of ethanol (96–100%) to the cleared lysate. Mix well by pipetting. Do not centrifuge.

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

- Pipet ▲ 3.0 ml or ● 12 ml of the sample, including any precipitate that may have formed, into an ▲ RNeasy Midi column or ● RNeasy Maxi column placed in a ▲ 15 ml or ● 50 ml centrifuge tube (supplied). Close the tube gently, and centrifuge for 5 min at 3000–5000 x g. Discard the flow-through.\*

Reuse the centrifuge tube in step 11.

- Repeat step 10, using another ▲ 3.0 ml or ● 12 ml of the sample. Discard the flow-through.\*

Reuse the centrifuge tube in step 12.

- Repeat step 10 again, using the remainder of the sample (approximately ▲ 3.0 ml or ● 9.5 ml). Discard the flow-through.\*

Reuse the centrifuge in step 13.

**Optional:** QIAGEN offers the RNase-Free DNase Set for convenient on-column DNase digestion during RNA purification. Generally, DNase digestion is not required, because the RNeasy silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). If using the RNase-Free DNase Set, follow the steps shown in Appendix E after performing this step.

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

13. Add ▲ 4.0 ml or ● 15 ml Buffer RW1 to the RNeasy column. Close the centrifuge tube gently, and centrifuge for 5 min at 3000–5000 x *g* to wash. Discard the flow-through.\*

Skip this step if performing the optional on-column DNase digestion (Appendix E). Reuse the centrifuge tube in step 14.

14. Add ▲ 2.5 ml or ● 10 ml of Buffer RPE to the RNeasy column. Close the centrifuge tube gently, and centrifuge for 2 min at 3000–5000 x *g* to wash the column. Discard the flow-through.

Reuse the centrifuge tube in step 15. In the RNeasy Midi procedure, the flow-through does not need to be discarded.

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

15. Add another ▲ 2.5 ml or ● 10 ml Buffer RPE to the spin column. Close the centrifuge tube gently, and centrifuge for ▲ 5 min or ● 10 min at 3000–5000 x *g* to dry the RNeasy membrane.

It is important to dry the RNeasy silica-gel membrane, because residual ethanol may interfere with downstream reactions. This centrifugation ensures that no ethanol is carried over during elution.

**Note:** After centrifugation, remove the RNeasy column from the centrifuge tube carefully so that the column does not come in contact with the flow-through, because this will result in carryover of ethanol.

16. To elute, transfer the RNeasy column to a new ▲ 15 ml or ● 50 ml collection tube (supplied). Pipet the appropriate volume of RNase-free water (see Table 16) directly onto the RNeasy silica-gel membrane. Close the tube gently. Let it stand for 1 min, and then centrifuge for 3 min at 3000–5000 x *g*.

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

**Table 16. RNase-free water volumes for RNeasy Midi/Maxi elution**

RNeasy column	Expected total RNA yield	RNase-free water
Midi ▲	≤150 µg	150 µl
Midi ▲	150 µg – 1 mg	250 µl
Maxi ●	≤1 mg	0.8 ml
Maxi ●	1–6 mg	1.2 ml

17. Repeat the elution step (step 16) as described with a second volume of RNase-free water.

To obtain a higher total RNA concentration, this second elution step may be performed using the first eluate (from step 16). The yield will be 15–30% less than the yield obtained using a second volume of RNase-free water, but the final concentration will be higher.

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## Appendix D: RNeasy Midi/Maxi Guidelines for Isolation of Total RNA from Plants

These guidelines have been adapted by customers from the “RNeasy Midi/Maxi Protocol for Isolation of Total RNA from Animal Tissues”. These guidelines should be considered as recommendations to help adapt the RNeasy Midi/Maxi protocol for total RNA isolation from plant cells and tissues.

For smaller amounts of plant material, up to 100 mg, the RNeasy Plant Mini Kit already comes complete with spin columns, RNase-free reagents, and detailed protocols. See “Ordering Information”.

### Important points before starting

- If using RNeasy Midi or Maxi Kits for the first time, read “Important Points Before Using RNeasy Kits”.
- If working with RNA for the first time, read Appendix A.
- Initially, do not use more than 500 mg (Midi) or 1000 mg (Maxi) plant material. With optimization, it may be possible to use larger amounts of starting material. RNA content of plant material can vary due to tissue type, developmental stage, growth conditions used, and other factors.
- Fresh or frozen tissue can be used. To freeze tissue for long-term storage, flash-freeze in liquid nitrogen and immediately transfer to  $-70^{\circ}\text{C}$ . Tissue can be stored for several months at  $-70^{\circ}\text{C}$ . To process, do not allow tissue to thaw during weighing or handling prior to disruption in Buffer RLT. Homogenized lysates (in Buffer RLT, step 3) can also be stored at  $-70^{\circ}\text{C}$  for several months. To process frozen lysates, thaw samples and incubate for 15–20 min at  $37^{\circ}\text{C}$  in a water bath to dissolve salts. Continue with step 4.

- $\beta$ -ME must be added to Buffer RLT before use.  $\beta$ -ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 10  $\mu$ l  $\beta$ -ME per 1 ml Buffer RLT. Buffer RLT is stable for 1 month after addition of  $\beta$ -ME.
- These guidelines for total RNA isolation from plants require more Lysis Buffer RLT than do other protocols in this handbook. Additional Buffer RLT is required for the maximum number of plant preparations. If necessary, additional Buffer RLT is available separately. See "Ordering Information".
- 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.
- Generally, DNase digestion is not required, because the RNeasy silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). In these cases, the small residual amounts of DNA remaining can be removed using the RNase-Free DNase Set for the optional on-column DNase digestion (see page 83) or by a DNase digestion after RNA isolation. For on-column DNase digestion with the RNase-Free DNase Set, prepare the DNase I stock solution as described in "Appendix E: Optional On-Column DNase Digestion with the RNase-Free DNase Set" before beginning the procedure.
- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature.
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. Guanidine is an irritant. Take appropriate safety measures and wear gloves when handling.
- All steps of these guidelines and of the RNeasy protocol should be performed at room temperature. During the procedure, work quickly.
- All centrifugation steps are performed at 20–25°C in a standard laboratory centrifuge with a swinging bucket rotor capable of  $\geq 3000 \times g$  (see page 23). Ensure that the centrifuge does not cool below 20°C.

- Blue (marked with a ▲) denotes ▲ RNeasy Midi prep volumes (for ≤500 mg plant material); red (marked with a ●) denotes ● RNeasy Maxi prep volumes (for ≤1 g plant material).

## Procedure

1. Grind sample under liquid nitrogen to a fine powder using mortar and pestle. (Initially, do not use more than ▲ 500 mg or ● 1 g plant material.) Transfer the powder and liquid nitrogen to an appropriate tube, and allow the liquid nitrogen to evaporate. Do not allow the sample to thaw. Continue immediately with lysis.

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

2. Lyse the sample by adding ▲ 5 ml or ● 20 ml Buffer RLT. Vortex vigorously.

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

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

3. Homogenize using a rotor–stator homogenizer until the sample is uniformly homogeneous (typically about 1 min).

See “Disruption and homogenization of starting material” for a more detailed description of homogenization methods.

4. Centrifuge the lysate for 10 min at room temperature at  $\geq 3000 \times g$  (do not exceed the recommended  $g$ -force limit of the centrifuge tube).

Cell debris should form a compact pellet. If some of the cell debris remains floating, remove the remaining debris by filtration through 2 or 3 layers of Miracloth (Calbiochem, cat. no. 475855).

5. Add 0.5 volumes of 96–100% ethanol to the cleared lysate and immediately mix well.

A precipitate may form after addition of the ethanol. If so, resuspend the precipitate by vigorous shaking.

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6. Apply the sample to an ▲ RNeasy Midi column or ● RNeasy Maxi column placed in a ▲ 15 ml or ● 50 ml centrifuge tube (supplied). Continue with step 7 of the “RNeasy Midi/Maxi Protocol for Isolation of Total RNA from Animal Tissues”.

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## Appendix E: Optional On-Column DNase Digestion with the RNase-Free DNase Set

The RNase-Free DNase Set provides efficient on-column digestion of DNA during RNA purification. The DNase is efficiently removed in subsequent wash steps.

**Note:** Standard DNase buffers are not compatible with on-membrane DNase digestion. Use of other buffers may affect the binding of the RNA to the RNeasy membrane, reducing the yield and integrity of the RNA.

Lysis and homogenization of the sample and binding of RNA to the silica-gel membrane are performed according to the standard protocols. After washing with a reduced volume of Buffer RW1, the RNA is treated with DNase I while bound to the silica-gel membrane. The DNase is removed by a second wash with Buffer RW1. Washing with Buffer RPE and elution are then performed according to the standard protocols.

- Generally, DNase digestion is not required, because the RNeasy silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). DNA can also be removed by a DNase digestion following RNA isolation.
- Prepare DNase I stock solution before using the RNase-Free DNase Set for the first time. Dissolve the solid DNase I (1500 Kunitz units) in 550  $\mu$ l of the RNase-free water provided. Take care that no DNase I is lost when opening the vial. Mix gently by inverting the tube. Do not vortex.
- **Do not vortex the reconstituted DNase I.** DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube.

- For long-term storage of DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at  $-20^{\circ}\text{C}$  for up to 9 months. Thawed aliquots can be stored at  $2-8^{\circ}\text{C}$  for up to 6 weeks. Do not refreeze the aliquots after thawing.
- Blue (marked with a ▲) denotes ▲ RNeasy Midi prep volumes; red (marked with a ●) denotes ● RNeasy Maxi prep volumes. The RNase-Free DNase Set contains RNase-free reagents and buffers for ▲ 25 RNA midipreps or ● 17 RNA maxipreps.

## Procedure

Carry out lysis, homogenization, and loading onto the RNeasy column as indicated in the individual protocols. Instead of continuing with the Buffer RW1 step, follow steps 1–4 below.

1. Pipet ▲ 2.0 ml or ● 7.5 ml Buffer RW1 into the RNeasy column. Centrifuge for 5 min at  $3000-5000 \times g$  to wash. Discard the flow-through.\*

Reuse the centrifuge tube in step 3.

2. Add ▲ 20  $\mu\text{l}$  or ● 30  $\mu\text{l}$  DNase I stock solution (see above) to ▲ 140  $\mu\text{l}$  or ● 210  $\mu\text{l}$  Buffer RDD. Mix by gently flicking the tube, and centrifuge briefly to collect residual liquid from the sides of the tube.

Buffer RDD is supplied with the RNase-Free DNase Set.

**Note:** DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently flicking the tube. Do not vortex.

3. Pipet the DNase I incubation mix (▲ 160  $\mu\text{l}$  or ● 240  $\mu\text{l}$ ) directly onto the RNeasy silica-gel membrane, and place on the benchtop ( $20-30^{\circ}\text{C}$ ) for 15 min.

**Note:** Make sure to pipet the DNase I incubation mix directly onto the RNeasy silica-gel membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the RNeasy column.

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

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4. Pipet ▲ 2.0 ml or ● 7.5 ml Buffer RW1 into the RNeasy column, place on the benchtop for 5 min, and then centrifuge for 5 min at 3000–5000 x *g*. Discard the flow-through.\* Continue with the first Buffer RPE wash step in the relevant protocol.

Reuse the centrifuge tube in the following Buffer RPE wash step in the relevant protocol.

**Note:** In most of the protocols, the Buffer RW1 wash step that immediately follows is skipped (as indicated in the protocol). Continue with the first Buffer RPE wash step.

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

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# Appendix F: Guidelines for Other RNA/*later* Applications

## RNA stabilization in cell-culture cells

### For RNA stabilization

Pellet cells by centrifugation at 300 x *g* for 5 min. Discard supernatant and wash cells (e.g., with PBS) to remove all medium. Resuspend cells in a small volume of PBS (e.g., 50–100  $\mu$ l PBS for 1 x 10<sup>6</sup> cells). Add 5–10 volumes of RNA/*later* RNA Stabilization Reagent.

**Note:** Do not add the RNA/*later* RNA Stabilization Reagent directly to the cell pellet. Always resuspend the cells first in a small volume of PBS.

### For RNA isolation

Pellet the cells in the RNA/*later* RNA Stabilization Reagent by centrifugation. Remove the supernatant completely and continue with step 2 of the “RNeasy Midi/Maxi Protocol for Isolation of Total RNA from Animal Cells”.

Because RNA/*later* RNA Stabilization Reagent has a higher density than most cell-culture media, higher centrifugal forces may be necessary for pelleting the cells. It is recommended to use small volumes of cells in the reagent (e. g., up to 500  $\mu$ l), because smaller volumes of cells pellet efficiently with lower centrifugal force. For example, 500  $\mu$ l suspensions of HeLa cells or macrophages in RNA/*later* RNA Stabilization Reagent will pellet efficiently at 3000 x *g*.

## RNA stabilization in white blood cells

RNA/*later* RNA Stabilization Reagent can be used to stabilize total RNA in white blood cells. However, it cannot be used for stabilization of RNA in whole blood, plasma, or sera. These

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materials will form an insoluble precipitate upon contact with the RNA/*later* RNA Stabilization Reagent. White blood cells must be separated from the red blood cells and sera prior to adding the reagent. RNA can be stabilized in the separated white blood cells following the guidelines for “RNA stabilization in cell-culture cells”, page 86.

# Appendix G: Guidelines for RT-PCR

To perform PCR using RNA as a starting template, the RNA must first be reverse transcribed into cDNA in a reverse transcription (RT) reaction. RT and PCR can be carried out either sequentially in the same tube (1-step RT-PCR) or separately (2-step RT-PCR).

One-step RT-PCR requires gene-specific primers. For this application, QIAGEN offers the QIAGEN OneStep Ahead RT-PCR Kit (see "Ordering Information"). Two-step RT-PCR is generally carried out using oligo-dT primers in the RT step and gene-specific primers in the PCR step (Table 17).

**Table 17. General guidelines for performing 2-step RT-PCR**

Reverse transcription	<p>QIAGEN offers Omniscript® and Sensiscript® RT Kits for reverse transcription (see "Ordering Information"). Omniscript RT is specially designed for all reverse transcription with any amount of RNA from 50 ng to 2 µg per reaction. Sensiscript RT is optimized for use with very small amounts of RNA (1 pg – 50 ng).</p> <p>Follow the detailed protocol in the accompanying handbook, or follow the supplier's instructions when using an enzyme from another supplier. The following guidelines may be helpful.</p> <ul style="list-style-type: none"><li>• Mix the following reagents in a microcentrifuge tube:<ul style="list-style-type: none"><li>2.0 µl 10x Buffer RT</li><li>2.0 µl dNTP Mix (5 mM each dNTP)</li><li>2.0 µl oligo-dT primer (10 µM)</li><li>1.0 µl RNase inhibitor (10 units/µl)</li><li>1.0 µl Omniscript or Sensiscript RT</li><li>x µl template RNA (up to 2 µg with Omniscript RT or up to 50 ng with Sensiscript RT)</li></ul></li><li>• Add RNase-free water to a final volume of 20 µl.</li></ul> <p>Incubate at 37°C for 60 min.*</p>
PCR	<p>Add an aliquot of the finished reverse-transcription reaction to the PCR mix. (No more than one-fifth of the final PCR volume should derive from the finished reverse-transcription reaction.)</p> <p>Carry out PCR with <i>Taq</i> DNA polymerase as recommended by the supplier. (We have consistently obtained excellent results using <i>Taq</i> DNA Polymerase or HotStarTaq® DNA Polymerase from QIAGEN. See "Ordering Information".)</p>

\* For other enzymes, refer to supplier's instructions.

# Appendix H: Protocol for Formaldehyde Agarose Gel Electrophoresis

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

## 1.2% FA gel preparation

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

- 1.2 g agarose
- 10 ml 10x FA gel buffer (see composition below)

Add RNase-free water to 100 ml

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

Heat the mixture to melt agarose. Cool to 65°C in a water bath. Add 1.8 ml of 37% (12.3 M) formaldehyde\* and 1 µl of a 10 mg/ml ethidium bromide\* stock solution. Mix thoroughly and pour onto gel support. Prior to running the gel, equilibrate in 1x FA gel running buffer (see “1x FA gel running buffer”, page 90) for at least 30 min.

## RNA sample preparation for FA gel electrophoresis

Add 1 volume of 5x loading buffer (see “5x RNA loading buffer”, page 90) per 4 volumes of RNA sample (for example 10 µl of loading buffer and 40 µl of RNA) and mix.

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

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

## Gel running conditions

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

## Composition of FA gel buffers

### 10x FA gel buffer

200 mM 3-[N-morpholino]propanesulfonic acid (MOPS) (free acid)

50 mM sodium acetate

10 mM EDTA

pH to 7.0 with NaOH

### 1x FA gel running buffer

100 ml 10x FA gel buffer

20 ml 37% (12.3 M) formaldehyde\*

880 ml RNase-free water

### 5x RNA loading buffer

16  $\mu$ l saturated aqueous 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 10 x FA gel buffer

RNase-free water to 10 ml

Stability: Approximately 3 months at 4°C

\* 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.

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# Appendix I: Equipment and Reagent Suppliers\*

Rotor–stator homogenizers can be purchased from:

- Kinematica AG, [www.kinematica.ch](http://www.kinematica.ch)
- IKA Works, IKA Analysentechnik GmbH (Ultra-Turrax®), [www.ika.com](http://www.ika.com)
- Silverson Machines, [www.silverson.com](http://www.silverson.com)
- VirTis Company, [www.spscientific.com/ProductCategory/104/VirTis/](http://www.spscientific.com/ProductCategory/104/VirTis/)

Bead-mill homogenizers can be purchased from:

- QIAGEN (Mixer Mill MM 300, see “Ordering Information”)

Glass and stainless steel beads can be purchased from:

- Retsch, [www.retsch.de](http://www.retsch.de)

Lyticase/zymolase can be purchased from:

- Sigma-Aldrich, [www.sigmaaldrich.com](http://www.sigmaaldrich.com)
- AMS Biotechnology, [www.amsbio.com](http://www.amsbio.com)

Substitutes for Nonidet™ P-40 can be purchased from:

- Sigma-Aldrich (Igepal® CA-630, cat. no. I 3021; Nonidet P-40 Substitute, cat. no. 74385), [www.sigmaaldrich.com](http://www.sigmaaldrich.com)

\* 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 Midi Kit – for isolation of up to 1 mg total RNA from animal cells or tissues, yeast, and bacteria</b>		
RNeasy Midi Kit (50)	50 RNeasy Midi Spin Columns, collection tubes (15 ml), and RNase-free reagents and buffers	75144
<b>RNeasy Maxi Kit – for isolation of up to 6 mg total RNA from animal cells or tissues, yeast, and bacteria</b>		
RNeasy Maxi Kit (12)	12 RNeasy Maxi Spin Columns, collection tubes (50 ml), and RNase-free reagents and buffers	75162
<b>RNA/<i>later</i> RNA Stabilization Reagent – for stabilization of RNA in animal tissues</b>		
RNA/ <i>later</i> RNA Stabilization Reagent (50 ml)	50 ml RNA/ <i>later</i> RNA Stabilization Reagent for stabilization of RNA in 25 x 200 mg tissue samples	76104
RNA/ <i>later</i> RNA Stabilization Reagent (250 ml)	250 ml RNA/ <i>later</i> RNA Stabilization Reagent for stabilization of RNA in 125 x 200 mg tissue samples	76106
<b>Accessories</b>		
<b>RNase-Free DNase Set – for DNase digestion during RNA purification</b>		
RNase-Free DNase Set (50)	1500 units RNase-free DNase I, RNase-free buffer, and RNase-free water for 50 RNA minipreps	79254
RNase-Free DNase Set (250)	5 x 1500 units RNase-free DNase I, RNase-free buffer, and RNase-free water for 250 RNA minipreps	79256

Product	Contents	Cat. no.
<b>Reagents and collection tubes</b>		
Buffer RLT (220 ml)	220 ml RNeasy Lysis Buffer	79216
Buffer EL (1000 ml)	1000 ml Erythrocyte Lysis Buffer	79217
QIAGEN Proteinase K (2 ml)	2 ml (>600 mAU/ml, solution)	19131
QIAGEN Proteinase K (10 ml)	10 ml (>600 mAU/ml, solution)	19133
Collection Tubes (2 ml)	1000 collection tubes (2 ml)	19201
<b>Related products</b>		
<b>RNeasy Plus Mini Kit — For purification of up to 100 µg total RNA from cells/tissues using gDNA Eliminator columns</b>		
RNeasy Plus Mini Kit (50)	50 RNeasy Mini Spin Columns, gDNA Eliminator Spin Columns, collection tubes, and RNase-free water and buffers	74134
RNeasy Plus Mini Kit (250)	250 RNeasy Mini Spin Columns, gDNA Eliminator Spin Columns, collection tubes, and RNase-free water and buffers	74136
<b>RNeasy Plant Mini Kit — for isolation of up to 100 µg total RNA from plants and fungi</b>		
RNeasy Plant Mini Kit (50)	50 RNeasy Mini Spin Columns, 50 QIAshredder™ Spin Columns, collection tubes (1.5 ml and 2 ml), and RNase-free reagents and buffers	74904
<b>RNeasy Plus 96 Kit — for 96-well purification of total RNA from cells using gDNA Eliminator plates</b>		
RNeasy Plus 96 Kit (12)	For 12 x 96 total RNA preps:	74192

Product	Contents	Cat. no.
	12 RNeasy 96 Plates, 12 gDNAEliminator 96 Plates, elution microtubes, caps, S-Blocks, AirPore tape sheets, and RNase-free reagents and buffers	
<b>QIAamp® RNA Blood Mini Kit – for isolation of total RNA from up to 1.5 ml whole blood</b>		
QIAamp RNA Blood Mini Kit (50)	For 50 RNA preps: 50 QIAamp Mini Spin Columns, 50 QIAshredder Spin Columns, collection tubes (1.5 ml and 2 ml), and RNase-free reagents and buffers	52304
<b>Oligotex® mRNA Kits* – for purification of mRNA from total RNA</b>		
Oligotex mRNA Mini Kit (12)	For 12 mRNA minipreps: 200 µl Oligotex Suspension, small spin columns, collection tubes (1.5 ml), and RNase-free reagents and buffers	70022
Oligotex mRNA Midi Kit (12)	For 12 mRNA midipreps: 700 µl Oligotex Suspension, small spin columns, collection tubes (1.5 ml), and RNase-free reagents and buffers	70042
Oligotex mRNA Maxi Kit (6)	For 6 mRNA maxipreps: 700 µl Oligotex Suspension, large spin columns, collection tubes (1.5 ml), and RNase-free reagents and buffers	70061
<b>Oligotex Suspension*</b>		
Oligotex Suspension (0.5 ml)	0.5 ml for mRNA isolation from up to 8 mg of total RNA	79000
<b>QuantiNova® RT PCR Kits – most advanced real-time PCR and RT-PCR Kits</b>		

\* Not available in Japan.

<b>Product</b>	<b>Contents</b>	<b>Cat. no.</b>
QuantiNova Reverse Transcription Kit (10)	For 10 x 20 µl reactions: 20 µl 8x gDNA Removal Mix, 10 µl Reverse Transcription Enzyme, 40 µl Reverse Transcription Mix (containing RT primers), 20 µl Internal Control RNA, 1.9 ml RNase-free water	205410
QuantiNova SYBR® Green PCR Kit (100)	For 100 x 20 µl reactions: 1 ml 2x QuantiNova SYBR® Green PCR Master Mix, 500 µl QuantiNova Yellow Template Dilution Buffer, 250 µl QN ROX™ Reference Dye, 1.9 ml Water	208052
QuantiNova Probe PCR Kit (100)	For 100 x 20 µl reactions: 1 ml 2x QuantiNova Probe PCR Master Mix, 250 µl QN ROX Reference Dye, 500 µl QuantiNova Yellow Template Dilution Buffer, 1.9 ml water	208252
QuantiNova Multiplex PCR Kit (100)	For 100 x 20µl reactions: 500 µl QuantiNova Multiplex PCR Mastermix, 500 µl yellow template dilution buffer, 250 µl ROX Reference Dye, 1.9 µl RNase-free water	208452
<b>Omniscript RT Kit – for reverse transcription using ≥50 ng RNA</b>		
Omniscript RT Kit (50)*	For 50 reverse-transcription reactions: 200 units Omniscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix, RNase-free water	205111
<b>Sensiscript RT Kit – for reverse transcription using &lt;50 ng RNA</b>		
Sensiscript RT Kit (50)*	For 50 reverse-transcription reactions: Sensiscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix, RNase-free water	205211
<b>QIAGEN OneStep RT-PCR Kit – for fast and efficient one-step RT-PCR</b>		

\* Other kit sizes and/or formats available; please inquire.

Product	Contents	Cat. no.
QIAGEN OneStep <i>Ahead</i> RT-PCR Kit (50) *	6 vials for 50 reactions: 1 x 500 µl OneStep <i>Ahead</i> RT-PCR Master Mix, 1 x 50 µl OneStep <i>Ahead</i> RT Mix, 1 x 200 µl Template Tracer, 1 x 50 µl Master Mix Tracer, 1 x 1.9 ml water, 1 x 400 µl Q-Solution	220211
<b>HotStarTaq DNA Polymerase — for hot-start PCR</b>		
HotStarTaq DNA Polymerase (250 U) *	250 units HotStarTaq DNA Polymerase, 10x PCR Buffer, 5x Q-Solution, 25 mM MgCl <sub>2</sub>	203203
AllTaq PCR Core Kit (250 U)	50 µl AllTaq Polymerase (5 U/µl), 1.2 ml AllTaq PCR Buffer (5x), 55 µl dNTP Mix (10 mM each), 200 µl Template Tracer (25x), 50 µl Master Mix Tracer (125x), 2 ml Q-Solution (5x), 1.2 ml MgCl <sub>2</sub> (25 mM), 1.9 ml RNase-free water	203123

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at [www.qiagen.com](http://www.qiagen.com) or can be requested from QIAGEN Technical Services or your local distributor.

\* Other kit sizes and/or formats available; please inquire.

# Document Revision History

Date	Changes
01/2019	Deleted the discontinued kits (RNeasy Protect Midi Kit and RNeasy Protect Maxi Kit) and details on RNeasy Protect Kits and RNA <sup>later</sup> Stabilization Reagent. Updated the Ordering Information section. Updated the template.
07/2019	Replaced "Handbook" with "Quick-Start Protocol" in "Kit Contents". Transferred all RNA <sup>later</sup> RNA Stabilization Reagent storage information to "Important points before starting" in protocols that use the product. Removed protocols for isolation of cytoplasmic RNA from animal cells, total RNA from bacteria, and total cellular RNA from whole blood (will be converted to supplementary protocols). In Table 4, "Comments" column, "Yeast" row, paragraph 1, removed all mention of "mortar and pestle". In Table 7 (formerly Table 9), corrected column 2 heading to "Amount of tissue (mg)" from "Number of cells". In Table 11 (formerly Table 15), corrected column 2 heading to "Number of yeast cells" from "Number of cells". In Table 13 (formerly Table 19), corrected column 2 heading from "Number of cells" to "Amount of RNA (mg)" and column 3 heading from "Buffer RLN (4°C)" to "Add RNase-free water to"; in column 4, values for rows 2 and 3 (i.e., 4.0 and 2.0, respectively) were interchanged (i.e., 2.0 and 4.0, respectively). In the "RNeasy Midi/Maxi Protocol for RNA Cleanup", step 3, "▲ 50 ml or ● 15 ml" was changed to "▲ 15 ml or ● 50 ml". In Appendix I, physical store addresses were replaced with manufacturer/vendor websites. Supplier info for lyticase, zymolase, and Nonidet-P40 substitute was updated. In "Ordering Information", cat. no. for RNeasy Plus Kit (12) was corrected; discontinued products (cat. nos. 52303, 79002) were deleted.
09/2019	Corrected unit of measure (old: µg; new: mg) for RNeasy Maxi column yield in Table 2.

## Limited License Agreement for RNeasy Midi/Maxi Handbook

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

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## Notes

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