Supplementary Protocol August 2018

# Purification of exosomal RNA from urine using the exoRNeasy Serum/Plasma Midi/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) from urine, using the exoRNeasy Serum/Plasma Midi Kit (cat. no. 77044) or the exoRNeasy Serum/Plasma Maxi Kit (cat. no. 77064).

For analysis of RNA specifically from EVs in urine, it is essential to remove residual cells and cell fragments by filtration or centrifugation (see recommendations for serum and plasma in Appendix A of the *exoRNeasy Serum/Plasma Handbook*, **www.qiagen.com/HB-1779**. In some cases, it may be necessary to limit the volume of urine used when isolating short RNAs to as little as 2 ml or, alternatively, to dilute eluates accordingly before analysis. This may be necessary when using first morning urine, which is generally more concentrated than urine collected later in the day. Urine may contain various metabolites that could interfere with RNA analysis using, for example, RT-PCR or RNA-Seq. To ensure optimal performance of the isolated RNA, we recommend separate isolation of long and short RNA species (larger or smaller than approximately 200 nt, respectively). For the isolation of short RNA, use Reagent UI (contact QIAGEN Technical Service for more information). To isolate both long and short RNA in separate fractions, the RNeasy® MinElute Cleanup Kit (cat. no. 74204) and additional Buffer RWT (cat. no. 1067933) are required.

## Description of protocols

This document contains 3 supplementary protocols for RNA purification from exosomes and other EVs from urine samples using the exoRNeasy Serum/Plasma Kits. The exoRNeasy Serum/Plasma Midi Kit can be used for the purification of up to 4 ml prefiltered urine, and the exoRNeasy Serum/Plasma Maxi Kit for up to 16 ml prefiltered urine. Protocols are provided for the isolation of long RNAs (>200 nt) only (page 3), for isolation of short RNAs (page 7) or for isolation of long and short RNAs in separate fractions (page 11). A short summary of the 3 protocols follows.

### Purification of RNA >200 nt from urine using the exoRNeasy Midi/Maxi Kit

Long RNA (including mRNA) from up to 4 ml (Midi) or up to 16 ml (Maxi) of prefiltered urine can be isolated using this protocol. The sample is mixed with Buffer XBP, allowing EVs to bind to the exoEasy Midi/Maxi Spin Column with high efficiency and specificity, leaving non-vesicular components in the column flow-through. After washing, vesicular content is eluted using QIAzol® Lysis Reagent. Following organic extraction with chloroform, long RNA is bound to RNeasy

MinElute columns, washed and eluted in RNase-free water. Binding long RNA only at lower alcohol concentration greatly reduces the risk of co-purifying metabolites that may act as enzymatic inhibitors. Due to low copy numbers of cell-free mRNA in urine, using less than 4 ml sample may prevent detection of any but the most abundant transcripts.

## Purification of short RNA, including miRNA, from urine using the exoRNeasy Midi/Maxi Kit (requires Regent UI)

Short RNA from up to 4 ml (Midi) or up to 16 ml (Maxi) prefiltered urine can be isolated. The sample is mixed with Buffer XBP, allowing EVs to bind to the exoEasy Midi/Maxi Spin Column with high efficiency and specificity, leaving non-vesicular components in the column flow-through. After washing, vesicular content is eluted using QIAzol Lysis Reagent. Following addition of Reagent UI (sold separately) and organic extraction with chloroform, total RNA is bound to RNeasy MinElute columns, washed and eluted in RNase-free water. Copy numbers of cell-free miRNA are generally higher than those for long RNA; therefore, isolation from smaller volumes of sample (or using less template in downstream assays) can still provide conclusive results. Technically, eluates obtained with this protocol also contain long RNA. However, to ensure optimal performance with enzymatic assays, it is not recommended to use this protocol for mRNA or other long RNA analyses.

## Purification of RNA >200 nt and short RNA (including miRNA) in separate fractions from urine samples using the exoRNeasy Midi/Maxi Kit (requires Regent UI)

Long and short RNA from up to 4 ml (Midi) or up to 16 ml (Maxi) prefiltered urine can be isolated in separate fractions. The sample is mixed with Buffer XBP, allowing EVs to bind to the exoEasy Midi/Maxi Spin Column with high efficiency and specificity, leaving non-vesicular components in the column flow-through. After washing, vesicular content is eluted using QIAzol Lysis Reagent. Following organic extraction with chloroform, long RNA is bound to RNeasy MinElute columns, washed and eluted in RNase-free water. After addition of Reagent UI (sold separately) and isopropanol to the first column flow-through, RNA smaller than approximately 200 nt is bound to a second RNeasy MinElute Spin Column (sold separately), washed and eluted in RNase-free water.

## Purification of RNA >200 nt from urine using the exoRNeasy Midi/Maxi Kit

This protocol is intended as a guideline for the purification of long RNA (approximately >200 nt) from exosomes and other EVs, from up to 4 ml (Midi) or up to 16 ml (Maxi) (2 exoEasy column loadings) urine using the exoRNeasy Midi/Maxi Kit. Processing of more than 4 ml (Midi) or 16 ml (Maxi) sample is not recommended, as the number of EVs and interfering substances introduced by larger sample volumes may exceed the binding capacity of the exoEasy Midi/Maxi Spin Column.

To isolate both long and short RNA in separate fractions, follow "Purification of RNA >200 nt and short RNA (including miRNA) in separate fractions from urine samples using the exoRNeasy Midi/Maxi Kit", page 7. To isolate both long and short RNA in one fraction, follow "Purification of short RNA, including miRNA, from urine using the exoRNeasy Midi/Maxi Kit ", page 11.

For recommendations on collection, preparation and storage of cell-free plasma and serum, see Appendix A of the *exoRNeasy Serum/Plasma Handbook*, **www.qiagen.com/HB-1779**.

### Important points before starting

- Although urine stored at 2–8°C for 1 week after collection has been used to produce meaningful results, we recommend using urine directly after collection or freezing it in aliquots at –15°C to –30°C or –65°C to –90°C for long-term storage. To process frozen samples, incubate at 37°C in a water bath until samples are completely thawed. Avoid prolonged incubation, which may compromise RNA yield and integrity.
- DNase I digestion is not recommended for urine samples, since the combined QIAzol and RNeasy technologies efficiently remove any DNA present in EVs. In addition, miRCURY LNA miRNA PCR 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 steps performed at 5000 x *g* down to a minimum force of 3000 x *g* without performance loss).
- The RNA purification part of the protocol (following step 7) is compatible with QIAGEN® MaXtract High Density Tubes (cat. no. 129056).

## Things to do before starting

- Buffer RWT and Buffer RPE are supplied as concentrates. Before using for the first time, add 30 ml ethanol (96–100%) to Buffer RWT and 44 ml ethanol (96–100%) to Buffer RPE to obtain a working solution.

## Procedure

1. It is recommended to only use pre-filtered urine, excluding particles larger than 0.8 µm with syringe filters (e.g., using Sartorius® Minisart NML®, cat. no. 16592, or Millipore® Millex®-AA, cat. no. SLAA033SB).

   **Note**: Alternatively, an additional centrifugation step can be performed to eliminate residual cellular material. See Appendix A of the *exoRNeasy Serum/Plasma Handbook* for detailed recommendations.
2. Add 1 volume Buffer XBP to 1 volume of sample. Mix well immediately by gently inverting the tube 5 times.
3. Add 4 (Midi) or 16 ml (Maxi) of the sample–Buffer XBP mix to the exoEasy Midi/Maxi Spin Column and spin the device for 1 min at 500 x *g*. Discard the flow-through and replace the column in the same collection tube.

   If the starting sample volume is larger than 2 ml (Midi) or 8 ml (Maxi), repeat this step until the entire volume has been passed through the column.

   **Note**: If liquid remains on the membrane, spin again for 1 min at 5000 x *g* to make sure all liquid has passed through the membrane.
4. Add 3.5 ml (Midi) or 10 ml (Maxi) Buffer XWP and spin 5 min at 5000 x *g* to wash the column and remove residual buffer. Discard the flow-through together with the collection tube.

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

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

   This step promotes dissociation of nucleoprotein complexes.
8. Add 90 µl chloroform to the tube containing the lysate and cap it securely. Shake vigorously for 15 s.

   Thorough mixing is important for subsequent phase separation.
9. Incubate at room temperature for 2–3 min.
10. Centrifuge for 15 min at 12,000 x *g* at 4°C. After centrifugation, heat the centrifuge to room temperature 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 thin, white interphase; and a lower, red organic phase. The volume of the aqueous phase should be approximately 400 µl.
11. Transfer the upper aqueous phase to a new collection tube (not supplied). Avoid transfer of interphase material. Add 1 volume of 70% ethanol (e.g., for 400 µl aqueous phase, add 400 µl 70% ethanol) and mix thoroughly by pipetting up and down several times or vortexing. Do not centrifuge. Continue without delay.

    A precipitate may form after addition of ethanol, but this will not affect the procedure.
12. Pipet up to 700 µl sample, including any precipitate that may have formed, into an RNeasy MinElute Spin Column in a 2 ml collection tube (supplied). Close the lid gently and centrifuge at ≥8000 x *g* for 15 s at room temperature. Discard the flow-through.*

    Reuse the collection tube in step 13–16.
13. Repeat step 12 using the remainder of the sample. Discard the flow-through.*
14. Add 700 µl Buffer RWT to the RNeasy MinElute Spin Column. Close the lid gently and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.*
15. Pipet 500 µl Buffer RPE into the RNeasy MinElute Spin Column. Close the lid gently and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.
16. Pipet 500 µl Buffer RPE into the RNeasy MinElute Spin Column. Close the lid, and centrifuge for 2 min at ≥8000 x *g*. 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 contact the flow-through. Otherwise, carryover of ethanol will occur.

* Flow-through contains a guanidine salt and is therefore not compatible with bleach.

17. 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 damage to the 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.

18. 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. Close the lid gently, let column stand for 1 min and then centrifuge for 1 min at full speed 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.

## Purification of short RNA, including miRNA, from urine using the exoRNeasy Midi/Maxi Kit

This protocol is intended as a guideline for the purification of short RNA from exosomes and other EVs, from up to 4 ml (Midi) or up to 16 ml (Maxi) (2 exoEasy column loadings) urine using the exoRNeasy Midi/Maxi Kit. Even though the protocol is for isolating total RNA, it is specifically recommended for isolation of miRNA (and other short RNA). Due to high copy numbers of short RNAs in urine, using more than 4 ml sample may be necessary only for very low abundant transcripts.

Processing more than 4 ml (Midi) or 16 ml (Maxi) sample is not recommended, as the number of EVs and interfering substances introduced by larger sample volumes may exceed the binding capacity of the exoEasy Midi/Maxi Spin Column, resulting in lower yields and purity.

To isolate both long and short RNA in separate fractions, follow protocol "Purification of RNA >200 nt and short RNA (including miRNA) in separate fractions from urine samples using the exoRNeasy Midi/Maxi Kit", page 11. To isolate long RNA only, follow protocol "Purification of RNA >200 nt from urine using the exoRNeasy Midi/Maxi Kit", page 3.

For recommendations on collection, preparation and storage of cell-free plasma and serum, see Appendix A of the *exoRNeasy Serum/Plasma Handbook*, **www.qiagen.com/HB-1779**.

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

- Although urine stored at 2–8°C for 1 week after collection has been used to produce meaningful results, we recommend using urine directly after collection or freezing it in aliquots at –15°C to –30°C or –65°C to –90°C for long-term storage. To process frozen samples, incubate at 37°C in a water bath until samples are completely thawed. Avoid prolonged incubation, which may compromise RNA yield and integrity.
- DNase I digestion is not recommended for urine samples, since the combined QIAzol and RNeasy technologies efficiently remove any DNA present in EVs. In addition, miRCURY LNA miRNA PCR 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 10), all protocol and centrifugation steps should be performed at room temperature.
- Centrifugation of the exoEasy spin columns are 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 steps performed at 5000 x *g* down to a minimum force of 3000 x *g* without performance loss).
- The RNA purification part of the protocol (following step 7) is compatible with QIAGEN® MaXtract High Density Tubes (cat. no. 129056).

## Things to do before starting

- Buffer RWT and Buffer RPE are supplied as concentrates. Before using for the first time, add 30 ml ethanol (96–100%) to Buffer RWT and 44 ml ethanol (96–100%) to Buffer RPE to obtain a working solution.
- Prepare a working solution of miRNeasy miRNeasy Serum/Plasma Spike-In Control as described in Appendix B of the *exoRNeasy Serum/Plasma Handbook*.

## Procedure

1. It is recommended to use only pre-filtered urine, excluding particles larger than 0.8 µm with syringe filters (e.g., using Sartorius Minisart NML, cat. no. 16592, or Millipore Millex-AA, cat. no. SLAA033SB).

   **Note**: Alternatively, an additional centrifugation step can be performed to eliminate residual cellular material. See Appendix A of the *exoRNeasy Serum/Plasma Handbook* for detailed recommendations.
2. Add 1 volume Buffer XBP to 1 volume of sample. Mix well immediately by gently inverting the tube 5 times.
3. Pipet up to 4 ml (Midi) or up to 16 ml (Maxi) of the sample–Buffer XBP mix into the exoEasy Midi/Maxi Spin Column and spin the device for 1 min at 500 x *g*. Discard the flow-through and replace the column in the same collection tube. If the starting sample volume is larger than 2 ml (Midi) or 8 ml (Maxi), repeat this step until the entire volume has been passed through the column.

   **Note**: If liquid remains on the membrane, spin again for 1 min at 5000 x *g* to make sure all liquid has passed through the membrane.

4. Add 3.5 ml (Midi) or 10 ml (Maxi) Buffer XWP and spin 5 min at 5000 x *g* to wash the column and remove residual buffer. Discard the flow-through together with the collection tube.

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

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

   This step promotes dissociation of nucleoprotein complexes.

   **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 *exoRNeasy Serum/Plasma Handbook*.

8. Add 90 µl chloroform to the tube containing the lysate and cap it securely. Shake vigorously for 15 s.

   Thorough mixing is important for subsequent phase separation.

9. Incubate at room temperature for 2–3 min.
10. Centrifuge for 15 min at 12,000 x *g* at 4°C. After centrifugation, heat the centrifuge to room temperature 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 thin, white interphase; and a lower, red organic phase. The volume of the aqueous phase should be approximately 400 µl.

11. Transfer the upper aqueous phase to a new collection tube (not supplied). Avoid transfer of interphase material. Add 1/50 volume Reagent UI (e.g., for 400 µl aqueous phase, add 8 µl) and 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 or vortexing. Do not centrifuge. Continue without delay.

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

12. Pipet up to 700 µl sample, including any precipitate that may have formed, into an RNeasy MinElute Spin Column in a 2 ml collection tube (supplied). Close the lid gently and centrifuge at ≥8000 x *g* for 15 s at room temperature. Discard the flow-through.*

    Reuse the collection tube in steps 13–16.

* Flow-through contains a guanidine salt and is therefore not compatible with bleach.

13. Repeat step 12 using the remainder of the sample. Discard the flow-through. *

14. Add 700 µl Buffer RWT to the RNeasy MinElute Spin Column. Close the lid gently and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.*

15. Pipet 500 µl Buffer RPE into the RNeasy MinElute Spin Column. Close the lid gently and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.

16. Pipet 500 µl Buffer RPE into the RNeasy MinElute Spin Column. Close the lid, and centrifuge for 2 min at ≥8000 x *g*. 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 contact the flow-through. Otherwise, carryover of ethanol will occur.

17. 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 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.

18. 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. Close the lid gently, let column stand for 1 min and then centrifuge for 1 min at full speed 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.

* Flow-through contains a guanidine salt and is therefore not compatible with bleach.

## Purification of RNA >200 nt and short RNA (including miRNA) in separate fractions from urine samples using the exoRNeasy Midi/Maxi Kit

This protocol is intended as a guideline for the purification of long RNA (approximately >200 nt) from exosomes and other EVs, from up to 4 ml (Midi) or up to 16 ml (Maxi) (2 exoEasy column loadings) urine using the exoRNeasy Midi/Maxi Kit together with short RNA (approximately <200 nt, including miRNA) in a separate fraction. Due to low copy numbers of cell-free mRNA in urine, using less than 4 ml sample may prevent detection of any but the most abundant transcripts. To isolate both long and short RNA in separate fractions, an RNeasy MinElute Cleanup Kit (cat. no. 74204) and additional Buffer RWT (cat. no. 1067933) are required.

We recommend using this protocol with the miRNeasy Serum/Plasma Spike-In Control (cat. no. 219610), which must be ordered separately.

Processing of more than 4 ml (Midi) or 16 ml (Maxi) sample is not recommended, as the number of EVs and interfering substances introduced by larger sample volumes may exceed the binding capacity of the exoEasy Midi/Maxi Spin Column, resulting in lower yields and purity.

To isolate both long and short RNA in one fraction, follow "Purification of short RNA, including miRNA, from urine using the exoRNeasy Midi/Maxi Kit", page 7. To isolate long RNA only, follow "Purification of RNA >200 nt from urine using the exoRNeasy Midi/Maxi Kit", page 3.

For recommendations on collection, preparation and storage of cell-free plasma and serum, see Appendix A of the *exoRNeasy Serum/Plasma Handbook*, **www.qiagen.com/HB-1779**.

Important points before starting

- Although urine stored at 2–8°C for 1 week after collection has been used to produce meaningful results, we recommend using urine directly after collection or freezing it in aliquots at –15°C to –30°C or –65°C to –90°C for long-term storage. To process frozen samples, incubate at 37°C in a water bath until samples are completely thawed. Avoid prolonged incubation, which may compromise RNA yield and integrity.
- DNase I digestion is not recommended for urine samples, since the combined QIAzol and RNeasy technologies efficiently remove any DNA present in EVs. In addition, miRCURY LNA miRNA PCR 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 10), all protocol and centrifugation steps should be performed at room temperature.
- Centrifugation of the exoEasy spin columns are 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 steps performed at 5000 x *g* down to a minimum force of 3000 x *g* without performance loss).
- The RNA purification part of the protocol (following step 7) is compatible with QIAGEN MaXtract High Density Tubes (cat. no. 129056).

## Things to do before starting

- Buffer RWT and Buffer RPE are supplied as concentrates. Before using for the first time, add 30 ml ethanol (96–100%) to Buffer RWT and 44 ml ethanol (96–100%) to Buffer RPE to obtain a working solution.
- Prepare a working solution of miRNeasy 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 urine, excluding particles larger than 0.8 µm with syringe filters (e.g., using Sartorius Minisart NML, cat. no. 16592, or Millipore Millex-AA, cat. no. SLAA033SB).

   **Note**: Alternatively, an additional centrifugation step can be performed to eliminate residual cellular material. See Appendix A of the *exoRNeasy Serum/Plasma Handbook* for detailed recommendations.
2. Add 1 volume Buffer XBP to 1 volume of sample. Mix well immediately by gently inverting the tube 5 times.

3. Pipet up to 4 ml (Midi) or up to 16 ml (Maxi) of the sample–Buffer XBP mix into the exoEasy Midi/Maxi Spin Column and spin the device for 1 min at 500 x *g*. Discard the flow-through and replace the column in the same collection tube. If the starting sample volume is larger than 2 ml (Midi) or 8 ml (Maxi), repeat this step until the entire volume has been passed through the column.

   **Note**: If liquid remains on the membrane, spin again for 1 min at 5000 x *g* to make sure all liquid has passed through the membrane.

4. Add 3.5 ml (Midi) or 10 ml (Maxi) Buffer XWP and spin 5 min at 5000 x *g* to wash the column and remove residual buffer. Discard the flow-through together with the collection tube.

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

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

   This step promotes dissociation of nucleoprotein complexes.

   **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 *exoRNeasy Serum/Plasma Handbook*.

8. Add 90 µl chloroform to the tube containing the lysate and cap it securely. Shake vigorously for 15 s.

   Thorough mixing is important for subsequent phase separation.

9. Incubate at room temperature for 2–3 min.
10. Centrifuge for 15 min at 12,000 x *g* at 4°C. After centrifugation, heat the centrifuge to room temperature 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 thin, white interphase; and a lower, red organic phase. The volume of the aqueous phase should be approximately 400 µl.

11. Transfer the upper aqueous phase to a new collection tube (not supplied). Avoid transfer of any interphase material. Add 1 volume of 70% ethanol (e.g., for 400 µl aqueous phase, add 400 µl 70% ethanol) and mix thoroughly by pipetting up and down several times or vortexing. Do not centrifuge. Continue without delay.

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

12. Pipet up to 700 µl sample, including any precipitate that may have formed, into an RNeasy MinElute Spin Column in a 2 ml collection tube (supplied). Close the lid gently and centrifuge at ≥8000 x *g* for 15 s at room temperature. Pipet the flow-through, which contains miRNA and other short RNA, into a new 2 ml reaction tube (not supplied) and keep on ice for up to 6 h.

    Reuse the collection tube in step 13.

13. Repeat step 12 using the remainder of the sample. Add the flow-through to the flow-through from step 12.*

## Purifying the long RNA fraction (approximately >200 nt)

14. Add 700 µl Buffer RWT to the RNeasy MinElute Spin Column. Close the lid gently and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.*

    Reuse the collection tube in steps 15 and 16.

15. Pipet 500 µl Buffer RPE into the RNeasy MinElute Spin Column. Close the lid gently and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.

16. Pipet 500 µl Buffer RPE into the RNeasy MinElute Spin Column. Close the lid, and centrifuge for 2 min at ≥8000 x *g*. 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 contact the flow-through. Otherwise, carryover of ethanol will occur.

17. 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 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.

18. 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. Close the lid gently, let column stand for 1 min and then centrifuge for 1 min at full speed to elute the RNA.

* Flow-through contains a guanidine salt and is therefore not compatible with bleach.

19. 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.

## Purifying the short RNA fraction (approximately <200 nt)

20. To the combined flow-through from steps 12 and 13, add 1/50 volume Reagent UI (e.g., for 800 µl flow-through, add 16 µl) and 0.7 volumes of 100% isopropanol and mix thoroughly by vortexing. Do not centrifuge. Proceed immediately to step 21.
21. Pipet up to 700 µl sample, including any precipitate that may have formed, into an RNeasy MinElute Spin Column in a 2 ml collection tube (supplied). Close the lid gently and centrifuge at ≥8000 x *g* for 15 s at room temperature. Discard the flow-through. *

    Reuse the collection tube in step 22–25.
22. Repeat step 21 using the remainder of the sample. Discard the flow-through.*
23. Add 700 µl Buffer RWT to the RNeasy MinElute Spin Column. Close the lid gently and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.*
24. Pipet 500 µl Buffer RPE into the RNeasy MinElute Spin Column. Close the lid gently and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.
25. Pipet 500 µl Buffer RPE into the RNeasy MinElute Spin Column. Close the lid, and centrifuge for 2 min at ≥8000 x *g*. 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 contact the flow-through. Otherwise, carryover of ethanol will occur.
26. 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 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.

* Flow-through contains a guanidine salt and is therefore not compatible with bleach.

27. 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. Close the lid gently, let column stand for 1min and then centrifuge for 1 min at full speed 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 |
| Buffer RWT | 80 ml Buffer RWT concentrate. For purification of RNA, including miRNA | 1067933 |
| miRNeasy Serum/Plasma Spike-In Control | 10 pmol lyophilized *C. elegans* miR-39 miRNA mimic | 219610 |
| RNeasy MinElute Cleanup Kit | For RNA cleanup and concentration with small elution volumes | 74204 |
| MaXtract High Density (200 x 2 ml) | 200 x 2 ml MaXtract High Density Tubes | 129056 |

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## Notes

## Notes