QIAGEN Supplementary Protocol:

Purification of cytoplasmic RNA from animal cells using the RNeasy® Mini Kit

This protocol requires the RNeasy Mini Kit.

IMPORTANT: Please consult the "Safety Information" and "Important Notes" sections in the *RNeasy Mini Handbook* (April 2006) 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.

This protocol is particularly advantageous for applications where unspliced or partially spliced RNA is not desirable, since the cytoplasm contains RNA in its mature form. Cytoplasmic RNA accounts for 85% of total cellular RNA. Since this protocol enables the removal of intact nuclei, it is also suitable for applications where the absence of DNA contamination is critical. Homogenization to shear genomic DNA is not required. Cultured cells are lysed in Buffer RLN (a buffer containing a nonionic detergent), which lyses the cell plasma membrane. Nuclei remain intact during lysis and are removed by centrifugation. Buffer RLT (a guanidine-thiocyanate–containing lysis buffer) and ethanol are added to the supernatant to provide optimal conditions for selective binding of RNA to the RNeasy membrane. The sample is then applied to the RNeasy Mini spin column. Cytoplasmic RNA binds to the membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in RNase-free water.

Equipment and reagents to be supplied by user

- Sterile, RNase-free pipet tips
- Microcentrifuge (with rotor for 2 ml tubes)
- 96–100% ethanol (do not use denatured alcohol, which contains other substances such as methanol or methylethylketone)
- Disposable gloves
- Ice
- Tris·Cl, NaCl, $MgCl_2$, Nonidet P-40, RNase inhibitor (optional), and DTT (optional) to prepare Buffer RLN

  Note: Nonidet P-40 is no longer manufactured. It can be replaced with Nonidet P 40 Substitute (Sigma-Aldrich, cat. no. 74385) or IGEPAL CA-630 (Sigma-Aldrich, cat. no. 56741).
- Optional: 14.3 M β-mercaptoethanol (β-ME) (commercially available solutions are usually 14.3 M)

Determining the correct amount of starting material

See "Determining the correct amount of starting material" on page 25 of the *RNeasy Mini Handbook* (April 2006).

Important points before starting

- If using the RNeasy Mini Kit for the first time, read "Important Notes" on page 18 of the *RNeasy Mini Handbook* (April 2006).
- If working with RNA for the first time, read Appendix A on page 63 of the the *RNeasy Mini Handbook* (April 2006).
- Only use freshly harvested cells. Ice crystals form during freezing and thawing and destroy the nuclear membranes, releasing DNA and other nuclear molecules.
- Homogenized cell lysates from step 4 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. If any insoluble material is visible, centrifuge for 5 min at 3000–5000 x *g*. Transfer supernatant to a new RNase-free glass or polypropylene tube, and continue with step 5.
- 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 page 8 of the *RNeasy Mini Handbook* (April 2006) for safety information.
- Cells are lysed on ice and the resulting lysate is centrifuged at 4°C. All subsequent steps of the procedure are performed at room temperature, and all subsequent centrifugation steps are performed at 20–25°C in a standard microcentrifuge (ensure that the centrifuge does not cool below 20°C). During the procedure, work quickly. With the exception of Buffer RLN, buffers should not be precooled.

Things to do before starting

- Prepare Buffer RLN and precool to 4°C:

  50 mM Tris·Cl, pH 8.0

  140 mM NaCl

  1.5 mM $MgCl_2$

  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-Mercaptoethanol (β-ME) may be optionally added to Buffer RLT before use. Add 10 µl β-ME per 1 ml Buffer RLT. 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 on page 69 of the *RNeasy Mini Handbook* (April 2006).

Procedure

1. Harvest cells according to step 1a or 1b.

1a. Cells grown in suspension (do not use more than 1 x 10^7 cells):

Determine the number of cells. Pellet the appropriate number of cells by centrifuging for 5 min at 300 x *g* in a centrifuge tube (not supplied). Carefully remove all supernatant by aspiration, and proceed to step 2.

Note: Incomplete removal of 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 10^7 cells):

Cells are trypsinized and collected as a cell pellet prior to lysis. Cells grown in a cell-culture dish of up to 3.5 cm diameter can alternatively be directly lysed in the dish.

To lyse cells directly (in a dish of up to 3.5 cm diameter):
Determine the number of cells. Completely aspirate the cell-culture medium, and proceed immediately to step 2.

Note: Incomplete removal of 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.

To trypsinize and collect cells:
Determine the number of cells. Aspirate the medium, and wash the cells with PBS. Aspirate the PBS, and add 0.1–0.25% trypsin in PBS. After the cells detach from the dish or flask, add medium (containing serum to inactivate the trypsin), transfer the cells to an RNase-free glass or polypropylene centrifuge tube (not supplied), and centrifuge at 300 x *g* for 5 min. Completely aspirate the supernatant, and proceed to step 2.

Note: Incomplete removal of 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.

2. Lyse the plasma membrane of the cells by adding Buffer RLN.

For pelleted cells, loosen the cell pellet thoroughly by flicking the tube. Carefully resuspend the cells in 175 µl of precooled (4°C) Buffer RLN. Incubate on ice for 5 min, and proceed to step 3.

The suspension should clear rapidly, indicating immediate lysis of the plasma membrane.

Note: Incomplete loosening of the cell pellet may lead to inefficient lysis and reduced RNA yields.

For direct lysis of cells grown in a dish of up to 3.5 cm diameter, add 175 µl of precooled (4°C) Buffer RLN. Gently detach the cells with a rubber policeman and transfer them to a microcentrifuge tube (not supplied). Incubate on ice for 5 min, and proceed to step 3.

The suspension should clear rapidly, indicating immediate lysis of the plasma membrane.

3. **Centrifuge the lysate at 4°C for 2 min at 300 x g. Transfer the supernatant to a new centrifuge tube (not supplied), and discard the pellet. If using the same centrifuge in the subsequent steps of this procedure, heat it to 20–25°C.**

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

4. **Add 600 µl Buffer RLT to the supernatant. Mix well by vigorously vortexing.**

   No further homogenization is required since genomic DNA was not released.

5. **Add 430 µl ethanol (96–100%) to the homogenized lysate, and mix well by pipetting. Do not centrifuge.**

   Note: When purifying RNA from certain cell lines, precipitates may be visible after addition of ethanol. This does not affect the procedure.

6. **Transfer up to 700 µl of the sample, 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.***

   Repeat this step with the remaining sample and the same spin column.

   Reuse the collection tube in step 7.

   Optional: If performing optional on-column DNase digestion (see "Eliminating genomic DNA contamination" in the *RNeasy Mini Handbook* [April 2006], page 23), follow steps D1–D4 on page 69 of the *RNeasy Mini Handbook* (April 2006) after performing this step.

7. **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 8.

   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.

8. **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 9.

* Flow-through contains Buffer RLT or Buffer RW1 and is therefore not compatible with bleach. See page 8 of the *RNeasy Mini Handbook* (April 2006) for safety information.

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 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.

10. **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 9.

11. **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.**

12. **If the expected RNA yield is >30 µg, repeat step 11 using another 30–50 µl RNase-free water, or using the eluate from step 11 (if high RNA concentration is required). Reuse the collection tube from step 11.**

    If using the eluate from step 11, 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.