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Supplementary Protocol

RNeasy® Midi/Maxi Protocol for Isolation of Total RNA from Bacteria

It is essential to use the correct number of bacteria in order to obtain optimal RNA yield and purity with RNeasy columns. A minimum amount of 5 x 10^8 or 5 x 10^9 bacteria can generally be processed with RNeasy Midi or Maxi columns, respectively. Two main criteria limit the maximum number of bacteria to use:

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

For bacterial cultures containing high levels of RNA, fewer bacteria should be used, so as not to exceed the RNA binding capacity of the RNeasy column. For bacterial cultures containing lower levels of RNA, the maximum number of bacteria can be used. In these cases, even though the RNA binding capacity of the column may not be reached, use of more cells would lead to incomplete lysis, resulting in lower RNA yield and purity. Average RNA yields that come from some bacterial species are given in Table 1 and can be used as a guide for calculating the number of bacteria to use.

Table 1. Average yields of total RNA isolated from bacteria using RNeasy Midi and Maxi columns

Bacteria	RNeasy Midi column		RNeasy Maxi column	
	No. of cells or mg of tissue	Yield (µg)*	No. of cells or grams of tissue	Yield (mg)*
E. coli	1 x 10^{10}	600	5 x 10^{10}	2.7
B. subtilis	1 x 10^{10}	320	5 x 10^{10}	1.5

If the bacterial species used is not shown in Table 1 and you have no information about the RNA content of your starting material, we recommend starting with no more than 5 x 10^9 bacteria per RNeasy Midi column or 2.5 x 10^{10} bacteria per RNeasy Maxi column. Depending on yield and purity, it may be possible to increase the number of bacteria in subsequent preparations.

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

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

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

The following values may be used as a rough guide. An *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 spectrophotometer or 0.125 with a Beckman DU-40 spectrophotometer.

The RNA content also varies greatly between bacterial strains and under different growth conditions: 5 x 10^9 bacteria correspond to approximately 100–800 µg total RNA; 1 x 10^{10} bacteria correspond to approximately 0.2–1.6 mg total RNA.

Equipment and reagents to be supplied by user

- 14.3 M β-mercaptoethanol (β-ME)* (stock solutions are usually 14.3 M)
- Sterile, RNase free pipette tips
- Laboratory centrifuge (capable of 3000–5000 x *g*)†
- Equipment for disruption and homogenization (see the *RNeasy Midi/Maxi Handbook*)
- Vessels for homogenization (e.g., 10–15 mL centrifuge tubes for the RNeasy Midi Kit; 50 mL centrifuge tubes for the RNeasy Maxi Kit)
- Ethanol (96–100%)
- Disposable gloves
- TE buffer (10 mM Tris·Cl; 1 mM EDTA, pH 8.0)
- Lysozyme (50 mg/mL in water)

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

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

Important points before starting

- If using RNeasy Midi or Maxi Kits for the first time, read "Important Points Before Using RNeasy Kits" in the *RNeasy Midi/Maxi Handbook*.
- If working with RNA for the first time, read Appendix A in the *RNeasy Midi/Maxi Handbook*.
- For RNA isolation, bacteria should be harvested in log phase growth.
- The optimal beads to use for bacteria are 0.1 mm (mean diameter) glass beads.
- Bacterial pellets can be stored at –70°C for later use or used directly in the procedure. Frozen bacterial pellets should be thawed slightly to allow pellets to be dislodged by flicking in step 2. Homogenized cell lysates (in Buffer RLT, step 3) can be stored at –70°C for several months. To process frozen lysates, thaw samples for 15–20 min at 37°C in a water bath to dissolve salts. Continue with step 4.
- **Prepare TE buffer, pH 8.0, with 1 mg/mL lysozyme for Gramnegative bacteria or 5 mg/mL lysozyme for Grampositive bacteria. TE buffer and lysozymes are not supplied with the kit.**
- **β-ME must be added to Buffer RLT before use. β-ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 10 µL β-ME per 1 mL Buffer RLT. Buffer RLT is stable for 1 month after addition of β-ME.**
- **Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.**
- Generally, DNase digestion is not required because the RNeasy silica membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RTPCR analysis with a low abundant target). In these cases, the small residual amounts of DNA remaining can be removed using the RNase-Free DNase Set (cat. no. 79254) for the optional on column DNase digestion (see Appendix E of the *RNeasy Midi/Maxi Handbook*) or by a DNase digestion after RNA isolation. For on column DNase digestion with the RNase-Free DNase Set, prepare the DNase I stock solution as described in Appendix E of the *RNeasy Midi/Maxi Handbook* before beginning the procedure.
- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature.
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. Guanidine is an irritant. Take appropriate safety measures and wear gloves when handling.
- The bacterial culture should be harvested at 4°C. All subsequent steps of the RNeasy protocol should be performed at room temperature. During the procedure, work quickly.
- After harvesting the cells, all centrifugation steps should be performed at 20–25°C in a standard laboratory centrifuge with a swinging bucket rotor capable of ≥3000 x *g* (see Equipment and reagents to be supplied by user). Ensure that the centrifuge does not cool below 20°C.
- In case of clogged RNeasy column, this may be due to centrifugation not having been performed before adding ethanol. Centrifuge lysate before adding ethanol and only use this supernatant in subsequent steps (see protocols). Pellets contain cell debris that can clog the RNeasy column.
- Blue (marked with a ▲) denotes ▲ RNeasy Midi prep volumes (for 5 x 10^8 to 1 x 10^{10} cells); red (marked with a ●) denotes ● RNeasy Maxi prep volumes (for 5 x 10^9 to 5 x 10^{10} cells).

Procedure

1. Harvest bacteria by centrifuging at 3000–5000 x *g* for 5 min at 4°C. (Do not use more than ▲ 1 x 10^{10} or ● 5 x 10^{10} bacteria.) Decant supernatant and carefully remove all remaining media by aspiration. After centrifuging, heat the centrifuge to 20–25°C if the same centrifuge is to be used in the following centrifugation steps of the protocol.

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

2. Loosen the bacterial pellet by flicking the bottom of the tube. Resuspend the bacteria thoroughly in the appropriate volume of lysozyme containing TE buffer (see Table 2). Incubate at room temperature for 2–5 min for Gramnegative bacteria or 5–10 min for Grampositive bacteria.

   Depending on the bacterial strain used, the amount of enzyme required and/or the incubation time may vary. For best results, follow the guidelines of the lysozyme supplier. Complete digestion of the cell wall is essential for efficient lysis.

3. Add the appropriate volume of Buffer RLT (see Table 2). Mix thoroughly by vortexing or shaking vigorously.

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

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

   Avoid transferring the pellet. This may reduce the amount of RNA that binds to the membrane and cause the spin column to clog.

5. Add the appropriate volume of ethanol (96–100%) to the lysate (see Table 2). Mix thoroughly by shaking. Do *not* centrifuge.

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

Table 2. Buffer volumes for RNeasy Midi/Maxi isolation of total RNA from bacteria

RNeasy column	Number of bacteria	TE buffer + lysozyme (mL)	Buffer RLT (mL)	Ethanol (96–100%) (mL)
Midi ▲	5 x 10^8 – 5 x 10^9	0.5	2.0	1.4
Midi ▲	5 x 10^9 – 1 x 10^{10}	1.0	4.0	2.8
Maxi ●	5 x 10^9 – 2.5 x 10^{10}	2.0	7.5	5.5
Maxi ●	2.5 x 10^{10} – 5 x 10^{10}	4.0	15.0	11.0

6. Apply the sample, including any precipitate that may have formed, to an ▲ RNeasy Midi column or ● RNeasy Maxi column placed in a ▲ 15 mL or ● 50 mL centrifuge tube (supplied). Maximum loading volume is ▲ 4.0 mL or ● 15 mL. Close the tube gently and centrifuge for 5 min at 3000–5000 x *g*. Discard the flow-through.*

   Reuse the collection tube in step 7.

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

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

   Optional: QIAGEN offers the RNase-Free DNase Set (cat. no. 79254) for convenient on column DNase digestion during RNA purification. Generally, DNase digestion is not required because the RNeasy silicamembrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive

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

to very small amounts of DNA (e.g., TaqMan RTPCR analysis with a low-abundant target). If using the RNase-Free DNase Set, follow the steps shown in Appendix E of the *RNeasy Midi/Maxi Handbook* after performing this step.

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

   Skip this step if performing the optional on column DNase digestion (Appendix E of the *RNeasy Midi/Maxi Handbook*). Reuse the collection tube in step 8.

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

   Reuse the collection tube in step 9. In the RNeasy Midi procedure, the flow-through does not need to be discarded.

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

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

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

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

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

Table 3. RNase free water volumes for RNeasy Midi/Maxi elution

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

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

    To obtain a higher total RNA concentration, this second elution step may be performed using the first eluate (from step 10). The yield will be 15–30% less than the yield obtained using a second volume of RNase free water, but the final concentration will be higher.

Document Revision History

Date	Changes
08/2019	Initial release
05/2023	Revised Table 1 heading to correct the unit of measurement used in Yield under RNeasy Maxi column from µg to mg. Changed document template to reflect QIAGEN's latest branding style.

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