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RNAprotect® Bacteria Reagent Handbook

RNAprotect Bacteria Reagent

For in vivo stabilization of total RNA in bacteria

RNeasy® Protect Bacteria Mini Kit
RNeasy Protect Bacteria Midi Kit

For in vivo stabilization of total RNA in bacteria and subsequent RNA purification

Sample to Insight

QIAGEN

Contents

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RNAprotect Bacteria Reagent **Catalog no.** **Volume of bacterial culture to stabilize**	 **76506** **100 ml**
RNAprotect Bacteria Reagent	2 x 100 ml
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RNeasy Protect Bacteria Kits **Catalog no.** **Number of preps**	**Mini (50)** **74524** **50**
RNeasy Mini Kit (50)	1
RNeasy Midi Kit (10)	–
RNAprotect Bacteria Reagent	2 x 100 ml
Quick-Start Protocol	1

Storage

Store RNAprotect Bacteria Reagent at room temperature (15–25°C). Under these conditions, the reagent is stable for at least 12 months, if not otherwise stated on the label. See the *RNeasy Mini Handbook* and the *RNeasy Midi/Maxi Handbook* for storage conditions for the RNeasy Mini Kit and the RNeasy Midi Kit, respectively.

Intended Use

RNAprotect Bacteria Reagent is intended for research use only. Prior to using it for other purposes, the user must validate the system in compliance with the applicable law, directives and regulations.

QIAcube® Connect is designed to perform fully automated purification of nucleic acids and proteins in molecular biology applications. The system is intended for use by professional users trained in molecular biological techniques and the operation of QIAcube Connect.

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

Safety Information

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

See the *RNeasy Mini Handbook* and the *RNeasy Midi/Maxi Handbook* for safety information on the RNeasy Mini Kit and the RNeasy Midi Kit, respectively.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of RNAprotect Bacteria Reagent is tested against predetermined specifications to ensure consistent product quality.

Introduction

To ensure accurate analysis of gene expression in bacteria, it is important to analyze RNA that truly represents in vivo gene expression. When using conventional methods to harvest bacterial cells, a combination of 2 major events greatly affects the gene expression profile. Firstly, RNA is enzymatically degraded, resulting in a reduction or loss of many transcripts. This is particularly significant for bacterial mRNAs, as they usually only have a very short half-life, often only a few minutes. Secondly, genes may be induced during handling and processing of bacterial cells, leading to higher expression of specific genes.

To ensure reliable gene expression analysis, RNA should ideally be stabilized in vivo, since changes in the gene expression profile occur during or directly after harvesting of bacterial cells. RNAprotect Bacteria Reagent uses a novel patent-pending technology to provide immediate stabilization of the in vivo gene expression profile in bacteria. This reagent prevents both degradation of RNA transcripts and induction of genes.

When using RNAprotect Bacteria Reagent, RNA is stabilized before bacterial cells are lysed. This allows sufficient time for efficient disruption of cells without the risk of distorting the gene expression profile. After cell lysis, the RNeasy Mini Kit or RNeasy Midi Kit can be used to purify total RNA. The resulting high-quality RNA reflects the true in vivo gene expression profile of bacteria and is suitable for use in a wide range of downstream applications. These include northern, dot and slot blotting; array analyses; quantitative, real-time RT-PCR, such as QuantiTect® technology; and other nucleic acid-based technologies, such as NASBA® and bDNA analyses.

Principle and procedure

RNAprotect Bacteria Reagent is suitable for use with both Gram-positive bacteria (e.g., *Staphylococcus aureus* and *Mycobacterium avium*) and Gram-negative bacteria (e.g., *Escherichia coli* and *Salmonella typhimurium*). We recommend growing bacteria in minimal

media to ensure reliable and reproducible gene expression. However, the RNAprotect Bacteria Reagent is compatible with both minimal and complex media.

Two volumes of RNAprotect Bacteria Reagent are added directly to one volume of bacterial culture, providing immediate stabilization of RNA (alternatively, two volumes of RNAprotect Bacteria Reagent are mixed with one volume of media or PBS, which is then applied to bacteria grown on solid media). The bacterial cells are then disrupted to release bacterial RNA. From the resulting lysed cells, RNA can be purified using either the RNeasy Mini Kit or RNeasy Midi Kit.

Description of protocols

This handbook contains 2 types of protocol. There are various different protocols for preparing lysates of bacterial cells (Protocols 1–6), and 2 different protocols for purifying total RNA from bacterial lysates (Protocols 7–8). You need to select and perform one protocol from Protocols 1–6, followed by another protocol from Protocols 7–8.

Each protocol for preparing lysates of bacterial cells (Protocols 1–6) provides instructions on stabilizing RNA followed by instructions on disrupting bacterial cells. The choice of protocol depends on the strength of the bacterial cell wall. Cell wall strength depends on various factors, including bacterial species, generation time and culture medium. Bacterial cells must be completely disrupted to ensure efficient RNA purification.

Each protocol for preparing lysates of bacterial cells (Protocols 1–6) includes one or more of the following disruption procedures:

- **Enzymatic lysis**: The cell wall is enzymatically digested by a lytic enzyme (e.g., lysozyme or lysostaphin). We recommend enzymatic lysis for both Gram-negative and Gram-positive bacteria.
- **Proteinase K digestion**: The large amount of protein in complex media is digested by proteinase K to improve the purity of the purified RNA. We recommend proteinase K digestion for any bacteria species grown in complex media. Proteinase K digestion usually improves RNA yields for Gram-positive bacteria. In addition, if purifying RNA from large amounts of starting material, proteinase K digestion may improve RNA yields.
- **Mechanical disruption**: The cell wall is mechanically disrupted using the TissueLyser II and glass beads. Mechanical disruption is suitable for a wide range of bacterial species. Mechanical disruption can be combined with enzymatic lysis to give higher RNA yields. Although the protocols in this handbook provide instructions on mechanical disruption using the TissueLyser II, other methods for mechanical disruption are possible.

Due to the wide range of bacteria and culture conditions, the optimal protocol for preparing lysates of bacterial cells (Protocols 1–6) must be carefully selected. The different protocols for preparing lysates of bacterial cells are described on page 9, and Table 1, page 11, provides an overview of these protocols to enable easy protocol selection.

Protocol 1: Enzymatic Lysis of Bacteria

This protocol involves enzymatic lysis only. We recommend this protocol for Gram-negative bacteria with short generation times that are grown in minimal media.

Protocol 2: Enzymatic Lysis and Mechanical Disruption of Bacteria

This protocol involves enzymatic lysis followed by mechanical disruption. We recommend this protocol for Gram-negative bacteria and easy-to-disrupt Gram-positive bacteria grown in minimal media.

Protocol 3: Mechanical Disruption of Bacteria

This protocol involves mechanical disruption only and is for rapid disruption of a wide range of bacterial species. However, RNA yields are generally lower than with the other protocols for disrupting bacterial cells.

Protocol 4: Enzymatic Lysis and Proteinase K Digestion of Bacteria

This protocol involves enzymatic lysis together with proteinase K digestion. We recommend this protocol for Gram-negative bacteria grown in complex media and for Gram-positive bacteria grown in minimal or complex media.

Protocol 5: Enzymatic Lysis, Proteinase K Digestion and Mechanical Disruption of Bacteria

This protocol involves enzymatic lysis together with proteinase K digestion, followed by mechanical disruption. We recommend this protocol for difficult-to-disrupt Gram-positive bacteria grown in minimal or complex media.

Protocol 6: Disruption of Bacteria Grown on Solid Media

This protocol is for bacteria grown on solid media. Bacterial cells are subjected to enzymatic lysis and, optionally, to proteinase K digestion and/or mechanical disruption.

Protocol 7: Purification of Total RNA from Bacterial Lysate using the RNeasy Mini Kit

This protocol provides instructions on using the RNeasy Mini Kit to purify up to 100 µg RNA per sample. The starting material for this protocol is the bacterial lysates prepared in Protocols 1–6.

Protocol 8: Purification of Total RNA from Bacterial Lysate using the RNeasy Midi Kit

This protocol provides instructions on using the RNeasy Midi Kit to purify up to 1 mg RNA per sample. The starting material for this protocol is the bacterial lysates prepared in Protocols 1–6.

Table 1. Overview of protocols for preparing lysates of bacterial cells

Bacteria	Culture	Protocol number	Enzymatic lysis	Proteinase K digestion	Mechanical disruption
Gram-negative	Minimal media	1*	Yes	No	No
		2	Yes	No	Yes
Gram-negative	Complex media	4	Yes	Yes	No
Gram-positive	Minimal media	2†	Yes	No	Yes
		4	Yes	Yes	No
		5	Yes	Yes	Yes
Gram-positive	Complex media	4‡	Yes	Yes	No
		5	Yes	Yes	Yes
Mixture of different species	Minimal media or complex media	3	No	No	Yes
Gram-negative or Gram-positive	Solid media	6§	Yes	Optional	Optional

* Choose Protocol 2 instead of Protocol 1 if generation times are long.

† Choose Protocol 2 if bacteria are relatively easy to disrupt, or choose Protocol 5 if bacteria are relatively difficult to disrupt. Otherwise, choose Protocol 4.

‡ Choose Protocol 5 if bacteria are relatively difficult to disrupt.

§ Proteinase K digestion is required when processing Gram-positive bacteria. Mechanical disruption may improve RNA yields for some bacteria species.

Note: For some bacterial species or culture conditions, using phenol-guanidine–based lysis buffer may improve RNA yields. The appendices of this handbook contain protocols which describe how to stabilize and purify bacterial RNA using RNAprotect Bacteria Reagent in combination with the RNeasy Lipid Tissue Mini Kit or RNeasy Plus Universal Kits (supplied with phenol-guanidine–based QIAzol Lysis Reagent). Appendix C (page 54) is intended for use with most bacteria, and describes enzymatic lysis and optional proteinase K digestion of bacteria followed by lysis in hot QIAzol Reagent and RNA purification.

Automated purification of RNA on QIAcube Instruments

Purification of RNA can be fully automated on QIAcube Connect or the classic QIAcube. The innovative QIAcube instruments use advanced technology to process QIAGEN spin columns, enabling seamless integration of automated, low-throughput sample prep into your laboratory workflow. Sample preparation using QIAcube instruments follows the same steps as the manual procedure (i.e., lyse, bind, wash and elute), enabling you to continue using the RNeasy Protect Bacteria Kits for purification of high-quality RNA.

QIAcube instruments are preinstalled with protocols for purification of plasmid DNA, genomic DNA, RNA, viral nucleic acids and proteins, plus DNA and RNA cleanup. The range of protocols available is continually expanding, and additional QIAGEN protocols can be downloaded free of charge at **www.qiagen.com/qiacubeprotocols**.

QIAcube Connect.

Equipment and Reagents to Be Supplied by User

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

For all protocols

- Sterile, RNase-free pipet tips
- Suitably sized tubes and microcentrifuge or centrifuge with appropriate rotors
- Disposable gloves
- Vortexer
- Shaker–incubator

For protocols requiring enzymatic lysis (see Table 1, page 11)

- Lysozyme (e.g., Sigma, cat. no. L7651) or appropriate lytic enzyme*
- Tris and EDTA for preparing TE buffer

For protocols requiring proteinase K digestion (see Table 1, page 11)

- QIAGEN Proteinase K (see "Ordering Information", starting on page 62)

For protocols requiring mechanical disruption (see Table 1, page 11)

- TissueLyser system (see "Ordering Information", starting on page 62)
- Glass beads (e.g., Sigma, cat. no. G1145, G1277 or G8772)*
- 2 ml Safe-Lock tubes (Eppendorf, cat. no. 0030 120.094)*

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

For protocols requiring RNeasy Kits (Protocols 7 and 8)

- 14.3 M β-mercaptoethanol (β-ME) (stock solutions are usually 14.3 M)
- RNeasy Mini Kit, RNeasy Midi Kit, RNeasy Protect Bacteria Mini Kit or RNeasy Protect Bacteria Midi Kit (see "Ordering Information", starting on page 62)
- Ethanol (96–100%), ethanol (80%) or ethanol (70%)*
- **Optional**: RNase-Free DNase Set (see "Ordering Information", starting on page 62)

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

Important Notes

Optimal culture conditions

To ensure accuracy and reproducibility in gene expression profiling, the following factors need to be considered:

- **Culture media**: We recommend using minimal media, since they are better defined and have less variation than complex media.
- **Time of cell harvest**: Cells should be harvested in mid-logarithmic growth. In this phase, culture conditions are most constant, cells are not nutrient depleted and RNA levels are at their highest due to high metabolic activity. In addition, when bacterial cells reach stationary phase, the cell wall becomes much harder to penetrate, which may reduce the speed and efficiency of RNA stabilization by RNAprotect Bacteria Reagent.

RNA yield is strongly dependent on the generation time of the bacterial cells. We therefore recommend the use of fresh cultures.

Determining the correct amount of starting material

When using RNeasy Kits to purify total RNA from bacterial lysates, the amount of starting material is critical and should be carefully calculated. The 2 main factors to consider are:

- **RNA binding capacity of the RNeasy spin column**: The maximum capacities are 100 µg per RNeasy Mini spin column, and 1 mg per RNeasy Midi spin column.
- **Presence of RNAprotect Bacteria Reagent in the cell culture and the volume of Buffer RLT required for efficient lysis**: Up to 7.5 x 10^8 cells per RNeasy Mini spin column or 5 x 10^8 – 7.5 x 10^9 cells per RNeasy Midi spin column can be used. These maximum cell numbers apply to cultures of *E. coli* grown in LB media. Since different bacterial species exhibit different morphological characteristics, which may also differ under different culture conditions, the maximum number of cells that can be used may also differ.

These limiting factors are illustrated in the following 2 examples, which show the calculation of the amount of *E. coli* to apply to an RNeasy Mini spin column.

***E. coli* grown in minimal medium**

RNA yield approximately 40 µg per 7.5 x 10^8 cells. Up to 7.5 x 10^8 cells can be used (use of higher numbers of cells results in inefficient lysis and reduced yield).

***E. coli* grown in LB medium**

RNA yield approximately 120 µg per 7.5 x 10^8 cells. Up to 6 x 10^8 cells can be used (100 µg RNA is the maximum binding capacity of the RNeasy Mini spin column).

Table 2 shows the typical RNA yields from bacterial cells grown in different culture media.

Note: If the RNA binding capacity of the RNeasy spin column is exceeded or, if cell lysis is incomplete due to the use of excess starting material, the yield and purity of the purified RNA will be significantly reduced.

Table 2. Typical yields of total RNA from two bacterial species grown in different culture media*

Bacterial species	Culture medium†	No. cells	RNA yield (µg)‡
E. coli	Minimal medium	5×10^8	25
E. coli	LB	5×10^8	70
B. subtilis	Minimal medium	1×10^8	8
B. subtilis	LB	1×10^8	15

* Bacterial cells disrupted according to Protocol 1.

† We recommend minimal media for growing bacteria.

‡ Yields can vary due to factors, such as generation time and growth conditions used. In addition, following the protocols for mechanical disruption of cells (Protocols 2, 3 and 5) may increase yields. Since the RNeasy procedure enriches for mRNA and other RNAs >200 nucleotides, the total RNA yield does not include quantitative amounts of 5S RNA, tRNA and other low-molecular-weight RNAs, which make up 15–20% of total cellular RNA.

If your starting material is neither *E. coli* nor *B. subtilis* and you do not know its RNA content, we recommend using no more than 2×10^8 cells per RNeasy Mini spin column or 2×10^9 cells per RNeasy Midi spin column in the first purification procedure. Depending on RNA yield and purity, it may be possible to increase the number of cells in subsequent procedures. To optimize RNA yields, we recommend performing pilot experiments in which RNA is purified from different amounts of cells.

Quantifying bacterial cells

Bacterial growth is usually measured using a spectrophotometer. However, it is very difficult to give reliable recommendations for the relationship between OD values and cell numbers in bacterial cultures. OD readings are influenced by factors, such as bacterial species and physiology, since OD readings measure light scattering rather than absorption. Measurements of light scattering depend on the distance between the sample and the detector, and readings from different types of spectrophotometer therefore vary. Furthermore, different species show

different OD values at certain wavelengths (e.g., 600 nm or 436 nm). Bacterial physiology can be influenced by various factors (e.g., culture media, temperature and shaker speed).

We therefore recommend calibrating your spectrophotometer by comparing OD readings at appropriate wavelengths with viable cell densities determined by plating experiments.* OD readings should be between 0.05 and 0.3 to ensure reliability. Samples with OD readings above 0.3 should be diluted so that the OD readings fall within this range; the dilution factors are used when calculating the number of cells per ml.

The following calculation may be helpful as a rough guide. An *E. coli* culture of 1 x 10^9 cells/ml is diluted 1:4 and gives OD600 readings of 0.25 with a Beckman DU®-7400 spectrophotometer or 0.125 with a Beckman DU-40 spectrophotometer. These correspond to calculated OD readings of 1.0 or 0.5, respectively, for 1 x 10^9 cells/ml.

Handling and storing starting material

RNAprotect Bacteria Reagent is added to bacterial cultures to immediately stabilize RNA. After RNA stabilization, bacterial cells can be pelleted by centrifugation. Pellets can be frozen and stored at –30°C to –15°C for up to 2 weeks or at –90°C to –65°C for up to 4 weeks.

DNase digestion

Generally, DNase digestion is not required when purifying RNA using RNeasy Kits, since RNeasy silica-membrane technology enables efficient removal of most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA-based applications that are sensitive to very small amounts of DNA. In these cases, residual amounts of DNA can be removed by on-column DNase digestion during the RNA purification procedure using the QIAGEN RNase-Free DNase Set or by DNase digestion after RNA purification.

* Ausubel, F. M. et al., eds (1991) Current Protocols in Molecular Biology. New York: Wiley Interscience.

Protocol 1: Enzymatic Lysis of Bacteria

Important points before starting

- Before growing and harvesting bacteria, read "Optimal culture conditions" (page 16).
- If preparing RNA for the first time, read "Appendix A" (page 48).
- Perform all steps of the procedure at room temperature (15–25°C) without interruption.
- If RNeasy Kits will not be used for RNA purification, follow the procedure up to step 7 only.
- If RNeasy Kits will be used for RNA purification, read "Determining the correct amount of starting material" and "Quantifying bacterial cells" (pages 17–18). Depending on the amount of starting material, choose whether to use the RNeasy Mini Kit or RNeasy Midi Kit.
- Bacterial lysis in this protocol consists of treatment with 1 mg/ml lysozyme for 5 min, which is optimal for *E. coli*. Since the conditions for enzymatic lysis are affected by bacterial species, cell number and culture medium, it may be necessary to adjust enzyme concentration, to adjust enzyme incubation time and/or to use a different enzyme.

Things to do before starting

- If using RNeasy Kits for RNA purification, add 10 µl β-mercaptoethanol per 1 ml Buffer RLT, and mix. Buffer RLT is stable for 1 month after addition of β-mercaptoethanol.

Procedure

1. Prepare TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) containing 1 mg/ml lysozyme.
2. Calculate the required volume of bacterial culture (1 volume).

   See "Determining the correct amount of starting material", page 17.

3. Pipet 2 volumes of RNAprotect Bacteria Reagent into a tube (not supplied).

   For example, if the volume of bacterial culture is 500 µl, add 1000 µl RNAprotect Bacteria Reagent. The volume of the tube must be 4-times that of the bacterial culture (e.g., if the volume of bacterial culture is 500 µl, use a 2 ml tube).

   Optional: Entire bacterial cultures can be stabilized by adding 2 volumes of RNAprotect Bacteria Reagent to the culture.

4. Add 1 volume of bacterial culture to the tube. Mix immediately by vortexing for 5 s. Incubate for 5 min at room temperature (15–25°C).

5. Centrifuge for 10 min at 5000 x *g*.

   A pellet may not be visible after centrifugation. This is due to an interaction between the cells and the stabilization reagent that causes a change in the optical density of the cells. The procedure is not affected.

   Note: For tubes larger than 50 ml, the centrifugal force or centrifugation time may be increased. However, using excessive centrifugal force may make resuspension of the pellet difficult.

6. Decant the supernatant. Remove residual supernatant by gently dabbing the inverted tube once onto a paper towel.

   After dabbing the tube onto a paper towel, do not remove the remaining supernatant by pipetting, as this may lead to loss of the pellet. If the tube is larger than 15 ml, we recommend removing residual supernatant by leaving the tube inverted on a paper towel for 10 s.

   Note: The remaining supernatant should not exceed approximately 80 µl per 100 µl TE buffer containing lysozyme used in step *7*.

   Optional: Pellets can be stored at –30°C to –15°C for up to 2 weeks or at –90°C to –65°C for up to 4 weeks. For subsequent RNA purification, thaw pellets at room temperature and proceed with step *7* of the procedure.

   Add the appropriate volume of TE buffer containing lysozyme (see Table 3).

Table 3. Reagent volumes for enzymatic lysis of bacteria

Number of bacteria*	RNeasy Spin column	TE buffer containing lysozyme (step 7)	Buffer RLT (step 9)	Ethanol (96–100%) (step 10)
<5 x 10^8	Mini	100 µl	350 µl	250 µl
5 x 10^8 – 7.5 x 10^8	Mini	200 µl	700 µl	500 µl
5 x 10^8 – <1 x 10^9	Midi	500 µl	2000 µl	1400 µl
1 x 10^9 – 7.5 x 10^9	Midi	1000 µl	4000 µl	2800 µl

* The cell numbers are optimized for *E. coli*, and may need to be optimized for other bacteria. See "Determining the correct amount of starting material", page 17.

7. Mix by vortexing for 10 s. Incubate at room temperature for 5 min. During incubation, incubate on a shaker–incubator, or vortex for 10 s at least every 2 min.

   Note: Since the RNA is stabilized, the incubation time can be extended without affecting the procedure, and may increase the RNA yield.

8. Add the appropriate volume of Buffer RLT (see Table 3) and vortex vigorously. If particulate material is visible, pellet it by centrifugation, and use only the supernatant in step 10.

   For tubes of up to 2 ml, centrifuge for 2 min at maximum speed in a microcentrifuge. For tubes larger than 2 ml, centrifuge for 5 min at 3000–5000 *x g*.

   Note: Ensure that β-mercaptoethanol is added to Buffer RLT before use (see "Important points before starting", page 20).

9. Add the appropriate volume of ethanol (96–100%) (see Table 3). Mix by pipetting (RNeasy Mini procedure) or by shaking vigorously (RNeasy Midi procedure). Do not centrifuge.

   After adding ethanol, a precipitate may form. This will not affect the RNeasy procedure.

10. If using the RNeasy Mini Kit, proceed to Protocol 7. If using the RNeasy Midi Kit, proceed to Protocol 8.

Protocol 2: Enzymatic Lysis and Mechanical Disruption of Bacteria

Important points before starting

- Before growing and harvesting bacteria, read "Optimal culture conditions" (page 16).
- If preparing RNA for the first time, read "Appendix A" (page 48).
- Perform all steps of the procedure at room temperature (15–25°C) without interruption.
- If RNeasy Kits will not be used for RNA purification, follow the procedure up to step 11 only.
- If RNeasy Kits will be used for RNA purification, read "Determining the correct amount of starting material" and "Quantifying bacterial cells" (pages 17–18). Depending on the amount of starting material, choose whether to use the RNeasy Mini Kit or RNeasy Midi Kit.
- Bacterial lysis in this protocol consists of treatment with 15 mg/ml lysozyme for 10 min, which is optimal for *B. subtilis*. Since the conditions for enzymatic lysis are affected by bacterial species, cell number and culture medium, it may be necessary to adjust enzyme concentration and/or to adjust enzyme incubation time. For some bacteria, other enzymes may be more effective (e.g., we recommend lysostaphin for disrupting the cell wall of *Staphylococcus aureus*).

Things to do before starting

- If using RNeasy Kits for RNA purification, add 10 µl β-mercaptoethanol per 1 ml Buffer RLT, and mix. Buffer RLT is stable for 1 month after addition of β-mercaptoethanol.

Procedure

1. Prepare TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) containing 15 mg/ml lysozyme.
2. For each sample, weigh 25–50 mg acid-washed glass beads (150–600 µm diameter) in a 2 ml Safe-Lock tube (not supplied) for use in step 11.
3. Calculate the required volume of bacterial culture (1 volume).

   See "Determining the correct amount of starting material", page 17.
4. Pipet 2 volumes of RNAprotect Bacteria Reagent into a tube (not supplied).

   For example, if the volume of bacterial culture is 500 µl, add 1000 µl RNAprotect Bacteria Reagent.

   The volume of the tube must be 4-times that of the bacterial culture (e.g., if the volume of bacterial culture is 500 µl, use a 2 ml tube).

   Optional: Entire bacterial cultures can be stabilized by adding 2 volumes of RNAprotect Bacteria Reagent to the culture.
5. Add 1 volume of bacterial culture to the tube. Mix immediately by vortexing for 5 s. Incubate for 5 min at room temperature (15–25°C).
6. Centrifuge for 10 min at 5000 x *g*.

   A pellet may not be visible after centrifugation. This is due to an interaction between the cells and the stabilization reagent that causes a change in the optical density of the cells. The procedure is not affected.

   Note: For tubes larger than 50 ml, the centrifugal force or centrifugation time may be increased. However, using excessive centrifugal force may make resuspension of the pellet difficult.

7. Decant the supernatant. Remove remaining supernatant by gently dabbing the inverted tube once onto a paper towel.

   After dabbing the tube onto a paper towel, do not remove the remaining supernatant by pipetting, as this may lead to loss of the pellet. If the tube is larger than 15 ml, we recommend removing residual supernatant by leaving the tube inverted on a paper towel for 10 s.

   Note: The remaining supernatant should not exceed approximately 80 µl per 100 µl TE buffer containing lysozyme used in step 8.

   Optional: Pellets can be stored at –30°C to –15°C for up to 2 weeks or at –90°C to –65°C for up to 4 weeks. For subsequent RNA purification, thaw pellets at room temperature and proceed with step 8 of the procedure.

8. Add the appropriate volume of TE buffer containing lysozyme (see Table 4).
9. Mix by vortexing for 10 s. Incubate at room temperature for 10 min. During incubation, incubate on a shaker–incubator, or vortex for 10 s at least every 2 min.

   Note: Since the RNA is stabilized, the incubation time can be extended without affecting the procedure, and may increase the RNA yield.

10. Add the appropriate volume of Buffer RLT (see Table 4). Vortex vigorously for 5–10 s.

    Note: Ensure that β-mercaptoethanol is added to Buffer RLT before use (see "Important points before starting", page 23).

    Ensure that the pellet is thoroughly resuspended in Buffer RLT.

Table 4. Reagent volumes for enzymatic lysis and mechanical disruption of bacteria

Number of bacteria*	RNeasy Spin column	TE buffer containing lysozyme (step 8)	Buffer RLT (step 10)	Buffer RLT (step 12)	Ethanol (96–100%) (step 13)
<5 x 10^8	Mini	100 µl	350 µl	–	220 µl
5 x 10^8 – 7.5 x 10^8	Mini	200 µl	700 µl	–	470 µl
5 x 10^8 – 7.5 x 10^8	Midi	200 µl	700 µl	3200 µl	–

* The cell numbers are optimized for *E. coli*, and may need to be optimized for other bacteria. See "Determining the correct amount of starting material", page 17.

11. Transfer the suspension into the 2 ml Safe-Lock tube containing the acid-washed glass beads prepared in step 2. Disrupt the cells in the TissueLyser II for 5 min at maximum speed.

    Other methods of mechanical disruption can be used instead. We recommend using the TissueLyser II.

12. Centrifuge for 10 s at maximum speed. Transfer supernatant (400 µl from starting material ≤5 x 10^8 cells or 850 µl from starting material ≥5 x 10^8 cells) into a new tube (not supplied). If using the RNeasy Midi Kit, add 3200 µl Buffer RLT to the supernatant, and mix thoroughly by shaking or vortexing vigorously for 5–10 s.

    Note: Ensure that β-mercaptoethanol is added to Buffer RLT before use (see "Important points before starting", page 23).

13. To the supernatant, add an appropriate volume of ethanol (96–100% for the RNeasy Mini procedure or 70% for the RNeasy Midi procedure) (see Table 4). Mix well by pipetting (RNeasy Mini procedure) or by shaking vigorously (RNeasy Midi procedure). Do not centrifuge.

    After adding ethanol, a precipitate may form. This will not affect the RNeasy procedure.

14. If using the RNeasy Mini Kit, proceed to Protocol 7. If using the RNeasy Midi Kit, proceed to Protocol 8.

Protocol 3: Mechanical Disruption of Bacteria

Important points before starting

- Before growing and harvesting bacteria, read "Optimal culture conditions" (page 16).
- If preparing RNA for the first time, read "Appendix A" (page 48).
- Perform all steps of the procedure at room temperature (15–25°C) without interruption.
- If RNeasy Kits will not be used for RNA purification, follow the procedure up to step 9 only.
- If RNeasy Kits will be used for RNA purification, read "Determining the correct amount of starting material" and "Quantifying bacterial cells" (pages 17–18). Depending on the amount of starting material, choose whether to use the RNeasy Mini Kit or RNeasy Midi Kit.

Things to do before starting

- If using RNeasy Kits for RNA purification, add 10 µl β-mercaptoethanol per 1 ml Buffer RLT, and mix. Buffer RLT is stable for 1 month after addition of β-mercaptoethanol.

Procedure

1. For each sample, weigh 25–50 mg acid-washed glass beads (150–600 µm diameter) in a 2 ml Safe-Lock tube (not supplied) for use in step 8.
2. Calculate the required volume of bacterial culture (1 volume).

   See "Determining the correct amount of starting material", page 17.

3. Pipet 2 volumes of RNAprotect Bacteria Reagent into a tube.

   For example, if the volume of bacterial culture is 500 µl, add 1000 µl RNAprotect Bacteria Reagent.

   The volume of the tube must be 4-times that of the bacterial culture (e.g., if the volume of bacterial culture is 500 µl, use a 2 ml tube).

   Optional: Entire bacterial cultures can be stabilized by adding 2 volumes of RNAprotect Bacteria Reagent to the culture.

4. Add 1 volume of bacterial culture to the tube. Mix immediately by vortexing for 5 s. Incubate for 5 min at room temperature (15–25°C).

5. Centrifuge for 10 min at 5000 *x g*.

   A pellet may not be visible after centrifugation. This is due to an interaction between the cells and the stabilization reagent that causes a change in the optical density of the cells. The procedure is not affected.

   Note: For tubes larger than 50 ml, the centrifugal force or centrifugation time may be increased. However, using excessive centrifugal force may make resuspension of the pellet difficult.

6. Decant the supernatant. Remove residual supernatant by gently dabbing the inverted tube once onto a paper towel.

   After dabbing the tube onto a paper towel, do not remove the remaining supernatant by pipetting, as this may lead to loss of the pellet. If the tube is larger than 15 ml, we recommend removing residual supernatant by leaving the tube inverted on a paper towel for 10 s.

   Note: The remaining supernatant should not exceed approximately 80 µl per 350 µl Buffer RLT used in step 7.

   Optional: Pellets can be stored at –30°C to –15°C for up to 2 weeks or at –90°C to –65°C for up to 4 weeks. For subsequent RNA purification, thaw pellets at room temperature and proceed with step 7 of the procedure.

7. Add the appropriate volume of Buffer RLT (see Table 5). Vortex vigorously for 5–10 s.

   Note: Ensure that β-mercaptoethanol is added to Buffer RLT before use (see "Important points before starting", page 27).

   Ensure that the pellet is thoroughly resuspended in Buffer RLT.

Table 5. Buffer RLT volumes for mechanical disruption of bacteria

Number of bacteria*	Buffer RLT (µl)
<5 x 10^8	350
5 x 10^8 – 1 x 10^9	700
>1 x 10^9	1800

* The cell numbers are optimized for *E. coli*, and may need to be optimized for other bacteria. See "Determining the correct amount of starting material", page 17.

8. Transfer the suspension into the 2 ml Safe-Lock tube containing the acid-washed beads prepared in step 1. Disrupt cells in the TissueLyser II for 5 min at maximum speed.

   Other methods of mechanical disruption can be used instead. We recommend using the TissueLyser II.

9. Centrifuge for 10 s at maximum speed. Transfer supernatant into a new tube (not supplied). If using the RNeasy Midi Kit, add Buffer RLT to the supernatant to a final volume of 4 ml. Vortex vigorously for 5–10 s.

   If using the RNeasy Mini Kit, the volume of the tube must be at least twice that of the Buffer RLT used. If using the RNeasy Midi Kit, the volume of the tube must be at least 10ml.

   Note: Ensure that β-mercaptoethanol is added to Buffer RLT before use (see "Important points before starting", page 27).

10. Determine the volume of supernatant. Add an equal volume of ethanol (70%), and mix well by pipetting (RNeasy Mini procedure) or by shaking vigorously (RNeasy Midi procedure). Do not centrifuge.

    After adding ethanol, a precipitate may form. This will not affect the RNeasy procedure.

11. If using the RNeasy Mini Kit, proceed to Protocol 7. If using the RNeasy Midi Kit, proceed to Protocol 8.

Protocol 4: Enzymatic Lysis and Proteinase K Digestion of Bacteria

Important points before starting

- Before growing and harvesting bacteria, read "Optimal culture conditions" (page 16).
- If preparing RNA for the first time, read "Appendix A" (page 48).
- Perform all steps of the procedure at room temperature (15–25°C) without interruption.
- If RNeasy Kits will not be used for RNA purification, follow the procedure up to step 7 only.
- If RNeasy Kits will be used for RNA purification, read "Determining the correct amount of starting material" and "Quantifying bacterial cells" (pages 17–18). Depending on the amount of starting material, choose whether to use the RNeasy Mini Kit or RNeasy Midi Kit.
- Bacterial lysis in this protocol consists of treatment with 15 mg/ml lysozyme for 10 min, which is optimal for *B. subtilis*. Since the conditions for enzymatic lysis are affected by bacterial species, cell number and culture medium, it may be necessary to adjust enzyme concentration and/or to adjust enzyme incubation time. For some bacteria, other enzymes may be more effective (e.g., we recommend lysostaphin for disrupting the cell wall of *Staphylococcus aureus*).

Things to do before starting

- If using RNeasy Kits for RNA purification, add 10 µl β-mercaptoethanol per 1 ml Buffer RLT, and mix. Buffer RLT is stable for 1 month after addition of β-mercaptoethanol.

Procedure

1. Prepare TE buffer (30 mM Tris·Cl, 1 mM EDTA, pH 8.0) containing 15 mg/ml lysozyme.
2. Calculate the required volume of bacterial culture (1 volume).

   See “Determining the correct amount of starting material”, page 17.
3. Pipet 2 volumes of RNAprotect Bacteria Reagent into a tube (not supplied).

   For example, if the volume of bacterial culture is 500 µl, add 1000 µl RNAprotect Bacteria Reagent.

   The volume of the tube must be 4-times that of the bacterial culture (e.g., if the volume of bacterial culture is 500 µl, use a 2 ml tube).

   Optional: Entire bacterial cultures can be stabilized by adding 2 volumes of RNAprotect Bacteria Reagent to the culture.
4. Add 1 volume of bacterial culture to the tube. Mix immediately by vortexing for 5 s. Incubate for 5 min at room temperature (15–25°C).
5. Centrifuge for 10 min at 5000 *x g*.

   A pellet may not be visible after centrifugation. This is due to an interaction between the cells and the stabilization reagent that causes a change in the optical density of the cells. The procedure is not affected.

   Note: For tubes larger than 50 ml, the centrifugal force or centrifugation time may be increased. However, using excessive centrifugal force may make resuspension of the pellet difficult.

6. Decant the supernatant. Remove residual supernatant by gently dabbing the inverted tube once onto a paper towel.

   After dabbing the tube onto a paper towel, do not remove the remaining supernatant by pipetting, as this may lead to loss of the pellet. If the tube is larger than 15 ml, we recommend removing residual supernatant by leaving the tube inverted on a paper towel for 10 s.

   Note: The remaining supernatant should not exceed approximately 80 µl per100 µl TE buffer containing lysozyme used in step 7.

   Optional: Pellets can be stored at –30°C to –15°C for up to 2 weeks or at –90°C to –65°C for up to 4 weeks. For subsequent RNA purification, thaw pellets at room temperature and proceed with step 7 of the procedure.

7. Add 10–20 µl QIAGEN Proteinase K to the appropriate volume of TE buffer containing lysozyme (see Table 6), and add the mixture to the pellet. Carefully resuspend the pellet by pipetting up and down several times.

   The amount of QIAGEN Proteinase K required depends on the bacterial species. If using the RNeasy Midi Kit for RNA purification, use 20 µl QIAGEN Proteinase K.

Table 6. Reagent volumes for enzymatic lysis and proteinase K digestion of bacteria

Number of bacteria*	RNeasy Spin column	TE buffer containing lysozyme (step 7)	Buffer RLT (step 9)	Ethanol (96–100%) (step 10)	Ethanol (80%) (step 10)
<1 x 10^8	Mini	100 µl	350 µl	250 µl	–
1 x 10^8 – 2.5 x 10^8	Mini	200 µl	700 µl	500 µl	–
2.5 x 10^8 – 1.5 x 10^8	Midi	200 µl	2000 µl	–	1750 µl
7.5 x 10^8 – 1.5 x 10^8	Midi	200 µl	4000 µl	–	3500 µl

* The cell numbers are optimized for *B. subtilis*, and may need to be optimized for other bacteria. See "Determining the correct amount of starting material", page 17.

8. Mix by vortexing for 10 s. Incubate at room temperature for 10 min. During incubation, incubate on a shaker–incubator, or vortex for 10 s at least every 2 min.

   Note: Since the RNA is stabilized, the incubation time can be extended without causing any adverse effects, and may increase the RNA yield.

9. Add the appropriate volume of Buffer RLT (see Table 6) and vortex vigorously. If particulate material is visible, pellet it by centrifugation, and use only the supernatant in step 10.

   For tubes of up to 2 ml, centrifuge for 2 min at maximum speed in a microcentrifuge. For tubes larger than 2 ml, centrifuge for 5 min at 3000– 5000 *x g*.

   Note: Ensure that β-mercaptoethanol is added to Buffer RLT before use (see "Important points before starting", page 30).

10. Add the appropriate volume of ethanol (96–100% for the RNeasy Mini procedure or 80% for the RNeasy Midi procedure) (see Table 6). Mix by pipetting (RNeasy Mini procedure) or by shaking vigorously (RNeasy Midi procedure). Do not centrifuge.

    After adding ethanol, a precipitate may form. This will not affect the RNeasy procedure.

11. If using the RNeasy Mini Kit, proceed to Protocol 7. If using the RNeasy Midi Kit, proceed to Protocol 8.

Protocol 5: Enzymatic Lysis, Proteinase K Digestion and Mechanical Disruption of Bacteria

Important points before starting

- Before growing and harvesting bacteria, read "Optimal culture conditions" (page 16).
- If preparing RNA for the first time, read "Appendix A" (page 48).
- Perform all steps of the procedure at room temperature (15–25°C) without interruption.
- If RNeasy Kits will not be used for RNA purification, follow the procedure up to step 11 only.
- If RNeasy Kits will be used for RNA purification, read "Determining the correct amount of starting material" and "Quantifying bacterial cells" (pages 17–18). Depending on the amount of starting material, choose whether to use the RNeasy Mini Kit or RNeasy Midi Kit.
- Bacterial lysis in this protocol consists of treatment with 15 mg/ml lysozyme for 10 min, which is optimal for *B. subtilis*. Since the conditions for enzymatic lysis are affected by bacterial species, cell number and culture medium, it may be necessary to adjust enzyme concentration and/or to adjust enzyme incubation time. For some bacteria, other enzymes may be more effective (e.g., we recommend lysostaphin for disrupting the cell wall of *Staphylococcus aureus*).

Things to do before starting

- If using RNeasy Kits for RNA purification, add 10 µl β-mercaptoethanol per 1 ml Buffer RLT, and mix. Buffer RLT is stable for 1 month after addition of β-mercaptoethanol.

Procedure

1. Prepare TE buffer (30 mM Tris·Cl, 1 mM EDTA, pH 8.0) containing 15 mg/ml lysozyme.
2. For each sample, weigh 25–50 mg acid-washed glass beads (150–600 µm diameter) in a 2 ml Safe-Lock tube (not supplied), for use in step 11.
3. Calculate the required volume of bacterial culture (1 volume).

   See "Determining the correct amount of starting material", page 17.
4. Pipet 2 volumes of RNAprotect Bacteria Reagent into a tube (not supplied).

   For example, if the volume of bacterial culture is 500 µl, add 1000 µl RNAprotect Bacteria Reagent. The volume of the tube must be 4-times that of the bacterial culture (e.g., if the volume of bacterial culture is 500 µl, use a 2 ml tube).

   Optional: Entire bacterial cultures can be stabilized by adding 2 volumes of RNAprotect Bacteria Reagent to the culture.
5. Add 1 volume of bacterial culture to the tube. Mix immediately by vortexing for 5 s. Incubate for 5 min at room temperature (15–25°C).
6. Centrifuge for 10 min at 5000 *x g*.

   A pellet may not be visible after centrifugation. This is due to an interaction between the cells and the stabilization reagent that causes a change in the optical density of the cells. The procedure is not affected.

   Note: For tubes larger than 50 ml, the centrifugal force or centrifugation time may be increased. However, using excessive centrifugal force may make resuspension of the pellet difficult.

7. Decant the supernatant. Remove remaining supernatant by gently dabbing the inverted tube once onto a paper towel.

   After dabbing the tube onto a paper towel, do not remove the remaining supernatant by pipetting, as this may lead to loss of the pellet. If the tube is larger than 15 ml, we recommend removing residual supernatant by leaving the tube inverted on a paper towel for 10 s.

   Note: The remaining supernatant should not exceed approximately 80 µl per 100 µl TE buffer containing lysozyme used in step 8.

   Optional: Pellets can be stored at –30°C to –15°C for up to 2 weeks or at –90°C to –65°C for up to 4 weeks. For subsequent RNA purification, thaw pellets at room temperature and proceed with step 8 of the procedure.

   Add 10–20 µl QIAGEN Proteinase K to the appropriate volume of TE buffer containing lysozyme (see Table 7), and add the mixture to the pellet. Carefully resuspend the pellet by pipetting up and down several times.

Table 7. Reagent volumes for enzymatic lysis, proteinase K digestion and mechanical disruption of bacteria

Number of bacteria*	RNeasy Spin column	TE buffer containing lysozyme (step 8)	Buffer RLT (step 10)	Ethanol (80%) (step 13)
<7.5 x 10^8	Mini	100 µl	700 µl	590 µl
5 x 10^8 – 1.5 x 10^9	Midi	200 µl	1500 µl	1300 µl

* The cell numbers are optimized for *B. subtilis*, and may need to be optimized for other bacteria. See "Determining the correct amount of starting material", page 17.

8. Mix by vortexing for 10 s. Incubate at room temperature for 10 min. During incubation, incubate on a shaker–incubator, or vortex for 10 s at least every 2 min.

   Note: Since the RNA is stabilized the incubation time can be extended without causing any adverse effects, and may increase the RNA yield.

9. Add the appropriate volume of Buffer RLT (see Table 7). Vortex vigorously for 5–10 s.

   Note: Ensure that β-mercaptoethanol is added to Buffer RLT before use (see "Important points before starting", page 34).

   Ensure that the pellet is thoroughly resuspended in Buffer RLT.

10. Transfer the suspension into the 2 ml Safe-Lock tube containing the acid-washed glass beads prepared in step 2. Disrupt the cells in the TissueLyser II for 5 min at maximum speed.

    Other methods of mechanical disruption can be used instead. We recommend using the TissueLyser II.

11. Centrifuge for 10 s at maximum speed. Transfer supernatant (760 µl for the RNeasy Mini procedure or 1670 µl for the RNeasy Midi procedure) into a new tube (not supplied).

12. To the supernatant, add an appropriate volume of ethanol (80%) (see Table 7). Mix well by pipetting (RNeasy Mini procedure) or by shaking vigorously (RNeasy Midi procedure). Do not centrifuge.

    After adding ethanol, a precipitate may form. This will not affect the RNeasy procedure.

13. If using the RNeasy Mini Kit, proceed to Protocol 7. If using the RNeasy Midi Kit, proceed to Protocol 8.

Protocol 6: Disruption of Bacteria Grown on Solid Media

Important points before starting

- If preparing RNA for the first time, read "Appendix A" (page 48).
- Perform all steps of the procedure at room temperature (15–25°C) without interruption.
- If RNeasy Kits will be used for RNA purification, read "Determining the correct amount of starting material" and "Quantifying bacterial cells" (pages 17–18). Depending on the amount of starting material, choose whether to use the RNeasy Mini Kit or RNeasy Midi Kit.
- This protocol needs to be optimized by the user for the bacterial species being grown.
- Depending on the bacterial species, Protocol 6 is followed by Protocol 1, 2, 4 or 5.
- Media or PBS are required to prepare the stabilization mix.

Procedure

1. Spread out 200 µl of freshly prepared overnight culture on an agar plate (9 cm diameter) using a sterile spreader.
2. Incubate the agar plate.

   The temperature and generation times are variable and determined by the user.
3. Prepare a stabilization mix by mixing 2 volumes of RNAprotect Bacteria Reagent with 500–1000 µl of media or PBS.
4. Pipet the stabilization mix onto the agar plate.
5. Carefully remove the bacterial lawn using the sterile spreader. Pipet the bacterial suspension into a tube. Mix immediately by vortexing for 5 s and incubate for 5 min at room temperature (15–25°C).

   Note: Avoid contamination with pieces of agar, otherwise RNA yields may be reduced.

6. Centrifuge for 10 min at 5000 *x g*.
7. Decant the supernatant. Remove residual supernatant by gently dabbing the inverted tube once onto a paper towel.
8. Depending on the bacterial species, follow Protocol 1, 2, 4 or 5.

   At least 200 µl TE buffer containing lysozyme has to be used. The lysozyme concentration, the need for proteinase K, and the volumes of Buffer RLT and ethanol required depend on which protocol will be carried out.

Protocol 7: Purification of Total RNA from Bacterial Lysate using the RNeasy Mini Kit

Important points before starting

- Carry out one of Protocols 1–6 before starting Protocol 7.

Things to do before starting

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

Procedure

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

   If the lysate exceeds 700 µl, centrifuge successive aliquots through the spin column. Discard the flow-through after each centrifugation.*

   Optional: The QIAGEN RNase-Free DNase Set provides convenient on-column DNase digestion during RNA purification. Generally, DNase digestion is not required, since RNeasy silica-membrane technology enables efficient removal of 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., RT-PCR analysis with a low-abundant target). If using the RNase-Free DNase Set, follow the relevant protocol steps in “Appendix B” (page 51) after performing this step.

* Flow-through contains Buffer RLT or Buffer RW1 and must not be mixed with bleach. See page 7 for “Safety Information”.

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

   Skip this step if performing the optional on-column DNase digestion ("Appendix B", page 51).

3. Place the RNeasy Mini spin column in a new 2 ml collection tube (supplied). Add 500 µl Buffer RPE to the RNeasy Mini spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 *x g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 4.

   Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see "Things to do before starting", page 40).

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

   This long centrifugation ensures that no ethanol is carried over during elution in step 5 (residual ethanol may interfere with downstream reactions).

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

   Optional: Place the spin column in a new 2 ml collection tube (not supplied), and discard the old collection tube containing the flow-through. Centrifuge at full speed for 1 min.

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

* Flow-through contains Buffer RLT or Buffer RW1 and must not be mixed with bleach. See page 7 for "Safety Information".

6. If the expected RNA yield is >30 µg, repeat step 5 using another 30–50 µl of RNase-free water or using the eluate from step 5 (if high RNA concentration is required). Reuse the collection tube from step 5.

   If using the eluate from step 5, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.

Protocol 8: Purification of Total RNA from Bacterial Lysate using the RNeasy Midi Kit

Important points before starting

- Carry out one of Protocols 1–6 before starting Protocol 8.

Things to do before starting

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

Procedure

1. Transfer up to 4 ml lysate, including any precipitate that may have formed, to an RNeasy Midi spin column placed in a 15 ml centrifuge tube (supplied). Close the lid gently, and centrifuge for 5 min at 3000–5000 *x g*. Discard the flow-through.* Reuse the collection tube in step 2.

   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 spin column. If the lysate exceeds 4 ml, centrifuge successive aliquots through the spin column. Discard the flow-through after each centrifugation.*

   Optional: The QIAGEN RNase-Free DNase Set provides convenient on-column DNase digestion during RNA purification. Generally, DNase digestion is not required, since RNeasy silica-membrane technology enables efficient removal of 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., RT-PCR analysis with a low-abundant target). If using the RNase-Free DNase Set, follow the relevant protocol steps in “Appendix B” (page 51) after performing this step.

* Flow-through contains Buffer RLT or Buffer RW1 and must not be mixed with bleach. See page 7 for “Safety Information”.

2. Add 4 ml Buffer RW1 to the RNeasy Midi spin column. Close the lid gently, and centrifuge for 5 min at 3000–5000 *x g* to wash the spin column membrane. Discard the flow-through.* Reuse the collection tube in step 3.

   Skip this step if performing the optional on-column DNase digestion ("Appendix B", page 51).

3. Add 2.5 ml Buffer RPE to the RNeasy Midi spin column. Close the lid gently, and centrifuge for 2 min at 3000–5000 *x g* to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 4.

   Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see "Things to do before starting", page 43).

4. Add 2.5 ml Buffer RPE to the RNeasy Midi spin column. Close the lid gently, and centrifuge for 5 min at 3000–5000 *x g* to wash the spin column membrane.

   This long centrifugation ensures that no ethanol is carried over during elution in step 5 (residual ethanol may interfere with downstream reactions).

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

   Place the RNeasy Midi spin column in a new 15 ml collection tube (supplied). Add the appropriate volume of RNase-free water (see Table 8) directly to the spin column membrane. Close the lid gently, wait for 1 min, and then centrifuge for 3 min at 3000–5000 *x g* to elute the RNA.

Table 8. RNase-free water volumes for eluting RNA from RNeasy Midi spin columns

Expected total RNA yield	RNase-free water
≤150 µg	150 µl
150 µg – 1 mg	250 µl

* Flow-through contains Buffer RLT or Buffer RW1 and must not be mixed with bleach. See page 7 for "Safety Information".

5. Repeat step 5 using a second volume of RNase-free water or using the eluate from step 5 (if high RNA concentration is required). Reuse the collection tube from step 5.

   If using the eluate from step 5, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: **www.qiagen.com/FAQ/FAQList.aspx**. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit **www.qiagen.com**).

	Comments and suggestions
Lysate contains particulate material after addition of Buffer RLT	
a) Cell pellet was not fully resuspended	After addition of Buffer RLT, centrifuge the lysate, and use only the supernatant in subsequent steps. For tubes up to 2 ml, centrifuge for 2 minutes at maximum speed in a microcentrifuge. For tubes larger than 2 ml, centrifuge for 5 minutes at 3000–5000 *x g*.
b) Incomplete removal of solution after centrifugation of RNAprotect Bacteria Reagent and bacterial culture	For subsequent preparations, remove supernatant (i.e., combined RNAprotect Bacteria Reagent and culture supernatant) by dabbing inverted tubes onto a paper towel exactly as described in the relevant protocol (i.e., Protocols 1–6).
c) Use of excess starting material	Repeat the procedure using the correct amount of starting material (see "Determining the correct amount of starting material", page 17).
Clogged RNeasy spin column	
a) Incomplete removal of solution after centrifugation of RNAprotect Bacteria Reagent and bacterial culture	For subsequent preparations, remove supernatant (i.e., combined RNAprotect Bacteria Reagent and culture supernatant) by dabbing inverted tubes onto a paper towel exactly as described in the relevant protocol (i.e., Protocols 1–6).
b) Use of excess starting material	Reduce amounts of starting material (see "Determining the correct amount of starting material", page 17).

		Comments and suggestions
Low RNA yields		
a)	The amount of starting material was incorrectly calculated	Repeat the procedure using the correct amount of starting material (see "Determining the correct amount of starting material", page 17).
b)	Incomplete disruption of cell walls	When digesting cell walls using lysozyme, it may be necessary to optimize lysozyme concentration and digestion time. When using enzymatic digestion, enzymes other than lysozyme may be needed to achieve efficient lysis in some bacterial species. Increase the enzyme concentration or increase the digestion time. Increasing the digestion time does not affect RNA stability. When using mechanical disruption, it may be necessary to lengthen the mechanical disruption step. Freezing and thawing of the stabilized cell pellets makes the cell walls easier to disrupt and cell pellets easier to resuspend.
c)	Incomplete resuspension of bacterial cell pellets	When centrifuging in conical tubes, vortex vigorously to resuspend the pellet. Freezing and thawing of the stabilized cell pellets makes the cell walls easier to disrupt and cell pellets easier to resuspend. The centrifugal force used to pellet the bacteria may be decreased to 2000 *x g*. However, depending on the culture volumes, density of bacterial cells and density of culture medium used, this may lead to incomplete sedimentation.
d)	Cells were grown past logarithmic phase	Harvest cells during the logarithmic growth phase to ensure the highest RNA yields and the most efficient RNA stabilization.

Note: Please refer to the *RNeasy Mini Handbook* or the *RNeasy Midi/Maxi Handbook* for further troubleshooting.

Appendix A: General Remarks on Handling RNA

Handling RNA

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

General handling

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

Disposable plasticware

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

Nondisposable plasticware

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

Glassware

Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent, thoroughly rinsed and oven baked at 240°C for four hours overnight, 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), leave overnight (12 hours) at 37°C, and then autoclave or heat to 100°C for 15 minutes to eliminate residual DEPC.

Electrophoresis tanks

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

Solutions

Solutions (water and other solutions)* should be treated with 0.1% DEPC. DEPC is a strong, but not absolute, inhibitor of RNases. It is commonly used at a concentration of 0.1% to inactivate RNases on glass or plasticware or to create RNase-free solutions and water. DEPC inactivates RNases by covalent modification. Add 0.1 ml DEPC to 100 ml of the solution to be treated and shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 hours at 37°C. Autoclave for 15 minutes to remove traces of DEPC. DEPC will react with primary amines and cannot be used directly to treat Tris* buffers. DEPC is highly unstable in

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

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

the presence of Tris buffers and decomposes rapidly into ethanol and CO_2. When preparing Tris buffers, treat water with DEPC first, then dissolve Tris to make the appropriate buffer. Trace amounts of DEPC will modify purine residues in RNA by carbethoxylation. Carbethoxylated RNA is translated with very low efficiency in cell-free systems. However, its ability to form DNA:RNA or RNA:RNA hybrids is not affected unless a large fraction of the purine residues has been modified. Residual DEPC must be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 minutes.

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

Appendix B: Optional On-Column DNase Digestion using the RNase-Free DNase Set

The QIAGEN RNase-Free DNase Set provides efficient on-column digestion of DNA during RNA purification using the RNeasy Mini or Midi Kit. The DNase is efficiently removed in subsequent wash steps.

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

Preparation of bacterial lysates and binding of RNA to the RNeasy spin column membrane are performed according to the protocols in this handbook. After washing with a reduced volume of Buffer RW1, RNA is treated with DNase I while bound to the spin column membrane. DNase I is removed by a second wash with Buffer RW1. Washing with Buffer RPE and elution are then performed according to the protocols in this handbook.

Important points before starting

- Generally, DNase digestion is not required since RNeasy silica-membrane technology enables efficient removal of 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., RT-PCR analysis with a low-abundant target). DNA can also be removed by a DNase digestion following RNA purification.
- 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 vial.

Things to do before starting

- 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 µl of the RNase-free water provided. To avoid loss of DNase I, do not open the vial. Inject RNase-free water into the vial using an RNase-free needle and syringe. Mix gently by inverting the vial. Do not vortex.
- For long-term storage of DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at –30°C to –15°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.

Procedure

Prepare bacterial lysates as described in Protocols 1–6. Then perform Protocol 7 (■) or Protocol 8 (▲). Instead of following step 2 of Protocol 7 or 8 (i.e., the wash with Buffer RW1), follow steps 1–4 below.

1. Add ■ 350 µl or ▲ 2 ml Buffer RW1 to the RNeasy spin column, and centrifuge for ■ 15 s at ≥8000 *x g* or ▲ 5 min at 3000–5000 *x g* to wash the spin column membrane. Discard the flow-through.* Reuse the collection tube in step 4.
2. Add ■ 10 µl or ▲ 20 µl DNase I stock solution (see above) to ■ 70 µl or ▲ 140 µl Buffer RDD. Mix by gently inverting 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 inverting the tube. Do not vortex.

* Flow-through contains Buffer RLT or Buffer RW1 and must not be mixed with bleach. See page 7 for "Safety Information".

3. Add the DNase I incubation mix (■ 80 µl or ▲ 160 µl) directly to the RNeasy spin column membrane, and incubate at room temperature (20–30°C) for 15 min.

   Note: Be sure to add the DNase I incubation mix directly to the RNeasy spin column membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.

4. Add ■ 350 µl or ▲ 2 ml Buffer RW1 to the RNeasy spin column, wait for 5 min, and then centrifuge for ■ 15 s at ≥8000 *x g* or ▲ 5 min at 3000–5000 *x g*. Discard the flow-through and collection tube.* Continue with step 3 of ■ Protocol 7 or ▲ Protocol 8 (i.e., the first wash with Buffer RPE).

* Flow-through contains Buffer RW1 and must not be mixed with bleach. See page 7 for "Safety Information".

Appendix C: Stabilization and Purification of Bacterial RNA using RNAprotect Bacteria Reagent and the RNeasy Lipid Tissue Mini Kit or RNeasy Plus Universal Kits

Important points before starting

- Carefully read the handbook supplied with the RNeasy Lipid Tissue Mini Kit or RNeasy Plus Universal Kits, including the safety information (see "Ordering Information", starting on page 62).
- Before growing and harvesting bacteria, read "Optimal culture conditions" (page 16).
- If preparing RNA for the first time, read "Appendix A" (page 48).
- Perform all steps of the procedure at room temperature (15–25°C) without interruption.
- Read "Determining the correct amount of starting material" and "Quantifying bacterial cells" (pages 17–18).
- Bacterial lysis in this protocol is optimized for *E. coli* (1 mg/ml lysozyme for 5 min) or *B. subtilis* (15 mg/ml lysozyme for 10 min). Since the conditions for enzymatic lysis are affected by bacterial species, cell number and culture medium, it may be necessary to adjust enzyme concentration and/or to adjust enzyme incubation time. For some bacteria, other enzymes may be more effective (e.g., we recommend lysostaphin for disrupting the cell wall of *Staphylococcus aureus*).

Things to do before starting

- Prepare TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) containing lysozyme. The lysozyme concentration should be 1 mg/ml for *E. coli* (Gram-negative) or 15 mg/ml for *B. subtilis* (Gram-positive).
- Preheat QIAzol Lysis Reagent to 65°C.

Procedure

1. Calculate the required volume of bacterial culture (1 volume).

   See “Determining the correct amount of starting material”, page 17.

2. Pipet 2 volumes of RNAprotect Bacteria Reagent into a tube (not supplied).

   For example, if the volume of bacterial culture is 500 µl, add 1000 µl RNAprotect Bacteria Reagent.

   The volume of the tube must be 4-times that of the bacterial culture (e.g., if the volume of bacterial culture is 500 µl, use a 2 ml tube).

   Optional: Entire bacterial cultures can be stabilized by adding 2 volumes of RNAprotect Bacteria Reagent to the culture.

3. Add 1 volume of bacterial culture to the tube. Mix immediately by vortexing for 5 s. Incubate for 5 min at room temperature (15–25°C).

4. Centrifuge for 10 min at 5000 *x g*.

   A pellet may not be visible after centrifugation. This is due to an interaction between the cells and the stabilization reagent that causes a change in the optical density of the cells. The procedure is not affected.

   Note: For tubes larger than 50 ml, the centrifugal force or centrifugation time may be increased. However, using excessive centrifugal force may make resuspension of the pellet difficult.

5. Decant the supernatant. Remove residual supernatant by gently dabbing the inverted tube once onto a paper towel.

   After dabbing the tube onto a paper towel, do not remove the remaining supernatant by pipetting, as this may lead to loss of the pellet. If the tube is larger than 15 ml, we recommend removing the supernatant by leaving the tube inverted on a paper towel for 10 s.

   Note: The remaining supernatant should not exceed approximately 80 µl per 100 µl TE buffer containing lysozyme used in step 6.

   Optional: Pellets can be stored at –30°C to –15°C for up to 2 weeks or at –90°C to –65°C for up to 4 weeks. For subsequent RNA purification, thaw pellets at room temperature and proceed with step 6 of the procedure.

6. Add 10–20 µl QIAGEN Proteinase K, if necessary, to 100 µl TE buffer containing lysozyme, and add the mixture to the pellet. Carefully resuspend the pellet by pipetting up and down several times.

   Bacteria grown in complex media may require treatment with Proteinase K. The amount of QIAGEN Proteinase K required depends on the bacterial species.

7. Mix by vortexing for 5 s. Incubate at room temperature for 5 min (Gram-negative bacteria) or 10 min (Gram-positive bacteria). During incubation, incubate on a shaker–incubator, or vortex for 10 s at least every 2 min.

   Note: Since the RNA is stabilized, the incubation time can be extended without causing any adverse effects, and may increase the RNA yield.

8. Add 1 ml QIAzol Lysis Reagent (preheated to 65°C), and mix by vortexing for 3 min. Incubate at room temperature for 5 min.

   Note: QIAzol Lysis Reagent must be preheated to 65°C to ensure successful bacterial lysis.

9. Add 200 µl chloroform, and mix by vigorously shaking the tube. Incubate at room temperature for 3 min.

10. Centrifuge at 12,000 *x g* for 15 min at 4°C. After centrifugation, heat the centrifuge to room temperature if it will be used in the subsequent steps of this procedure.

    There are 3 phases after centrifugation: an upper, colorless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase. 11.

11. Transfer the upper, aqueous phase (approximately 700 µl) to a new tube, add 500 µl ethanol (80%), and mix thoroughly by vortexing.

12. Continue from step 9 of the protocol in the *RNeasy Lipid Tissue Handbook* (February 2009) *or* step 11 in the *RNeasy Plus Universal Handbook* (September 2010).

    Note: All centrifugation steps must be performed at room temperature.

Appendix D: Stabilization and Purification of Bacterial RNA using RNAprotect Bacteria Reagent, the RNeasy Lipid Tissue Mini Kit or RNeasy Plus Universal Kits and the TissueLyser II

Important points before starting

- Carefully read the handbook supplied with the RNeasy Lipid Tissue Mini Kit or RNeasy Plus Universal Kits, including the safety information (see "Ordering Information", starting on page 62).
- Before growing and harvesting bacteria, read "Optimal culture conditions" (page 16).
- If preparing RNA for the first time, read "Appendix A" (page 48).
- Perform all steps of the procedure at room temperature (15–25°C) without interruption.
- Read "Determining the correct amount of starting material" and "Quantifying bacterial cells" (pages 17–18).
- Bacterial lysis in this protocol is optimized for *E. coli* (1 mg/ml lysozyme for 5 min) or *B. subtilis* (15 mg/ml lysozyme for 10 min). Since the conditions for enzymatic lysis are affected by bacterial species, cell number and culture medium, it may be necessary to adjust enzyme concentration and/or to adjust enzyme incubation time. For some bacteria, other enzymes may be more effective (e.g., we recommend lysostaphin for disrupting the cell wall of *Staphylococcus aureus*).

Things to do before starting

- Prepare TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) containing lysozyme. The lysozyme concentration should be 1 mg/ml for *E. coli* (Gram-negative) or 15 mg/ml for *B. subtilis* (Gram-positive).
- Preheat QIAzol Lysis Reagent to 65°C.

Procedure

1. For each sample, weigh 25–50 mg acid-washed glass beads (150–600 µm diameter) in a 2 ml Safe-Lock tube (not supplied), for use in step 10.
2. Calculate the required volume of bacterial culture (1 volume).

   See “Determining the correct amount of starting material”, page 17.
3. Pipet 2 volumes of RNAprotect Bacteria Reagent into a tube (not supplied).

   For example, if the volume of bacterial culture is 500 µl, add 1000 µl RNAprotect Bacteria Reagent. The volume of the tube must be 4-times that of the bacterial culture (e.g., if the volume of bacterial culture is 500 µl, use a 2 ml tube).

   Optional: Entire bacterial cultures can be stabilized by adding 2 volumes of RNAprotect Bacteria Reagent to the culture.
4. Add 1 volume of bacterial culture to the tube. Mix immediately by vortexing for 5 s. Incubate for 5 min at room temperature (15–25°C).
5. Centrifuge for 10 min at 5000 *x g*.

   A pellet may not be visible after centrifugation. This is due to an interaction between the cells and the stabilization reagent that causes a change in the optical density of the cells. The procedure is not affected.

   Note: For tubes larger than 50 ml, the centrifugal force or centrifugation time may be increased. However, using excessive centrifugal force may make resuspension of the pellet difficult.

6. Decant the supernatant. Remove residual supernatant by gently dabbing the inverted tube once onto a paper towel.

   After dabbing the tube onto a paper towel, do not remove the remaining supernatant by pipetting, as this may lead to loss of the pellet. If the tube is larger than 15 ml, we recommend removing the supernatant by leaving the tube inverted on a paper towel for 10 s.

   Note: The remaining supernatant should not exceed approximately 80 µl per 100 µl TE buffer containing lysozyme used in step 7.

   Optional: Pellets can be stored at –30°C to –15°C for up to 2 weeks or at –90°C to –65°C for up to 4 weeks. For subsequent RNA purification, thaw pellets at room temperature and proceed with step 7 of the procedure.

7. Add 10–20 µl QIAGEN Proteinase K, if necessary, to 100 µl TE buffer containing lysozyme, and add the mixture to the pellet. Carefully resuspend the pellet by pipetting up and down several times.

   Bacteria grown in complex media may require treatment with Proteinase K. The amount of QIAGEN Proteinase K required depends on the bacterial species.

8. Mix by vortexing for 5 s. Incubate at room temperature for 5 min (Gram-negative bacteria) or 10 min (Gram-positive bacteria). During incubation, incubate on a shaker–incubator, or vortex for 10 s at least every 2 min.

   Note: Since the RNA is stabilized, the incubation time can be extended without causing any adverse effects, and may increase the RNA yield.

9. Add 1 ml QIAzol Lysis Reagent (preheated to 65°C), and mix by vortexing for 3 min. Incubate at room temperature for 5 min.

   Note: QIAzol Lysis Reagent must be preheated to 65°C to ensure successful bacterial lysis.

10. Transfer the sample into the 2 ml Safe-Lock tube containing the acid-washed glass beads prepared in step 1. Disrupt the cells in the TissueLyser II for 5 min at 30 Hz.

    Other methods of mechanical disruption can be used instead. We recommend using the TissueLyser II.

11. Add 200 µl chloroform, and mix by vigorously shaking the tube. Incubate at room temperature for 3 min.
12. Centrifuge at 12,000 *x g* for 15 min at 4°C. After centrifugation, heat the centrifuge to room temperature if it will be used in the subsequent steps of this procedure.

    There are 3 phases after centrifugation: an upper, colorless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase.
13. Transfer the upper, aqueous phase (approximately 700 µl) to a new tube, add 500 µl ethanol (80%), and mix thoroughly by vortexing.
14. Continue from step 9 of the protocol in the *RNeasy Lipid Tissue Handbook* (February 2009) or step 11 in the *RNeasy Plus Universal Handbook* (September 2010).

    Note: All centrifugation steps must be performed at room temperature.

Ordering Information

Product	Contents	Cat. no.
RNAprotect Bacteria Reagent	RNAprotect Bacteria Reagent (2 x 100 ml)	76506
RNeasy Protect Bacteria Mini Kit (50)*	RNeasy Mini Kit (50) and RNAprotect Bacteria Reagent (2 x 100 ml)	74524
RNeasy Plus Universal Mini Kit (50)	RNeasy Mini Spin Columns, gDNA Eliminator Solution, Collection Tubes, RNase-Free Water and Buffers	73404
RNeasy Plus Universal Midi Kit (10)	RNeasy Midi Spin Columns, gDNA Eliminator Solution, Collection Tubes, RNase-Free Water and Buffers	73442
Accessories		
Buffer RLT (220 ml)	220 ml Buffer RLT	79216
QIAGEN Proteinase K (2 ml)	2 ml (>600 mAU/ml, solution)	19131
QIAGEN Proteinase K (10 ml)	10 ml (>600 mAU/ml, solution)	19133
TissueLyser II	Universal laboratory mixer-mill disruptor	Inquire
TissueLyser Adapter Set 2 x 24	2 sets of Adapter Plates and 2 racks for use with 2 ml microcentrifuge tubes on the TissueLyser II	69982
RNase-Free DNase Set (50)	For 50 RNA minipreps, 25 midipreps or 17 maxipreps: 1500 units RNase-Free DNase I, RNase-Free Buffer RDD and RNase-Free Water	79254
RNeasy Lipid Tissue Mini Kit (50)	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), QIAzol Lysis Reagent, RNase-Free Reagents and Buffers	74804
QIAzol Lysis Reagent (200 ml)	200 ml QIAzol Lysis Reagent	79306

Product	Contents	Cat. no.
QIAcube Connect — for fully automated nucleic acid extraction with QIAGEN spin-column kits		
QIAcube Connect†	Instrument, connectivity package, 1-year warranty on parts and labor	Inquire
Starter Pack, QIAcube	Filter-tips, 200 µl (1024), 1000 µl filter-tips (1024), 30 ml reagent bottles (12), rotor adapters (240), elution tubes (240), rotor adapter holder	990395
Related products		
QIAGEN OneStep RT-PCR Kit — for fast and successful one-step RT-PCR		
QIAGEN OneStep RT-PCR Kit (25)‡	For 25 x 50 µl reactions: QIAGEN OneStep RT-PCR Enzyme Mix, 5x QIAGEN OneStep PCR Buffer (contains 12.5 mM $MgCl_2$), dNTP Mix (contains 10 mM each dNTP), 5x Q-Solution, RNase-Free Water	210210
Omniscript® RT Kit — for efficient and sensitive reverse transcription using 50 ng to 2 µg RNA per reaction		
Omniscript RT Kit (50)‡	For 50 x 20 µl reactions: Omniscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix (contains 5 mM each dNTP), RNase-Free Water	205111
Sensiscript® RT Kit — for efficient and sensitive reverse transcription using less than 50 ng RNA per reaction		
Sensiscript RT Kit (50)‡	For 50 x 20 µl reactions: Sensiscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix (contains 5 mM each dNTP), RNase-Free Water	205211
QuantiTect Reverse Transcription Kit — for fast cDNA synthesis for sensitive real-time two-step RT-PCR		
QuantiTect Rev. Transcription Kit (50)	For 50 x 20 µl reactions: gDNA Wipeout Buffer, Quantiscript® Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix and RNase-Free Water	205311

Product	Contents	Cat. no.
QuantiTect SYBR® Green PCR Kit — for quantitative, real-time PCR and two-step RT-PCR using SYBR Green I		
QuantiTect SYBR Green PCR Kit (200)‡	For 200 x 50 µl reactions: 3 x 1.7 ml 2x QuantiTect SYBR Green PCR Master Mix (providing a final concentration of 2.5 mM $MgCl_2$), 2 x 2 ml RNase-Free Water	204143
QuantiTect SYBR Green RT-PCR Kit — for quantitative, real-time one-step RT-PCR using SYBR Green I		
QuantiTect SYBR Green RT-PCR Kit (200)‡	For 200 x 50 µl reactions: 3 x 1.7 ml 2x QuantiTect SYBR Green RT-PCR Master Mix (providing a final concentration of 2.5 mM $MgCl_2$), 100 µl QuantiTect RT Mix, 2 x 2 ml RNase-Free Water	204243
QuantiTect Probe PCR Kit — for quantitative, real-time PCR and two-step RT-PCR using sequence-specific probes		
QuantiTect Probe PCR Kit (200)‡§	For 200 x 50 µl reactions: 3 x 1.7 ml 2x QuantiTect Probe PCR Master Mix (providing a final concentration of 4 mM $MgCl_2$), 2 x 2 ml RNase-Free Water	204343
QuantiTect Probe RT-PCR Kit — for quantitative, real-time one-step RT-PCR using sequence-specific probes		
QuantiTect Probe RT-PCR Kit (200)‡§	For 200 x 50 µl reactions: 3 x 1.7 ml 2x QuantiTect Probe RT-PCR Master Mix (providing a final concentration of 4 mM $MgCl_2$), 100 µl QuantiTect RT Mix, 2 x 2 ml RNase-Free Water	204443
QuantiTect Multiplex PCR Kits — for quantitative, multiplex, real-time PCR and two-step RT-PCR using sequence-specific probes		
QuantiTect Multiplex PCR Kit (200)‡¶	For 200 x 50 µl reactions: 3 x 1.7 ml 2x QuantiTect Multiplex PCR Master Mix (with ROX dye), 2 x 2 ml RNase-Free Water	204543

Product	Contents	Cat. no.
QuantiTect Multiplex PCR NoROX Kit (200)*	For 200 x 50 µl reactions: 3 x 1.7 ml 2x QuantiTect Multiplex PCR NoROX Master Mix (without ROX dye), 2 x 2 ml RNase-Free Water	204743
QuantiTect Multiplex RT-PCR Kits — for quantitative, multiplex, real-time, one-step RT-PCR using sequence-specific probes		
QuantiTect Multiplex RT-PCR Kit (200)‡§	For 200 x 50 µl reactions: 3 x 1.7 ml 2x QuantiTect Multiplex RT-PCR Master Mix (with ROX dye), 100 µl QuantiTect Multiplex RT Mix, 2 x 2 ml RNase-Free Water	204643
QuantiTect Multiplex RT-PCR NR Kit (200)‡§	For 200 x 50 µl reactions: 3 x 1.7 ml 2x QuantiTect Multiplex RT-PCR NoROX Master Mix (without ROX dye), 100 µl QuantiTect Multiplex RT Mix, 2 x 2 ml RNase-Free Water	204843

* QIAGEN offers a wide range of RNA purification kits for different sample types, sizes and throughputs. Robotic workstations for automated RNA purification are also available. For details, visit www.qiagen.com or contact your local QIAGEN office.

† All QIAcube Connect instruments are provided with a region-specific connectivity package, including tablet and equipment necessary to connect to the local network. Further, QIAGEN offers comprehensive instrument service products, including service agreements, installation, introductory training and preventive subscription. Contact your local sales representative to learn about your options.

‡ Larger kit sizes available; for details, visit www.qiagen.com.

§ Visit www.qiagen.com/goto/assays to design and order QuantiTect Custom Assays (custom designed primer–probe sets).

¶ Recommended for use with ABI PRISM® and Applied Biosystems® cyclers.

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Document Revision History

Date	Changes
January 2020	Updated text, ordering information and intended use for QIAcube Connect.