Supplementary Protocol April 2015

# Purification of exosomal RNA, including miRNA, from cell culture supernatants using the exoRNeasy Serum/Plasma Maxi Kit

This protocol is intended as a guideline for the purification of total RNA including mRNA, miRNA and other non-coding RNAs from exosomes and other extracellular vesicles (EVs) in cell culture supernatant, using the exoRNeasy Serum/Plasma Maxi Kit (cat. no. 77064). Processing of up to 32 ml of sample has been successfully tested. However, the concentration of vesicles in supernatants depends strongly on the cell type and culture conditions; therefore, we recommend starting with no more than 16 ml of supernatant for sample material that has not been tested with the kit previously. We recommend using this protocol with the miRNeasy Serum/Plasma Spike-In Control (cat. no. 219610), which must be ordered separately.

## Important points before starting

- For isolation of vesicular RNA from cell culture supernatant, use either serum-free culture medium or medium prepared with vesicle-free serum.
- After collection and centrifugation or filtration, cell culture supernatants can be stored at 2–8°C for up to 6 hours or used directly in the procedure. For long-term storage, freeze in aliquots at –20°C or –65°C to –90°C. To process frozen samples, incubate at 37°C in a water bath until samples are completely thawed. Avoid prolonged incubation as this may compromise RNA yield and integrity.
- DNase I digestion is not recommended for cell culture supernatants since the combined QIAzol® and RNeasy® technologies efficiently remove DNA present in EVs. In addition, miScript® Primer Assays and most other assays for mature miRNA are not affected by the presence of small amounts of genomic DNA. On-column DNase treatment may reduce recovery of small RNA from EVs.
- 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.
- Except for phase separation (step 11), all protocol and centrifugation steps should be performed at room temperature.

- Centrifugation of the exoEasy spin columns is performed in a standard laboratory centrifuge with a swinging bucket rotor, preferably capable of up to 5000 x *g*. It is possible to reduce the centrifugation speed from 5000 x *g* down to a minimum force of 3000 x *g* without loss of performance.
- The RNA purification step in this protocol (step 7) is compatible with QIAGEN MaXtract™ High Density Tubes (cat. no. 129056).

## Things to do before starting

- Buffers RWT and RPE are supplied as concentrates. Before use, add the required volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- Prepare a working solution of miRNeasy Serum/Plasma Spike-In Control as described in Appendix B of the *exoRNeasy Serum/Plasma Handbook*.

## Procedure

1. It is recommended to only use pre-filtered cell culture supernatants. Supernatants should be filtered to exclude particles larger than 0.8 µm (e.g., using Sartorius® Minisart® NML (cat. no. 16592) or Millipore® Millex®-AA (cat. no. SLAA033SB) syringe filters).

   **Note**: Alternatively, an additional centrifugation step can be performed to eliminate residual cellular material (apply the same conditions as for serum or plasma in Appendix A of the *exoRNeasy Serum/Plasma Handbook*).
2. Add 1 volume buffer XBP to 1 volume of sample. Mix well by gently inverting the tube 5 times.
3. Add the sample/XBP mix onto the exoEasy spin column (up to 16 ml) and centrifuge the device at 500 x *g* for 1 min. Discard the flow-through and place the column back into the same collection tube. Repeat this step until the entire sample has been passed through the column.

   **Optional**: To remove residual liquid from the membrane, centrifuge at 5000 x *g* for 1 min.
4. Add 10 ml XWP and centrifuge at 5000 x *g* for 5 min to wash the column and remove residual buffer. Discard the flow-through together with the collection tube.

   **Note**: It is possible to reduce the centrifugation speed from 5000 x *g* down to a minimum force of 3000 x *g* without loss of performance. After centrifugation, carefully remove the exoEasy spin column from the collection tube so that the column does not come into contact with the flow-through.
5. Transfer the spin column to a fresh collection tube.

6. Add 700 µl QIAzol to the membrane. Centrifuge at 5000 x *g* for 5 min to collect the lysate. Transfer the lysate to a 2 ml tube (not supplied).
7. Briefly vortex the lysate and incubate at room temperature (15–25°C) for 5 min.

   This step promotes the dissociation of nucleoprotein complexes.
8. **Optional**: Add 3.5 µl miRNeasy Serum/Plasma Spike-In Control (1.6 x $10^8$ copies/µl working solution).

   For details on making appropriate stocks and working solutions of miRNeasy Serum/Plasma Spike-In Control, see Appendix B of the e*xoRNeasy Serum/Plasma Handbook*.
9. Add 90 µl chloroform to the lysate. Tightly cap the tube and shake vigorously for 15 s.

   Thorough mixing is important for subsequent phase separation.
10. Incubate at room temperature (15–25°C) for 2–3 min.
11. Centrifuge at 12,000 x *g* for 15 min at 4°C. After centrifugation, heat the centrifuge to room temperature (15–25°C) if using the same centrifuge for the following centrifugation steps.

    After centrifugation, the sample separates into 3 phases: an upper, colorless aqueous phase containing RNA; a thin, white interphase; and a lower, red organic phase. The volume of the aqueous phase should be approximately 400 µl.
12. Transfer the upper aqueous phase to a new collection tube (not supplied). Avoid transfer of any interphase material. Add 2 volumes of 100% ethanol (e.g., for 400 µl aqueous phase, add 800 µl ethanol) and mix thoroughly by pipetting up and down several times. Do not centrifuge. Immediately continue to step 13.

    A precipitate may form after addition of ethanol, but this will not affect the procedure.
13. Pipet a maximum of 700 µl sample, including any precipitate that may have formed, onto an RNeasy MinElute spin column in a 2 ml collection tube (supplied). Gently close the lid 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 14.
14. Repeat step 13 using the remainder of the sample. Discard the flow-through.*

    Reuse the collection tube in step 15.
15. Add 700 µl Buffer RWT to the RNeasy MinElute spin column. Gently close the lid and centrifuge at ≥8000 x *g* (≥10,000 rpm) for 15 s. Discard the flow-through.*

    Reuse the collection tube in step 16.

* Flow-through contains QIAzol Lysis Reagent or Buffer RWT and is therefore not compatible with bleach.

16. Pipet 500 µl Buffer RPE onto the RNeasy MinElute spin column. Gently close the lid and centrifuge at ≥8000 x *g* (≥10,000 rpm) for 15 s. Discard the flow-through.

    Reuse the collection tube in step 17.

17. Pipet 500 µl Buffer RPE onto the RNeasy MinElute spin column. Close the lid and centrifuge at ≥8000 x *g* (≥10,000 rpm) for 2 min. Discard the collection tube with the flow-through.

    **Note**: After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not come into contact with the flow-through and cause carryover of ethanol.

18. Place the RNeasy MinElute spin column into a new 2 ml collection tube (supplied). Open the lid of the spin column and centrifuge at full speed for 5 min to dry the membrane. Discard the collection tube with the flow-through.

    To avoid damaging the spin column lids, place the spin columns into the centrifuge with at least one empty position between columns. Position 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, because residual ethanol may interfere with downstream reactions. Centrifugation with open lids ensures that no ethanol is carried over during RNA elution.

19. Place the RNeasy MinElute spin column in a new 1.5 ml collection tube (supplied). Add 14 µl RNase-free water directly to the center of the spin column membrane. Gently close the lid, let the column stand for 1min and then centrifuge at full speed for 1 min to elute the RNA.

    As little as 10 µl RNase-free water can be used for elution if a higher RNA concentration is required, but the yield will be reduced by approximately 20%. Do not elute with less than 10 µl RNase-free water, as the spin column membrane will not be sufficiently hydrated.

    The dead volume of the RNeasy MinElute spin column is 2 µl; elution with 14 µl RNase-free water results in a 12 µl eluate.

## Ordering Information

| Product | Contents | Cat. no. |
|---|---|---|
| exoRNeasy Serum/Plasma Maxi Kit | For 50 RNA preps: 50 exoEasy Maxi and RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml, 2 ml and 50 ml), QIAzol Lysis Reagent, Ce_miR-39_1 miScript Primer Assay, RNase-free Reagents and Buffers | 77064 |
| exoRNeasy Serum/Plasma Midi Kit | For 50 RNA preps: 50 exoEasy Midi and RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml, 2 ml and 50 ml), QIAzol Lysis Reagent, Ce_miR-39_1 miScript Primer Assay, RNase-free Reagents and Buffers | 77044 |
| exoRNeasy Serum/Plasma Starter Kit | For 20 RNA preps: 10 exoEasy Maxi, 10 exoEasy Midi, 20 RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml, 2 ml and 50 ml), QIAzol Lysis Reagent, Ce_miR-39_1 miScript Primer Assay, RNase-free Reagents and Buffers | 77023 |
| **Related products** | | |
| exoEasy Maxi Kit | For 20 vesicle preps: 20 exoEasy Maxi Spin Columns, Collection Tubes (50 ml), Reagents and Buffers | 76064 |
| miRNeasy Serum/Plasma Spike-In Control | 10 pmol lyophilized C. elegans miR-39 miRNA mimic | 219610 |
| MaXtract High Density (200 x 2 ml) | 200 x 2 ml MaXtract High Density Tubes | 129056 |
| **Related Products for quantitative, real-time RT-PCR** | | |
| miScript II RT Kit (12) | For 12 cDNA synthesis reactions: miScript Reverse Transcriptase Mix, 10x miScript Nucleics Mix, 5x miScript HiSpec Buffer, 5x miScript HiFlex Buffer, RNase-Free Water | 218160 |
| miScript II RT Kit (50) | For 50 cDNA synthesis reactions: miScript Reverse Transcriptase Mix, 10x miScript Nucleics Mix, 5x miScript HiSpec Buffer, 5x miScript HiFlex Buffer, RNase-Free Water | 218161 |
| miScript SYBR® Green PCR Kit (200) | For 200 reactions: QuantiTect SYBR Green PCR Master Mix, miScript Universal Primer | 218073 |
| miScript SYBR Green PCR Kit (1000) | For 1000 reactions: QuantiTect SYBR Green PCR Master Mix, miScript Universal Primer | 218075 |
| miScript Primer Assay (100) | miRNA-specific primer; available via GeneGlobe | Varies* |

* Visit **www.qiagen.com/GeneGlobe** to search for and order these products.

| Product | Contents | Cat. no. |
|---|---|---|
| miScript PreAMP PCR Kit (12) | For 12 preamplification reactions: 5x miScript PreAMP Buffer, HotStarTaq DNA Polymerase (2 U/µl), miScript PreAMP Universal Primer, 4 miScript Primer Assays, RNase-Free Water | 331451 |
| miScript PreAMP PCR Kit (60) | For 60 preamplification reactions: 5x miScript PreAMP Buffer, HotStarTaq DNA Polymerase (2 U/µl), miScript PreAMP Universal Primer, 4 miScript Primer Assays, RNase-Free Water | 331452 |
| miScript PreAMP Primer Mix | Primer mix for preamplification; for use with corresponding miScript miRNA PCR Array | Varies* |
| Pathway-Focused miScript miRNA PCR Array | Array of assays for a pathway, disease, or gene family for human, mouse, rat, dog, or rhesus macaque miRNAs; available in 96-well, 384-well, or Rotor-Disc 100 format | Varies* |
| miScript miRNA HC PCR Array | High-content array of assays for a pathway, disease, or gene family miRNAs; available in 384-well format | Varies* |
| miRNome miScript miRNA PCR Array | Assays for the complete human, mouse, rat, dog, or rhesus macaque miRNome; available in 96-well, 384-well or Rotor-Disc 100 format | Varies* |

* Visit **www.qiagen.com/GeneGlobe** to search for and order these products

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