QIAGEN Supplementary Protocol:

Purification of total RNA from bacteria using the RNeasy® Mini Kit

Up to 1 x 10^9 bacteria are disrupted and homogenized by bead-milling in a guanidine-thiocyanate–containing lysis buffer. After addition of ethanol, the sample is loaded onto an RNeasy Mini spin column. Total RNA binds to the RNeasy silica-membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in RNase-free water.

Note: Cross-references to the *RNeasy Mini Handbook* in this protocol refer to the fourth edition of the handbook (April 2006).

For optimal results, we strongly recommend using the RNeasy Mini Kit in combination with RNAprotect® Bacteria Reagent (cat. no. 76506). This reagent provides in vivo stabilization of RNA in bacteria to ensure reliable gene expression analysis. Protocols for stabilizing and purifying RNA from bacteria, with appropriate disruption methods for different bacterial species, are included in the *RNAprotect Bacteria Reagent Handbook*. This handbook is supplied with RNAprotect Bacteria Reagent and can also be downloaded at www.qiagen.com/HB/RNAprotectBacteriaReagent . The RNeasy Mini Kit and RNAprotect Bacteria Reagent can also be purchased together as the RNeasy Protect Bacteria Mini Kit (cat. no. 74524).

IMPORTANT: Please consult the "Safety Information" section in the *RNeasy Mini Handbook* before beginning this procedure. For safety information on the additional chemicals mentioned in this protocol, please consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Equipment and reagents to be supplied by user

- 14.3 M β-mercaptoethanol (β-ME) (commercially available solutions are usually 14.3 M)
- Sterile, RNase-free pipet tips
- Microcentrifuge (with rotor for 2 ml tubes)
- 96–100% and 70% ethanol (do not use denatured alcohol, which contains other substances such as methanol or methylethylketone)
- Disposable gloves
- TissueLyser (cat. no. 85200 [100 V, 50/60 Hz; Japan], cat. no. 85210 [120 V, 50/60 Hz; Canada and USA], or cat. no. 85220 [220–240 V, 50/60 Hz; Australia and Europe])
- TissueLyser Adapter Set 2 x 24 (cat. no. 69982)

- Glass beads (e.g., Sigma, cat. no. G1145, G1277, or G8772)*
- 2 ml Safe-Lock tubes (Eppendorf, cat. no. 0030 120.094)*
- Optional: RNase-Free DNase Set (cat. no. 79254)

Determining the correct amount of starting material

It is essential to use the correct number of bacteria in order to obtain optimal RNA yield and purity. The maximum number that can be used is determined by:

- The RNA binding capacity of the RNeasy spin column (100 µg RNA)
- The volume of Buffer RLT required for efficient lysis (the maximum volume of Buffer RLT that can be used limits the maximum amount of starting material to 1 x 10^9 bacteria)

When processing cultures containing high amounts of RNA, fewer bacteria should be used, so that the RNA binding capacity of the RNeasy spin column is not exceeded. When processing cultures containing lower amounts of RNA, the maximum number of bacteria can be used. However, even though the RNA binding capacity of the RNeasy spin column is not reached, using more bacteria would lead to incomplete lysis, resulting in lower RNA yield and purity.

RNA content varies greatly between bacterial strains and under different growth conditions: 1 x 10^9 bacteria corresponds to approximately 20–160 µg total RNA. Typical yields of total RNA from 1 x 10^9 *E. coli* and 1 x 10^9 *B. subtilis* are 55 µg and 33 µg, respectively. If there is no information about the RNA content of your starting material, we recommend starting with no more than 5 x 10^8 bacteria. Depending on RNA yield and purity, it may be possible to increase the number of bacteria in subsequent preparations.

Do not overload the RNeasy spin column, as this will significantly reduce RNA yield and purity.

Bacterial growth is usually measured using a spectrophotometer. However, it is very difficult to give specific and reliable recommendations for the relationship between OD values and cell numbers in bacterial cultures. Cell density is influenced by a variety of factors (e.g., species, media, incubation time, and shaker speed), and OD readings of cultures measure light scattering rather than absorption. Measurements of light scattering are highly dependent on the distance between the sample and the detector. Therefore, readings vary between different types of spectrophotometer. In addition, different bacterial species show different OD values at the same wavelength.

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

The following values may be used as a rough guide. An *E. coli* culture containing 1 x 10^9 cells per milliliter, diluted 1 in 4, gives an OD_{600} value of approximately 0.25 with a Beckman DU®-7400

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

spectrophotometer or 0.125 with a Beckman DU-40 spectrophotometer. These correspond to calculated OD values of 1 or 0.5, respectively, for 1 x 10^9 bacteria per milliliter.

Important points before starting

- If working with RNA for the first time, read Appendix A (page 63) in the *RNeasy Mini Handbook*.
- Bacteria should be harvested in log-phase growth.
- Bacterial pellets can be stored at –70°C for later use or used directly in the procedure. Frozen bacterial pellets should be thawed slightly so that they can be dislodged by flicking the tube in step 3. Homogenized bacterial lysates from step 5 can be stored at –70°C for several months. Frozen lysates should be incubated at 37°C in a water bath until completely thawed and salts are dissolved. Avoid prolonged incubation, which may compromise RNA integrity. Continue with step 6.
- Generally, DNase digestion is not required since RNeasy silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan® RT-PCR analysis with a low-abundant target). In these cases, residual DNA can be removed by optional on-column DNase digestion using the RNase-Free DNase Set (see Appendix D [page 69] in the *RNeasy Mini Handbook*). The DNase is efficiently removed in subsequent wash steps. Alternatively, residual DNA can be removed by a DNase digestion after RNA purification (see Appendix E [page 71] in the *RNeasy Mini Handbook*). The DNase digestion can then be cleaned up, if desired, using the RNA cleanup protocol (page 56) in the *RNeasy Mini Handbook*.
- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature (15–25°C).
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See "Safety Information" in the *RNeasy Mini Handbook*.
- The bacterial culture should be harvested at 4°C. Perform all subsequent steps of the procedure at room temperature. During the procedure, work quickly.
- After harvesting the cells, perform all centrifugation steps at 20–25°C in a standard microcentrifuge. Ensure that the centrifuge does not cool below 20°C.

Things to do before starting

- Add 10 µl β-mercaptoethanol (β-ME) per 1 ml Buffer RLT, and mix. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT containing β-ME can be stored at room temperature for up to 1 month.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- If performing optional on-column DNase digestion, prepare DNase I stock solution as described in Appendix D (page 69) of the *RNeasy Mini Handbook*.

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 4.**
2. **Harvest bacteria by centrifuging at 5000 x *g* for 5 min at 4°C (do not use more than 1 x 10^9 bacteria). Decant the supernatant, and carefully remove any remaining media by aspiration. If the centrifuge will be used later in this procedure, heat it to 20–25°C.**

   Note: Incomplete removal of medium will inhibit lysis and dilute the lysate, affecting the conditions for binding of RNA to the RNeasy membrane. Both effects may reduce RNA yield.
3. **Add the appropriate volume of Buffer RLT (see table below). Vortex vigorously for 5–10 s.**

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

   Ensure that the pellet is thoroughly resuspended in Buffer RLT.

Number of bacteria*	Buffer RLT (µl)
$<5 \times 10^8$	350
$5 \times 10^8 – 1 \times 10^9$	700

   * 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".
4. **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.
5. **Centrifuge for 10 s at maximum speed. Transfer supernatant into a new tube (not supplied).**

   The volume of the tube must be at least twice that of the Buffer RLT used.
6. **Determine the volume of supernatant. Add an equal volume of ethanol (70%), and mix well by pipetting. Do not centrifuge.**

   After adding ethanol, a precipitate may form. This will not affect the RNeasy procedure.
7. **Transfer up to 700 µl lysate, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.***

   Reuse the collection tube in step 8.

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

* Flow-through contains Buffer RLT and is therefore not compatible with bleach. See the safety information in the *RNeasy Mini Handbook*.

Optional: If performing optional on-column DNase digestion (see "Important points before starting"), follow steps D1–D4 in Appendix D (page 69) of the *RNeasy Mini Handbook* after performing this step.

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

   Reuse the collection tube in step 9.

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

   Skip this step if performing optional on-column DNase digestion.

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

   Reuse the collection tube in step 10.

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

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

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

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

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

    Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 10.

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

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

    If using the eluate from step 12, 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.

* Flow-through contains Buffer RW1 and is therefore not compatible with bleach. See the safety information in the *RNeasy Mini Handbook*.

QIAGEN kit handbooks can be requested from QIAGEN Technical Service or your local QIAGEN distributor.
Selected kit handbooks can be downloaded from www.qiagen.com/literature .
Material safety data sheets (MSDS) for any QIAGEN product can be downloaded from www.qiagen.com/ts/msds.asp .