QIAGEN Supplementary Protocol:

Purification of miRNA from animal cells using the RNeasy® Plus Mini Kit and RNeasy MinElute® Cleanup Kit

There are two protocols: follow Protocol 1 if you want to purify total RNA containing miRNA, or follow Protocol 2 if you want to purify small RNA (includes miRNA, 5S rRNA, and tRNA) and larger RNA (>200 nt) separately.

IMPORTANT: Please consult the "Safety Information" and "Important Notes" sections in the *RNeasy Plus Mini Handbook* and the *RNeasy MinElute Cleanup 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.

miRNA Purification Procedures

Protocol 1: Purification of total RNA containing miRNA

Disrupt and homogenize cells in Buffer RLT Plus
↓
Centrifuge through gDNA Eliminator spin column
↓
Add 1.5 volumes of 100% ethanol
↓
Apply to RNeasy Mini spin column
↓
Wash spin column membrane
↓
Elute total RNA containing miRNA

Protocol 2: Separate purification of small RNA (containing miRNA) and larger RNA

Disrupt and homogenize cells in Buffer RLT Plus
↓
Centrifuge through gDNA Eliminator spin column
↓
Add 1 volume of 70% ethanol
↓
Apply to RNeasy Mini spin column
↙ ↘

Add 0.65 volumes of 100% ethanol to flow-through
↓
Apply to RNeasy MinElute spin column
↓
Wash spin column membrane
↓
Elute small RNA (contains miRNA)

Wash spin column membrane
↓
Elute total RNA >200 nucleotides

Protocol 1: Purification of total RNA containing miRNA from animal cells using the RNeasy Plus Mini Kit

Equipment and reagents to be supplied by user

- RNeasy Plus Mini Kit (50) (cat. no. 74134)
- Ethanol (100%)

Important points before starting

- This protocol is for use with up to 5 x 10^6 cells.
- All centrifugation steps are carried out at room temperature (15–25°C).
- Read the handbook supplied with the RNeasy Plus Mini Kit.

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. **Add 350 µl Buffer RLT Plus to the sample, and disrupt and homogenize immediately.**

   For details on disrupting and homogenizing samples, refer to the *RNeasy Plus Mini Handbook*.

2. **Transfer the homogenate to a gDNA Eliminator spin column placed in a 2 ml collection tube. Centrifuge for 30 s at ≥8000 x *g* (≥10,000 rpm). Discard the column, and save the flow-through.**

   Note: If necessary, repeat the centrifugation until all liquid has passed through the membrane.

3. **Add 1.5 volumes (usually 525 µl) of 100% ethanol to the flow-through, and mix thoroughly by vortexing. Do not centrifuge. Proceed immediately to step 4.**

4. **Transfer 700 µl of the sample, including any precipitate that may have formed, to an RNeasy Mini spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.**

   Repeat step 4 until the whole sample has passed through the membrane. Discard the flow-through each time.

5. **Place the RNeasy Mini spin column in a new 2 ml collection tube. Add 500 µl Buffer RPE to the 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.**

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

6. **Add 500 µl Buffer RPE to the RNeasy Mini 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 and the collection tube.**

7. **Place the RNeasy Mini spin column in a new 2 ml collection tube. Close the lid, and centrifuge at full speed for 1 min.**
8. **Place the RNeasy Mini spin column in a 1.5 ml collection tube. 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 (total RNA containing miRNA).**

   If the expected RNA yield is >30 µg, repeat step 8 with a second volume of RNase-free water. Elute into the same collection tube.

Protocol 2: Separate purification of small RNA (containing miRNA) and larger RNA from animal cells using the RNeasy Plus Mini Kit and RNeasy MinElute Cleanup Kit

Equipment and reagents to be supplied by user

- RNeasy Plus Mini Kit (50) (cat. no. 74134)
- RNeasy MinElute Cleanup Kit (50) (cat. no. 74204)
- Ethanol (70%, 80%, and 100%)
- Collection tubes (2 ml and 1.5 ml) are supplied with both RNeasy Kits; if necessary, extra 2 ml collection tubes can be purchased separately (cat. no. 19201)

Important points before starting

- This protocol is for use with up to 5 x 10^6 cells.
- All centrifugation steps are carried out at room temperature (15–25°C).
- Read the handbooks supplied with the RNeasy Plus Mini Kit and the RNeasy MinElute Cleanup Kit.

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

Separating small RNA from total RNA using the RNeasy Plus Mini Kit

1. **Add 350 µl Buffer RLT Plus to the sample, and disrupt and homogenize immediately.**

   For details on disrupting and homogenizing samples, refer to the *RNeasy Plus Mini Handbook*.
2. **Transfer the homogenate to a gDNA Eliminator spin column placed in a 2 ml collection tube. Centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the column, and save the flow-through.**

   Note: If necessary, repeat the centrifugation until all liquid has passed through the membrane.

3. **Add 1 volume (usually 350 µl) of 70% ethanol, and mix thoroughly by vortexing. Do not centrifuge. Proceed immediately to step 4.**
4. **Transfer the sample, including any precipitate that may have formed, to an RNeasy Mini spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm).**
5. **If purifying small RNA (containing miRNA) only, discard the RNeasy Mini spin column and follow steps 6 to 12 only.**

   If purifying both small RNA (containing miRNA) and larger RNA (>200 nt), save the RNeasy Mini spin column for use in step 13 (the spin column can be stored at 4°C or at room temperature [15–25°C], but not for long periods). Follow steps 6 to 12 to purify small RNA, and then steps 13 to 17 to purify larger RNA.

Purifying small RNA (containing miRNA) using the RNeasy MinElute Cleanup Kit

6. **Transfer the flow-through from step 4 (which contains miRNA) to a 2 ml reaction tube (not supplied).**
7. **Add 0.65 volumes (usually 455 µl) of 100% ethanol, and mix thoroughly by vortexing. Do not centrifuge. Proceed immediately to step 8.**
8. **Transfer 700 µl of the sample to an RNeasy MinElute spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.**

   Repeat step 8 until the whole sample has passed through the membrane. Discard the flow-through each time.

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

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

10. **Add 500 µl of 80% ethanol to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through and the collection tube.**
11. **Place the RNeasy MinElute spin column in a new 2 ml collection tube. Open the lid, and centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm).**
12. **Place the RNeasy MinElute spin column in a 1.5 ml collection tube. Add 14 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 (small RNA containing miRNA).**

    This RNA eluate is enriched in various RNAs of <200 nucleotides, including miRNA, 5S rRNA, and tRNA. For this reason, the miRNA yield cannot be quantified by OD measurement or fluorogenic assays. To determine yield, we recommend using quantitative, real-time RT-PCR assays specific for the type of small RNA under study. For example, to estimate miRNA yield, an assay directed against any miRNA known to be adequately expressed in the samples being processed may be used.

Purifying larger RNA (>200 nt) using the RNeasy Plus Mini Kit

13. **Add 700 µl Buffer RW1 to the RNeasy Mini spin column from step 4. 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 and the collection tube.**
14. **Place the RNeasy Mini spin column in a new 2 ml collection tube. Add 500 µl Buffer RPE to the 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.**

    Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see "Things to do before starting").
15. **Add 500 µl Buffer RPE to the RNeasy Mini 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 and the collection tube.**
16. **Place the RNeasy Mini spin column in a new 2 ml collection tube. Open the lid, and centrifuge at full speed for 1 min.**

    To avoid damage to their lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counterclockwise).

    It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.
17. **Place the RNeasy Mini spin column in a 1.5 ml collection tube. 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 (>200 nt).**

    If the expected RNA yield is >30 µg, repeat step 17 with a second volume of RNase-free water. Elute into the same collection tube.

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 .