

# RNAprotect<sup>®</sup> Bacteria Reagent Handbook

RNAprotect Bacteria Reagent  
For in vivo stabilization of total RNA  
in bacteria

RNeasy<sup>®</sup> Protect Bacteria Mini Kit  
RNeasy Protect Bacteria Midi Kit  
For in vivo stabilization of total RNA  
in bacteria and subsequent RNA  
purification



Trademarks: QIAGEN®, Omniscript®, Quantiscript®, QuantiTect®, RNAprotect®, RNeasy®, Sensiscript® (QIAGEN Group); ABI PRISM®, Applied Biosystems® (Applied Biosystems Corporation or its subsidiaries); DU® (Beckman Instruments, Inc.); NASBA® (Organon Teknica); SYBR® (Molecular Probes, Inc.).

Purchase of QIAGEN products for PCR containing HotStarTaq DNA Polymerase is accompanied by a limited license to use them in the polymerase chain reaction (PCR) process for research and development activities in conjunction with a thermal cycler whose use in the automated performance of the PCR process is covered by the up-front license fee, either by payment to Applied Biosystems or as purchased, i.e. an authorized thermal cycler. The PCR process is covered by the foreign counterparts of U. S. Patents Nos. 4,683,202 and 4,683,195 owned by F. Hoffmann-La Roche Ltd.

The 5' nuclease process is covered by patents owned by Roche Molecular Systems, Inc. and F. Hoffmann-La Roche Ltd.

Patents of third parties in certain countries may cover the process of multiplex PCR or of certain applications.

QIAzol Lysis Reagent is a subject of US Patent No. 5,346,994 and foreign equivalents.

QuantiTect Custom Assays or portions hereof are subject to proprietary rights of Nanogen, Inc. and are made and sold under license from Nanogen under the patents and patent applications as may be designated by Nanogen from time to time set forth, including one or more of the following: U. S. Patent Nos. 5,801,155, 6,084,102, 6,312,894, 6,426,408, and 6,127,121, and applications currently pending. Purchase of this product carries with it a limited, non-transferable, non-exclusive (without the right to resell, repackage, or sublicense) license under U. S. Patent Nos. 6,030,787; 5,723,591; and 5,876,930, and corresponding foreign patents.

© 2001–2005 QIAGEN, all rights reserved.

# Contents

<b>Kit Contents</b>	<b>5</b>
<b>Storage</b>	<b>5</b>
<b>Quality Control</b>	<b>5</b>
<b>Product Use Limitations</b>	<b>6</b>
<b>Product Warranty and Satisfaction Guarantee</b>	<b>6</b>
<b>Technical Assistance</b>	<b>6</b>
<b>Safety Information</b>	<b>7</b>
<b>Introduction</b>	<b>8</b>
Principle and procedure	8
Description of protocols	9
<b>Equipment and Reagents to Be Supplied by User</b>	<b>12</b>
<b>Important Notes</b>	<b>13</b>
Optimal culture conditions	13
Determining the correct amount of starting material	13
Quantifying bacterial cells	14
Handling and storing starting material	15
DNase digestion	15
<b>Protocols</b>	
■ <b>1: Enzymatic Lysis of Bacteria</b>	<b>16</b>
■ <b>2: Enzymatic Lysis and Mechanical Disruption of Bacteria</b>	<b>19</b>
■ <b>3: Mechanical Disruption of Bacteria</b>	<b>22</b>
■ <b>4: Enzymatic Lysis and Proteinase K Digestion of Bacteria</b>	<b>25</b>
■ <b>5: Enzymatic Lysis, Proteinase K Digestion, and Mechanical Disruption of Bacteria</b>	<b>28</b>
■ <b>6: Disruption of Bacteria Grown on Solid Media</b>	<b>31</b>
■ <b>7: Purification of Total RNA from Bacterial Lysate Using the RNeasy Mini Kit</b>	<b>32</b>
■ <b>8: Purification of Total RNA from Bacterial Lysate Using the RNeasy Midi Kit</b>	<b>34</b>
<b>Troubleshooting Guide</b>	<b>36</b>

<b>Appendix A: General Remarks on Handling RNA</b>	<b>38</b>
<b>Appendix B: Optional On-Column DNase Digestion Using the RNase-Free DNase Set</b>	<b>40</b>
<b>Appendix C: Stabilization and Purification of Bacterial RNA Using RNAprotect Bacteria Reagent and the RNeasy Lipid Tissue Mini Kit</b>	<b>41</b>
<b>Appendix D: Stabilization and Purification of Bacterial RNA Using RNAprotect Bacteria Reagent, the RNeasy Lipid Tissue Mini Kit, and the TissueLyser</b>	<b>44</b>
<b>References</b>	<b>46</b>
<b>Ordering Information</b>	<b>47</b>
<b>QIAGEN Distributors</b>	<b>51</b>

## Kit Contents

<b>RNAprotect Bacteria Reagent</b>	
<b>Catalog no.</b>	<b>76506</b>
<b>Volume of bacterial culture to stabilize</b>	<b>100 ml</b>
RNAprotect Bacteria Reagent	2 x 100 ml
RNAprotect Bacteria Reagent Handbook	1

<b>RNeasy Protect Bacteria Kits</b>	<b>Mini (50)</b>	<b>Midi (10)</b>
<b>Catalog no.</b>	<b>74524</b>	<b>75552</b>
<b>Number of preps</b>	<b>50</b>	<b>10</b>
RNeasy Mini Kit (50)	1	–
RNeasy Midi Kit (10)	–	1
RNAprotect Bacteria Reagent	2 x 100 ml	2 x 100 ml
RNeasy Mini Handbook	1	–
RNeasy Midi/Maxi Handbook	–	1
RNAprotect Bacteria Reagent Handbook	1	1

## Storage

Store RNAprotect Bacteria Reagent at room temperature (15–25°C). Under these conditions, the reagent is stable for at least 12 months. 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.

## 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.

## Product Use Limitations

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.

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.

## Product Warranty and Satisfaction Guarantee

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

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

## Technical Assistance

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

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques. For technical assistance and more information please call one of the QIAGEN Technical Service Departments or local distributors (see back cover).

## Safety Information

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

The following risk and safety phrases apply to RNAprotect Bacteria Reagent.

### RNAprotect Bacteria Reagent

Contains tetradecyltrimethylammonium bromide: irritant. Risk and safety phrases:\*  
R36/38, S13-26-36-46

### 24-hour emergency information

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

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

\* R36/38: Irritating to eyes and skin; S13: Keep away from food, drink, and animal feedingstuffs; S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice; S36: Wear suitable protective clothing; S46: If swallowed, seek medical advice immediately and show this container or label.

## 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 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, 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. Page 10 describes the different protocols for preparing lysates of bacterial cells, while 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 are 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 are 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 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 RNeasy Protect Bacteria Reagent in combination with the RNeasy Lipid Tissue Mini Kit (supplied with phenol-guanidine-based QIAzol Lysis Reagent). Appendix C (page 41) 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. Appendix D (page 44) is intended for use with certain Gram-positive bacteria, and describes enzymatic lysis, proteinase K digestion, and mechanical disruption of bacteria in hot QIAzol Reagent followed by RNA purification.

## Equipment and Reagents to Be Supplied by User

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

### For all protocols

- 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, page 47)

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

- TissueLyser system (see ordering information, page 47)
- Glass beads (e.g., Sigma, cat. no. G1145, G1277, or G8772)\*
- 2 ml Safe-Lock tubes (Eppendorf, cat. no. 0030 120.094)\*

### For protocols requiring RNeasy Kits (Protocols 7 and 8)

- 14.3 M  $\beta$ -mercaptoethanol ( $\beta$ -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, page 47)
- Ethanol (96–100%), ethanol (80%), or ethanol (70%)<sup>†</sup>
- Optional: RNase-Free DNase Set (see ordering information, page 47)

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

<sup>†</sup> 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  $7.5 \times 10^8$  cells per RNeasy Mini spin column or  $5 \times 10^8 - 7.5 \times 10^9$  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:

<i>E. coli</i> grown in minimal medium	RNA yield approximately 40 µg per $7.5 \times 10^8$ cells. Up to $7.5 \times 10^8$ cells can be used (use of higher numbers of cells results in inefficient lysis and reduced yield).
<i>E. coli</i> grown in LB medium	RNA yield approximately 120 µg per $7.5 \times 10^8$ cells. Up to $6 \times 10^8$ 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 <sup>†</sup>	No. cells	RNA yield (µg) <sup>‡</sup>
<i>E. coli</i>	Minimal medium	$5 \times 10^8$	25
<i>E. coli</i>	LB	$5 \times 10^8$	70
<i>B. subtilis</i>	Minimal medium	$1 \times 10^8$	8
<i>B. subtilis</i>	LB	$1 \times 10^8$	15

\* Bacterial cells disrupted according to Protocol 1.

<sup>†</sup> We recommend minimal media for growing bacteria.

<sup>‡</sup> 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, rRNA, 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  $2 \times 10^8$  cells per RNeasy Mini spin column or  $2 \times 10^9$  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  $1 \times 10^9$  cells/ml is diluted 1:4, and gives OD<sub>600</sub> 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  $1 \times 10^9$  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  $-20^{\circ}\text{C}$  for up to 2 weeks, or at  $-70^{\circ}\text{C}$  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 13).
- If preparing RNA for the first time, read Appendix A (page 38).
- 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 13–14). 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 13.
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 –20°C for up to 2 weeks or at –70°C for up to 4 weeks. For subsequent RNA purification, thaw pellets at room temperature (15–25°C) and proceed with step 7 of the procedure.

7. **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)
$<5 \times 10^8$	Mini	100 µl	350 µl	250 µl
$5 \times 10^8 - 7.5 \times 10^8$	Mini	200 µl	700 µl	500 µl
$5 \times 10^8 - <1 \times 10^9$	Midi	500 µl	2000 µl	1400 µl
$1 \times 10^9 - 7.5 \times 10^9$	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 13.

- 8. Mix by vortexing for 10 s. Incubate at room temperature (15–25°C) 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.

- 9. 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 × *g*.

**Note:** Ensure that β-mercaptoethanol is added to Buffer RLT before use (see “Important points before starting”, page 16).

- 10. 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.

- 11. 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 13).
- If preparing RNA for the first time, read Appendix A (page 38).
- 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 13–14). 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  $\mu$ l  $\beta$ -mercaptoethanol per 1 ml Buffer RLT, and mix. Buffer RLT is stable for 1 month after addition of  $\beta$ -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  $\mu$ m 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 13.

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

For example, if the volume of bacterial culture is 500  $\mu$ l, add 1000  $\mu$ l 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  $\mu$ l, 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  $\mu$ l per 100  $\mu$ l TE buffer containing lysozyme used in step 8.

**Optional:** Pellets can be stored at –20°C for up to 2 weeks or at –70°C for up to 4 weeks. For subsequent RNA purification, thaw pellets at room temperature (15–25°C) 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 (15–25°C) 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  $\beta$ -mercaptoethanol is added to Buffer RLT before use (see “Important points before starting”, page 19).

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)	Ethanol (70%) (step 13)
$<5 \times 10^8$	Mini	100 $\mu$ l	350 $\mu$ l	–	220 $\mu$ l	–
$5 \times 10^8$ – $7.5 \times 10^8$	Mini	200 $\mu$ l	700 $\mu$ l	–	470 $\mu$ l	–
$5 \times 10^8$ – $7.5 \times 10^9$	Midi	200 $\mu$ l	700 $\mu$ l	3200 $\mu$ l	–	4000 $\mu$ l

\* 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 13.

- 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 for 5 min at maximum speed.

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

- Centrifuge for 10 s at maximum speed. Transfer supernatant (400  $\mu$ l from starting material  $\leq 5 \times 10^8$  cells or 850  $\mu$ l from starting material  $\geq 5 \times 10^8$  cells) into a new tube (not supplied). If using the RNeasy Midi Kit, add 3200  $\mu$ l Buffer RLT to the supernatant, and mix thoroughly by shaking or vortexing vigorously for 5–10 s.

**Note:** Ensure that  $\beta$ -mercaptoethanol is added to Buffer RLT before use (see “Important points before starting”, page 19).

- 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.

- 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 13).
- If preparing RNA for the first time, read Appendix A (page 38).
- 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 13–14). 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  $\mu$ l  $\beta$ -mercaptoethanol per 1 ml Buffer RLT, and mix. Buffer RLT is stable for 1 month after addition of  $\beta$ -mercaptoethanol.

### Procedure

- 1. For each sample, weigh 25–50 mg acid-washed glass beads (150–600  $\mu$ m 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 13.
- 3. Pipet 2 volumes of RNAprotect Bacteria Reagent into a tube.**  
For example, if the volume of bacterial culture is 500  $\mu$ l, add 1000  $\mu$ l 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  $\mu$ l, 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  $\mu$ l per 350  $\mu$ l Buffer RLT used in step 7.

**Optional:** Pellets can be stored at  $-20^{\circ}\text{C}$  for up to 2 weeks or at  $-70^{\circ}\text{C}$  for up to 4 weeks. For subsequent RNA purification, thaw pellets at room temperature ( $15$ – $25^{\circ}\text{C}$ ) 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  $\beta$ -mercaptoethanol is added to Buffer RLT before use (see "Important points before starting", page 22).

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 ( $\mu$ l)
$<5 \times 10^8$	350
$5 \times 10^8 - 1 \times 10^9$	700
$>1 \times 10^9$	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 13.

- 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 for 5 min at maximum speed.**

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

- 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 10 ml.

**Note:** Ensure that  $\beta$ -mercaptoethanol is added to Buffer RLT before use (see "Important points before starting", page 22).

- 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 13).
- If preparing RNA for the first time, read Appendix A (page 38).
- 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 13–14). 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 13.

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

For example, if the volume of bacterial culture is 500  $\mu$ l, add 1000  $\mu$ l 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  $\mu$ l, 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  $\mu$ l per 100  $\mu$ l TE buffer containing lysozyme used in step 7.

**Optional:** Pellets can be stored at –20°C for up to 2 weeks or at –70°C for up to 4 weeks. For subsequent RNA purification, thaw pellets at room temperature (15–25°C) and proceed with step 7 of the procedure.

**7. Add 10–20  $\mu$ l 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  $\mu$ l 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)
$<1 \times 10^8$	Mini	100 $\mu$ l	350 $\mu$ l	250 $\mu$ l	–
$1 \times 10^8 - 2.5 \times 10^8$	Mini	200 $\mu$ l	700 $\mu$ l	500 $\mu$ l	–
$<2.5 \times 10^8 - <1.5 \times 10^9$	Midi	200 $\mu$ l	2000 $\mu$ l	–	1750 $\mu$ l
$7.5 \times 10^8 - 1.5 \times 10^9$	Midi	200 $\mu$ l	4000 $\mu$ l	–	3500 $\mu$ l

\* 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 13.

- 8. Mix by vortexing for 10 s. Incubate at room temperature (15–25°C) 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  $\times g$ .

**Note:** Ensure that  $\beta$ -mercaptoethanol is added to Buffer RLT before use (see “Important points before starting”, page 25).

- 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 13).
- If preparing RNA for the first time, read Appendix A (page 38).
- 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 13–14). 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  $\mu$ l  $\beta$ -mercaptoethanol per 1 ml Buffer RLT, and mix. Buffer RLT is stable for 1 month after addition of  $\beta$ -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  $\mu$ m 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 13.

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

For example, if the volume of bacterial culture is 500  $\mu$ l, add 1000  $\mu$ l 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  $\mu$ l, 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  $\mu$ l per 100  $\mu$ l TE buffer containing lysozyme used in step 8.

**Optional:** Pellets can be stored at –20°C for up to 2 weeks or at –70°C for up to 4 weeks. For subsequent RNA purification, thaw pellets at room temperature (15–25°C) and proceed with step 8 of the procedure.

**8. Add 10–20  $\mu$ l 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)
$<7.5 \times 10^8$	Mini	100 $\mu$ l	700 $\mu$ l	590 $\mu$ l
$5 \times 10^8 - 1.5 \times 10^9$	Midi	200 $\mu$ l	1500 $\mu$ l	1300 $\mu$ l

\* 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 13.

- Mix by vortexing for 10 s. Incubate at room temperature (15–25°C) 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.

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

**Note:** Ensure that  $\beta$ -mercaptoethanol is added to Buffer RLT before use (see "Important points before starting", page 28).

Ensure that the pellet is thoroughly resuspended in Buffer RLT.

- 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 for 5 min at maximum speed.**

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

- Centrifuge for 10 s at maximum speed. Transfer supernatant (760  $\mu$ l for the RNeasy Mini procedure, or 1670  $\mu$ l for the RNeasy Midi procedure) into a new tube (not supplied).**

- 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.

- 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 38).
- 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 13–14). 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  $\mu$ l 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  $\geq 8000 \times g$  ( $\geq 10,000$  rpm). Discard the flow-through.\* Reuse the collection tube in step 2.**

If the lysate exceeds 700  $\mu$ l, 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 40) after performing this step.

2. **Add 700  $\mu$ l Buffer RW1 to the RNeasy Mini spin column. Close the lid gently, and centrifuge for 15 s at  $\geq 8000 \times g$  ( $\geq 10,000$  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 40).

\* Flow-through contains Buffer RLT or Buffer RW1 and must not be mixed with bleach. See page 7 for safety information.

3. Place the RNeasy Mini spin column in a new 2 ml collection tube (supplied). Add 500  $\mu$ l Buffer RPE to the RNeasy Mini spin column. Close the lid gently, and centrifuge for 15 s at  $\geq 8000 \times g$  ( $\geq 10,000$  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 32).

4. Add 500  $\mu$ l Buffer RPE to the RNeasy Mini spin column. Close the lid gently, and centrifuge for 2 min at  $\geq 8000 \times g$  ( $\geq 10,000$  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  $\mu$ l RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to elute the RNA.
6. If the expected RNA yield is  $>30 \mu\text{g}$ , repeat step 5 using another 30–50  $\mu$ l 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 40) after performing this step.

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 40).

\* Flow-through contains Buffer RLT or Buffer RW1 and must not be mixed with bleach. See page 7 for safety information.

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 34).

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.

5. 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

6. 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. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or molecular biology applications (see back cover for contact information).

### Comments and suggestions

---

#### 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 $\times g$ . |
| b) | Incomplete removal of solution after centrifugation of RNeasy Protect Bacteria Reagent and bacterial culture | For subsequent preparations, remove supernatant (i.e., combined RNeasy Protect 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 13).  |

#### Clogged RNeasy spin column

- |    |  |  |
|----|--|--|
| a) | Incomplete removal of solution after centrifugation of RNeasy Protect Bacteria Reagent and bacterial culture | For subsequent preparations, remove supernatant (i.e., combined RNeasy Protect 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 13).  |

### 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 13).
- 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. In order to create and maintain an RNase-free environment, the following precautions must be taken during pretreatment and use of disposable and nondisposable vessels and solutions while working with RNA.

## General handling

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

## Disposable plasticware

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

## Nondisposable plasticware

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

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

## Glassware

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

## Electrophoresis tanks

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

## Solutions

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

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

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

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

## 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  $\mu$ l 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  $-20^{\circ}\text{C}$  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.

## 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 B1–B4 below.

**B1.** Add ■ 350  $\mu$ l or ▲ 2 ml Buffer RW1 to the RNeasy spin column, and centrifuge for ■ 15 s at  $\geq 8000 \times g$  or ▲ 5 min at 3000–5000  $\times g$  to wash the spin column membrane. Discard the flow-through.\* Reuse the collection tube in step B4.

**B2.** Add ■ 10  $\mu$ l or ▲ 20  $\mu$ l DNase I stock solution (see above) to ■ 70  $\mu$ l or ▲ 140  $\mu$ l 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.

**B3.** Add the DNase I incubation mix (■ 80  $\mu$ l or ▲ 160  $\mu$ l) 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.

**B4.** Add ■ 350  $\mu$ l or ▲ 2 ml Buffer RW1 to the RNeasy spin column, wait for 5 min, and then centrifuge for ■ 15 s at  $\geq 8000 \times g$  or ▲ 5 min at 3000–5000  $\times g$ . 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).

## Appendix C: Stabilization and Purification of Bacterial RNA Using RNAprotect Bacteria Reagent and the RNeasy Lipid Tissue Mini Kit

### Important points before starting

- Carefully read the handbook supplied with the RNeasy Lipid Tissue Mini Kit, including the safety information (see ordering information, page 47).
- Before growing and harvesting bacteria, read “Optimal culture conditions” (page 13).
- If preparing RNA for the first time, read Appendix A (page 38).

\* Flow-through contains Buffer RW1 and must not be mixed with bleach. See page 7 for safety information.

- 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 13–14).
- 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 13.

#### 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  $\mu$ l per 100  $\mu$ l TE buffer containing lysozyme used in step 6.

**Optional:** Pellets can be stored at  $-20^{\circ}\text{C}$  for up to 2 weeks or at  $-70^{\circ}\text{C}$  for up to 4 weeks. For subsequent RNA purification, thaw pellets at room temperature ( $15$ – $25^{\circ}\text{C}$ ) and proceed with step 6 of the procedure.

- 6. Add 10–20  $\mu$ l QIAGEN Proteinase K, if necessary, to 100  $\mu$ l 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 ( $15$ – $25^{\circ}\text{C}$ ) 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  $65^{\circ}\text{C}$ ), and mix by vortexing for 3 min. Incubate at room temperature ( $15$ – $25^{\circ}\text{C}$ ) for 5 min.**

**Note:** QIAzol Lysis Reagent must be preheated to  $65^{\circ}\text{C}$  to ensure successful bacterial lysis.

- 9. Add 200  $\mu$ l chloroform, and mix by vigorously shaking the tube. Incubate at room temperature ( $15$ – $25^{\circ}\text{C}$ ) for 3 min.**

- 10. Centrifuge at  $12,000 \times g$  for 15 min at  $4^{\circ}\text{C}$ . After centrifugation, heat the centrifuge to room temperature ( $15$ – $25^{\circ}\text{C}$ ) 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. Transfer the upper, aqueous phase (approx. 700  $\mu$ l) to a new tube, and add 500  $\mu$ l ethanol (80%).**

- 12. Continue from step 10 of the protocol in the *RNeasy Lipid Tissue Mini Handbook* (January 2003).**

**Note:** All centrifugation steps must be performed at room temperature ( $15$ – $25^{\circ}\text{C}$ ).

# Appendix D: Stabilization and Purification of Bacterial RNA Using RNAprotect Bacteria Reagent, the RNeasy Lipid Tissue Mini Kit, and the TissueLyser

## Important points before starting

- Carefully read the handbook supplied with the RNeasy Lipid Tissue Mini Kit, including the safety information (see ordering information, page 47).
- Before growing and harvesting bacteria, read “Optimal culture conditions” (page 13).
- If preparing RNA for the first time, read Appendix A (page 38).
- 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 13–14).
- 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 13.
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 –20°C for up to 2 weeks or at –70°C for up to 4 weeks. For subsequent RNA purification, thaw pellets at room temperature (15–25°C) 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 (15–25°C) 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 (15–25°C) 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 for 5 min at 30 Hz.**

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

11. **Add 200  $\mu$ l chloroform, and mix by vigorously shaking the tube. Incubate at room temperature (15–25°C) for 3 min.**
12. **Centrifuge at 12,000  $\times$  g for 15 min at 4°C. After centrifugation, heat the centrifuge to room temperature (15–25°C) 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 (approx. 700  $\mu$ l) to a new tube, and add 500  $\mu$ l ethanol (80%).**
14. **Continue from step 10 of the protocol in the *RNeasy Lipid Tissue Mini Handbook* (January 2003).**

**Note:** All centrifugation steps must be performed at room temperature (15–25°C).

## References

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

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

## 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 Protect Bacteria Midi Kit (10)*	RNeasy Midi Kit (10) and RNAprotect Bacteria Reagent (2 x 100 ml)	75552
<b>Accessories</b>		
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	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	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

\* 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](http://www.qiagen.com) or contact your local QIAGEN office.

## Ordering Information

Product	Contents	Cat. no.
<b>Related products</b>		
<b>QIAGEN OneStep RT-PCR Kit — for fast and successful one-step RT-PCR</b>		
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 <sub>2</sub> ), dNTP Mix (contains 10 mM each dNTP), 5x Q-Solution, RNase-Free Water	210210
<b>Omniscript® RT Kit — for efficient and sensitive reverse transcription using 50 ng to 2 µg RNA per reaction</b>		
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
<b>Sensiscript® RT Kit — for efficient and sensitive reverse transcription using less than 50 ng RNA per reaction</b>		
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
<b>QuantiTect Reverse Transcription Kit — for fast cDNA synthesis for sensitive real-time two-step RT-PCR</b>		
QuantiTect Reverse 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
<b>QuantiTect SYBR® Green PCR Kit — for quantitative, real-time PCR and two-step RT-PCR using SYBR Green I</b>		
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 <sub>2</sub> ), 2 x 2 ml RNase-Free Water	204143

\* Larger kit sizes available; for details, visit [www.qiagen.com](http://www.qiagen.com).

# Ordering Information

Product	Contents	Cat. no.
<b>QuantiTect SYBR Green RT-PCR Kit — for quantitative, real-time one-step RT-PCR using SYBR Green I</b>		
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 <sub>2</sub> ), 100 µl QuantiTect RT Mix, 2 x 2 ml RNase-Free Water	204243
<b>QuantiTect Probe PCR Kit — for quantitative, real-time PCR and two-step RT-PCR using sequence-specific probes</b>		
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 <sub>2</sub> ), 2 x 2 ml RNase-Free Water	204343
<b>QuantiTect Probe RT-PCR Kit — for quantitative, real-time one-step RT-PCR using sequence-specific probes</b>		
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 <sub>2</sub> ), 100 µl QuantiTect RT Mix, 2 x 2 ml RNase-Free Water	204443
<b>QuantiTect Multiplex PCR Kits — for quantitative, multiplex, real-time PCR and two-step RT-PCR using sequence-specific probes</b>		
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
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

\* Larger kit sizes available; for details, visit [www.qiagen.com](http://www.qiagen.com).

† Visit [www.qiagen.com/goto/assays](http://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.

§ Recommended for use with all other cyclers.

## Ordering Information

Product	Contents	Cat. no.
<b>QuantiTect Multiplex RT-PCR Kits — for quantitative, multiplex, real-time, one-step RT-PCR using sequence-specific probes</b>		
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

\* Larger kit sizes available; for details, visit [www.qiagen.com](http://www.qiagen.com).

† Recommended for use with ABI PRISM and Applied Biosystems cyclers.

‡ Recommended for use with all other cyclers.

# QIAGEN Companies

Please see the back cover for contact information for your local QIAGEN office.

## QIAGEN Distributors

### Argentina

Tecnolab S. A.  
Tel: (011) 4555 0010  
Fax: (011) 4553 3331  
E-mail: info@tecnolab.com.ar  
Web site: www.tecnolab.com.ar

### Brazil

Uniscience do Brasil  
Tel: 011 3622 2320  
Fax: 011 3622 2323  
E-mail: info@uniscience.com  
Web site: www.uniscience.com

### Chile

Biosonda SA  
Tel: 562 209 6770  
Fax: 562 274 5462  
E-mail: ventas@biosonda.cl  
Web site: www.biosonda.cl

### China

Gene Company Limited  
Tel: (852) 28 96-6283  
Fax: (852) 25 15-9371  
E-mail:  
Hang Kong: info@genehk.com  
Beijing: info\_bj@genecompany.com  
Shanghai: info\_sh@genecompany.com  
Chengdu: gene@public.cd.sc.cn  
Guangzhou: info\_gz@genecompany.com

### Croatia

INEL Medicinska Tehnika d.o.o.  
Tel: (01) 2984-898  
Fax: (01) 6520-966  
E-mail: inel-medicinska-tehnika@zg.htnet.hr

### Cyprus

Scientronics Ltd  
Tel: 02-357 22 765416  
Fax: 02-357 22 764614  
E-mail: a.sarpetsas@biotronics.com.cy

### Czech Republic

BIO-CONSULT spol. s.r.o.  
Tel/Fax: (420) 2 417 29 792  
E-mail: info@bioconsult.cz  
Web site: www.bioconsult.cz

### Egypt

Clinilab  
Tel: 52 57 212  
Fax: 52 57 210  
E-mail: Clinilab@link.net

### Estonia

PEAL-Est OÜ  
Tel: (051) 65 830  
Fax: (07) 383 360  
E-mail: langel@ut.ee

### Greece

BioAnalytica S. A.  
Tel: (210)-640 03 18  
Fax: (210)-646 27 48  
E-mail: bioanalyt@hol.gr  
Web site: www.bioanalytica.gr

### Hungary

Kasztel-Med Co. Ltd.  
Tel: (01) 385 3887  
Fax: (01) 381 0695  
E-mail: info@kasztel.hu  
Web site: www.kasztel.hu

### India

Genetix  
Tel: (011)-2542 1714  
or (011)-2515 9346  
Fax: (011)-2546 7637  
E-mail: genetix@nda.vsnl.net.in

### Israel

Westburg (Israel) Ltd.  
Tel: 08-6900655  
or 1-800 20 22 20 (toll free)  
Fax: 08-6900650  
E-mail: info@westburg.co.il  
Web site: www.westburg.co.il

### Korea

LRS Laboratories, Inc.  
Tel: (02) 924-86 97  
Fax: (02) 924-86 96  
E-mail: webmaster@lrslab.co.kr  
Web site: www.lrslab.co.kr

### Malaysia

RESEARCH BIOLABS SDN. BHD.  
Tel: (603)-8070 3101  
Fax: (603)-8070 5101  
E-mail: biolabs@tm.net.my  
Web site: www.researchbiolabs.com

### Mexico

Quimica Valaner S. A. de C. V.  
Tel: (55) 55 25 57 25  
Fax: (55) 55 25 56 25  
E-mail: ventas@valaner.com  
Web site: www.valaner.com

### New Zealand

Biolab Ltd  
Tel: (09) 980 6700  
or 0800 933 966  
Fax: (09) 980 6788  
E-mail: biosciences@nzl.biolabgroup.com  
Web site: www.biolabgroup.com/nzl

### Poland

Syngen Biotech Sp.z.o.o.  
Tel: (071) 798 58 50 - 52  
Fax: (071) 798 58 53  
E-mail: info@syngen.pl  
Web site: www.syngen.pl

### Portugal

IZASA PORTUGAL, LDA  
Tel: (21) 424 7312  
Fax: (21) 417 2674  
E-mail: consultasbiotec@izasa.es

### Saudi Arabia

Abdulla Fouad Co. Ltd.  
Tel: (03) 8324400  
Fax: (03) 8346174  
E-mail: sadiq.omar@abdulla-fouad.com

### Singapore

Research Biolabs Pte Ltd  
Tel: 6777 5366  
Fax: 6778 5177  
E-mail: sales@researchbiolabs.com  
Web site: www.researchbiolabs.com

### Slovak Republic

BIO-CONSULT Slovakia spol. s.r.o.  
Tel/Fax: (02) 5022 1336  
E-mail: bio-cons@cdicon.sk  
Web site: www.bioconsult.cz

### Slovenia

MEDILINE d.o.o.  
Tel: (01) 830-80-40  
Fax: (01) 830-80-70  
or (01) 830-80-63  
E-mail: mediline@siol.net

### South Africa

Southern Cross Biotechnology (Pty) Ltd  
Tel: (021) 671 5166  
Fax: (021) 671 7734  
E-mail: info@scb.co.za

### Spain

IZASA, S. A.  
Tel: (93) 902.20.30.90  
Fax: (93) 902.22.33.66  
E-mail: consultasbiotec@izasa.es

### Taiwan

TAIGEN Bioscience Corporation  
Tel: (02) 2880 2913  
Fax: (02) 2880 2916  
E-mail: taigen@ms10.hinet.net

### Thailand

Theera Trading Co. Ltd.  
Tel: (02) 412-5672  
Fax: (02) 412-3244  
E-mail: theetrad@samart.co.th

### Turkey

Medek Medikal Ürünler ve Sağlık Hizmetleri A. S.  
Tel: (216) 302 15 80  
Fax: (216) 302 15 88  
E-mail: makialp@med-ek.com

### All other countries

QIAGEN GmbH, Germany

**Australia** = Orders 03-9840-9800 = Fax 03-9840-9888 = Technical 1-800-243-066  
**Austria** = Orders: 0800/28-10-10 = Fax: 0800/28-10-19 = Technical: 0800/28-10-11  
**Belgium** = Orders 0800-79612 = Fax 0800-79611 = Technical 0800-79556  
**Canada** = Orders 800-572-9613 = Fax 800-713-5951 = Technical 800-DNA-PREP (800-362-7737)  
**China** = Orders 021-51345678 = Fax 021-51342500 = Technical 021-51345678  
**Denmark** = Orders 80-885945 = Fax 80-885944 = Technical 80-885942  
**Finland** = Orders 0800-914416 = Fax 0800-914415 = Technical 0800-914413  
**France** = Orders 01-60-920-920 = Fax 01-60-920-925 = Technical 01-60-920-930  
**Germany** = Orders 02103-29-12000 = Fax 02103-29-22000 = Technical 02103-29-12400  
**Ireland** = Orders 1800 555 049 = Fax 1800 555 048 = Technical 1800 555 061  
**Italy** = Orders 02-33430411 = Fax 02-33430426 = Technical 800 787980  
**Japan** = Telephone 03-5547-0811 = Fax 03-5547-0818 = Technical 03-5547-0811  
**Luxembourg** = Orders 8002-2076 = Fax 8002-2073 = Technical 8002-2067  
**The Netherlands** = Orders 0800-0229592 = Fax 0800-0229593 = Technical 0800-0229602  
**Norway** = Orders 800-18859 = Fax 800-18817 = Technical 800-18712  
**Sweden** = Orders 020-790282 = Fax 020-790582 = Technical 020-798328  
**Switzerland** = Orders 055-254-22-11 = Fax 055-254-22-13 = Technical 055-254-22-12  
**UK** = Orders 01293-422-911 = Fax 01293-422-922 = Technical 01293-422-999  
**USA** = Orders 800-426-8157 = Fax 800-718-2056 = Technical 800-DNA-PREP (800-362-7737)

