QIAGEN Supplementary Protocol

Purification of total RNA, including small RNAs, from serum or plasma using the miRNeasy Mini Kit

This protocol is intended as a guideline for the purification of total RNA, including small RNAs (e.g., miRNAs), from serum and plasma using the miRNeasy Mini Kit (cat. no. 217004).

IMPORTANT: Please read the *miRNeasy Mini Handbook*, paying careful attention to the "Safety Information" and "Important Notes" sections, before beginning these procedures. Handbooks can be found at www.qiagen.com/handbooks. miRNeasy Kits are intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

Equipment and reagents to be supplied by user

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- Chloroform (without added isoamyl alcohol)
- Ethanol (70% and 96–100%); do not use denatured alcohol, which contains other substances such as methanol and methylethylketone
- Sterile, RNase-free pipet tips
- 1.5 ml or 2 ml microcentrifuge tubes
- Microcentrifuge(s) (with rotor for 2 ml tubes) for centrifugation at 4°C and at room temperature (15–25°C)
- Disposable gloves
- Optional (if using *C. elegans* miRNA for normalization and/or as an internal control):
  - RNase-free TE buffer
  - Syn-cel-miR-39 miScript miRNA Mimic (cat. no. MSY0000010)
  - Method for *C. elegans* miRNA detection (e.g., miScript PCR System in combination with the Ce_miR-39_1 miScript Primer Assay [cat. no. MS00019789])

Important points before starting

- If using the miRNeasy Mini Kit for the first time, read "Important Notes" in the *miRNeasy Mini Handbook*.
- If working with RNA for the first time, read Appendix E in the *miRNeasy Mini Handbook*.

- After collection and centrifugation, plasma or serum can be stored at 2–8°C for up to 6 hours or used directly in the procedure. For long-term storage, freezing at –20°C or –80°C in aliquots is recommended. To process frozen, homogenized lysates, incubate at 37°C in a water bath until samples are completely thawed and salts are dissolved. Avoid prolonged incubation, which may compromise RNA integrity.
- DNase I digestion is not recommended for plasma or serum samples. The combined QIAzol® and RNeasy® technologies efficiently remove most of the trace amounts of DNA in plasma and serum. In addition, miScript Primer Assays and most other assays for mature miRNA are not affected by the presence of small amounts of genomic DNA. In some cases, on-column DNase treatment may reduce recovery of small RNA from plasma or serum.
- Buffer RWT may form a precipitate upon storage. If necessary, redissolve by warming and then place at room temperature (15–25°C).
- QIAzol Lysis Reagent and Buffer RWT contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See the "Safety Information" in the *miRNeasy Mini Handbook*.
- Except for phase separation (step 7), all protocol and centrifugation steps should be performed at room temperature.
- The procedure is suitable for use with serum samples or with plasma samples containing either citrate or EDTA. Plasma samples containing heparin should not be used because this anticoagulant can interfere with downstream assays, such as RT-PCR.

Things to do before starting

- Buffers RWT and RPE are supplied as concentrates. Before using for the first time, add the required volumes of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.
- Optional: If desired, synthetic *C. elegans* miRNA can be added to samples to control for variations during the preparation of total RNA and subsequent steps. After purification, real-time RT-PCR detection of the *C. elegans* miRNA can be performed and these results can be used for normalization of real-time RT-PCR results of endogenous miRNAs in the sample. This corrects for variations during RNA preparation, cDNA synthesis, and real-time PCR.
  We recommend Syn-cel-miR-39 miScript miRNA Mimic (cat. no. MSY0000010) for this purpose, as it shows no sequence homology to any known human, mouse, or rat miRNA. miScript miRNA Mimics are provided lyophilized. Prepare miScript miRNA Mimic stock and working solution as follows.
  Briefly centrifuge the miScript miRNA Mimic tube prior to opening as some of the product may have been dislodged during shipping. Resuspend with an appropriate volume of RNase-free water (provided) to obtain a 1 µM stock solution. For example, resuspend 1 nmol miScript miRNA Mimic in 1 ml RNase-free water. To obtain a 5 nM working solution, further dilute 5 µl of 1 µM stock solution in 995 µl RNase-free TE

buffer. Aliquot stock and working solution and store at –20°C for future use. Aliquots are stable for 18 months.

Procedure

1. **Prepare serum or plasma or thaw frozen samples.**
2. **Add 5 volumes QIAzol Lysis Reagent (see table 1 for guidelines). Mix by vortexing or pipetting up and down.**

Table 1. Reagent volumes for various starting volumes of serum/plasma

Serum/plasma (µl)	Protocol step 2: QIAzol Lysis Reagent (µl)	Protocol step 4: Chloroform (µl)	Protocol step 8: Approx. volume of upper aqueous phase (µl)	Protocol step 8: 100% Ethanol (µl)
≤50	250	50	150	225
100	500	100	300	450
200	1000	200	600	900

Note: If the volume of plasma or serum is not limited, we recommend using 100–200 µl per RNA preparation.

Note: After addition of QIAzol Lysis Reagent, lysates can be stored at –70°C for several months.

3. **Place the tube containing the homogenate on the benchtop at room temperature (15–25°C) for 5 min.**

   This step promotes dissociation of nucleoprotein complexes.

4. **Optional: Add 5 µl of 5 nM Syn-cel-miR-39 miScript miRNA Mimic.**

   Details of how to prepare a 5 nM miScript miRNA Mimic working solution are provided in "Things to do before starting".

5. **Add 1 volume chloroform to the tube containing the homogenate and close securely (see table 1 for guidelines). Vortex the tube vigorously for 15 s.**

   Thorough mixing is important for subsequent phase separation.

6. **Place the tube containing the homogenate on the benchtop at room temperature for 2–3 min.**

7. **Centrifuge for 15 min at 12,000 x *g* at 4°C. After centrifugation, heat the centrifuge up to room temperature (15–25°C) if the same centrifuge will be used for the next centrifugation steps.**

   After centrifugation, the sample separates into 3 phases: an upper, colorless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase. For the approximate volume of the aqueous phase, see Table 1.

8. **Transfer the upper aqueous phase to a new collection tube (supplied). Avoid transfer of any interphase material. Add 1.5 volumes of 100% ethanol and mix thoroughly by pipetting up and down several times. Do not centrifuge. Continue without delay with step 9.**

   For the approximate volume of the aqueous phase and the volume of ethanol to add, see Table 1.

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

9. **Pipet up to 700 µl of the sample, including any precipitate that may have formed, into an RNeasy Mini spin column in a 2 ml collection tube (both supplied). Close the lid gently and centrifuge at ≥8000 x *g* (≥10,000 rpm) for 15 s at room temperature (15–25°C). Discard the flow-through.***

   Reuse the collection tube in step 10.

10. **Repeat step 9 using the remainder of the sample. Discard the flow-through.***

    Reuse the collection tube in step 11.

11. **Add 700 µl Buffer RWT 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 column. Discard the flow-through.***

    Reuse the collection tube in step 12.

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

    Reuse the collection tube in step 13.

13. **Repeat step 12.**

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

* Flow-through contains QIAzol Lysis Reagent or Buffer RWT and is therefore not compatible with bleach. See the "Safety Information" in the *miRNeasy Mini Handbook*.

14. **Place the RNeasy Mini spin column into a new 2 ml collection tube (not supplied), and discard the old collection tube with the flow-through. Centrifuge in a microcentrifuge at full speed for 2 min.**

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

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

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