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Supplementary Protocol

RNeasy® Midi/Maxi Protocol for Isolation of Cytoplasmic RNA from Animal Cells

It is essential to use the correct number of cells in order to obtain optimal RNA yield and purity with RNeasy columns. A minimum amount of 5 x 10^6 or 5 x 10^7 cells can generally be processed with RNeasy Midi or Maxi columns, respectively. The maximum number of cells that can be used depends on the specific RNA content of the cell line used, which varies greatly between cell types. Read the "RNeasy Midi/Maxi Protocol for Isolation of Total RNA from Animal Cells" in the *RNeasy Midi/Maxi Handbook* to determine the correct number of cells to use.

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
- Buffer RLN‡ 50 mM Tris·Cl, pH 8.0
140 mM NaCl
1.5 mM MgCl2
0.5% (v/v) Nonidet™ P-40 (1.06 g/ml)§
- Just before use, add: 1000 U/ml RNase inhibitor (optional)
1 mM DTT (optional)

*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.

‡The use of molecular biology grade reagents is recommended.

§Nonidet P-40 is no longer manufactured. It can be replaced with Nonidet P-40 Substitute (Fluka®, cat. no. 74385) or Igepal® CA-630 (Sigma-Aldrich[4], cat. no. I3021).

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*.
- Only use freshly harvested cells. Ice crystals form during freezing and thawing and destroys the nuclear membranes, releasing DNA and other nuclear molecules. After addition of Buffer RLT (step 4), samples can be stored at –70°C for several months. To process, frozen lysates should be thawed and incubated at 37°C for 10 min to ensure that all salts have been dissolved. Vortex vigorously. If any insoluble material remains, centrifuge for 2 min and use the supernatant. Continue with step 5.
- Prepare Buffer RLN* and precool to 4°C.
- **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.**
- Using the cytoplasmic protocol, DNase digestion is generally not required. Most of the DNA is removed by pelleting the nuclei during the procedure, and the RNeasy silica-membrane technology efficiently removes nearly all of the remaining small amounts of DNA without DNase treatment. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan® RT-PCR analysis with a low-abundant target). In these cases, the small residual amounts of DNA remaining can be removed using the RNase-Free DNase Set (cat. no. 79254) for the optional on-column DNase digestion (see Appendix E) 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 before beginning the procedure.
- No homogenization is needed for cytoplasmic protocol.
- 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.
- Cell lysis is performed on ice, and the following centrifugation is done at 4°C. All subsequent steps of the RNeasy protocol should be performed at room temperature. With the exception of Buffer RLN, buffers should not be precooled.

* The use of molecular biology grade reagents is recommended.

- After cell harvesting and pelleting of nuclei, all centrifugation steps are 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.
- Blue (marked with a ▲) denotes ▲ RNeasy Midi prep volumes (for 5 x 10^6 to 1 x 10^8 cells); red (marked with a ●) denotes ● RNeasy Maxi prep volumes (for 5 x 10^7 to 5 x 10^8 cells).

Procedure

1. Harvest cells.

   1a. Cells grown in suspension (Do not use more than ▲ 1 x 10^8 or ● 5 x 10^8 cells.) Determine the number of cells. Pellet the appropriate number of cells for 5 min at 300 x *g* in an RNase-free glass or polypropylene centrifuge tube (not supplied). Carefully remove all supernatant by aspiration, and continue with step 2 of the protocol.

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

   1b. Cells grown in a monolayer (Do not use more than ▲ 1 x 10^8 or ● 5 x 10^8 cells.) Cells grown in a monolayer in cell-culture vessels can either be lysed directly in the culture dish or trypsinized and collected as a cell pellet prior to lysis. Cells grown in a monolayer in cell-culture flasks should always be trypsinized.

   To lyse cells directly in culture dish (≤10 cm in diameter): Determine the number of cells. (Table 1 gives the approximate cell numbers for various cell-culture vessels.) Completely aspirate the cell-culture medium and continue immediately with step 2 of the protocol.

   To trypsinize cells: Determine the number of cells. (Table 1 gives the approximate cell numbers for various cell-culture vessels.) Aspirate medium, and wash cells with PBS. Aspirate PBS, and add 0.10–0.25% trypsin in PBS to trypsinize the cells. After cells detach from the dish or flask, add medium (containing serum to inactivate the trypsin), transfer cells to an RNase-free glass or polypropylene centrifuge tube (not supplied), and pellet by centrifugation at 300 x *g* for 5 min. Completely aspirate supernatant, and continue with step 2 of the protocol.

   Note: Incomplete removal of the 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.

Table 1. Growth area and number of HeLa cells in various culture vessels

Cell culture vessel	Growth area (cm^2)*	No. of cells†
Dishes		
100 mm	56	7×10^6
145–150 mm	145	2×10^7
Flasks		
40–50 ml	25	3×10^6
250–300 ml	75	1×10^7
650–750 ml	162–175	2×10^7
900 ml	225	3×10^7

* Varies slightly depending on the supplier.

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

2. Add buffer RLN to lyse plasma membrane.

   For pelleted cells, loosen the cell pellet thoroughly by flicking the tube. Carefully resuspend cells in the appropriate volume of cold (4°C) Buffer RLN (see Table 2). Incubate on ice for 5 min. Proceed to step 3.

   The suspension should clear rapidly, indicating lysis of the plasma membrane, which occurs almost immediately.

   Note: Incomplete loosening of the cell pellet may lead to inefficient lysis and reduced yields. For isolation of cytoplasmic RNA, freshly harvested cells must be used.

   For direct lysis of cells in cell-culture dishes (≤10 cm diameter), add ▲ 1.0 ml of cold Buffer RLN (4°C). Detach cells gently using a rubber policeman, and transfer to a centrifuge tube (not supplied). Incubate on ice for 5 min. Proceed to step 3.

   The suspension should clear rapidly, indicating lysis of the plasma membrane, which occurs almost immediately.

Table 2. Buffer volumes for RNeasy Midi/Maxi isolation of cytoplasmic RNA from animal cells

RNeasy column	Number of cells	Buffer RLN (4°C) (ml)	Buffer RLT (ml)	Ethanol (96–100%) (ml)
Midi ▲	Cell-culture dish (≤10 cm diameter)*	1.0	4.0	2.8
Midi ▲	$5 \times 10^6 – 5 \times 10^7$	0.5	2.0	1.4
Midi ▲	$5 \times 10^7 – 1 \times 10^8$	1.0	4.0	2.8
Maxi ●	$5 \times 10^7 – 2.5 \times 10^8$	2.0	7.5	5.5
Maxi ●	$2.5 \times 10^8 – 5 \times 10^8$	4.0	15.0	11.0

* For direct lysis of cells in culture dishes (≤10 cm), use the indicated amounts, regardless of cell number.

3. Centrifuge lysate at 4°C for 5 min at 300–500 x *g*. Transfer supernatant to an RNase-free ▲ 10–15 ml or ● 50 ml centrifuge tube (not supplied), and discard the pellet. 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.

   The supernatant contains the cytoplasmic extract. It is generally slightly cloudy and yellow-white, depending on the cell type used. The pellet contains the nuclei and cell debris. The pellet is white and considerably smaller than the whole cell pellet obtained during harvesting in step 1.

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

   No further homogenization is required, because genomic DNA is not released.

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

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

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

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 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 the centrifugation time to 10 min to allow the lysate to completely pass through the column.

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

   Optional: QIAGEN offers the RNase-Free DNase Set (cat. no. 79254) for convenient oncolumn DNase digestion during RNA purification. Using the cytoplasmic protocol, DNase digestion is generally not required. Most of the DNA is removed by pelleting the nuclei during the procedure, and the RNeasy silica-membrane technology efficiently removes nearly all of the remaining small amounts of DNA without DNase treatment. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). If using the RNase-Free DNase Set, follow the steps shown on 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 centrifuge tube in step 8.

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

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

   Reuse the centrifuge tube in step 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 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 spin-column 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 the 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

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