August 2019

exoRNeasy Midi/Maxi Handbook

exoRNeasy Maxi Kit

exoRNeasy Midi Kit

exoRNeasy Serum/Plasma Starter Kit

For purification of total RNA, including miRNA, from exosomes and other extracellular vesicles (EVs)

Sample to Insight

QIAGEN

Contents

Kit Contents

exoRNeasy Maxi Kit	**(50)**
Catalog no.	**77164**
Number of preps	**50**
exoEasy Maxi Spin Columns (in 50 ml Collection Tube)	50
Collection tubes (50 ml)	50
Buffer XBP	2 x 250 ml
Buffer XWP	1000 ml
RNeasy® MinElute® Spin Columns (in 2 ml Collection Tube)	50
Collection tubes (1.5 ml)	50
Collection tubes (2 ml)	50
QIAzol® Lysis Reagent*	50 ml
Buffer RWT*†	15 ml
Buffer RPE‡	11 ml
RNase-free water	10 ml
Quick-Start Protocol	2

* Contains a guanidine salt. Not compatible with disinfectants containing bleach.

† Buffer RWT is supplied as a concentrate. Before using for the first time, add 2 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.

‡ 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.

exoRNeasy Midi Kit	**(50)**
Catalog no.	**77144**
Number of preps	**50**
exoEasy Midi Spin Columns (in 50 ml Collection Tube)	50
Collection tubes (15 ml)	50
Buffer XBP	2 x 55 ml
Buffer XWP	200 ml
RNeasy MinElute Spin Columns (in 2 ml Collection Tube)	50
Collection tubes (1.5 ml)	50
Collection tubes (2 ml)	50
QIAzol Lysis Reagent*	50 ml
Buffer RWT*†	15 ml
Buffer RPE‡	11 ml
RNase-free water	10 ml
Quick-Start Protocol	2

* Contains a guanidine salt. Not compatible with disinfectants containing bleach.

† Buffer RWT is supplied as a concentrate. Before using for the first time, add 2 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.

‡ 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.

exoRNeasy Serum/Plasma Starter Kit	**(20)**
Catalog no.	**77023**
Number of preps	**20**
exoEasy Maxi Spin Columns (in 50 ml Collection Tube)	10
exoEasy Midi Spin Columns (in 15 ml Collection Tube)	10
Collection Tubes (50 ml)	10
Collection Tubes (15 ml)	10
Buffer XBP	55 ml
Buffer XWP	200 ml
RNeasy MinElute Spin Columns (in 2 ml Collection Tube)	20
Collection Tubes (1.5 ml)	50
Collection Tubes (2 ml)	50
QIAzol Lysis Reagent*	50 ml
Buffer RWT*†	15 ml
Buffer RPE‡	11 ml
RNase-free water	10 ml
Quick-Start Protocol	2

* Contains a guanidine salt. Not compatible with disinfectants containing bleach.

† Buffer RWT is supplied as a concentrate. Before using for the first time, add 2 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.

‡ 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.

Shipping and Storage

The exoRNeasy Kits (cat. no. 77164, 77144, 77023) are shipped at ambient temperature. Store the RNeasy MinElute spin columns immediately at 2–8°C. QIAzol Lysis Reagent can be stored at room temperature (15–25°C) or at 2–8°C. Store the remaining components dry at room temperature. All kit components are stable for at least 9 months upon arrival under these conditions if not otherwise stated on the label.

Intended Use

The exoRNeasy Kits are intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

QIAcube® Connect is designed to perform fully automated purification of nucleic acids and proteins in molecular biology applications. The system is intended for use by professional users trained in molecular biological techniques and the operation of QIAcube Connect.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety** where you can find, view, and print the SDS for each QIAGEN kit and kit component.

CAUTION	
	DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

Buffer RWT and QIAzol Lysis Reagent contain guanidinium thiocyanate, which can form highly reactive compounds when combined with bleach.

If liquid containing these buffers is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of exoRNeasy Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

Recent years have seen an increased interest in the significance of RNA and other molecules carried by exosomes and other extracellular vesicles (EVs). Specifically, these vesicles may be the key to identifying circulating biomarkers. Until now, methods for purifying exosomes for RNA isolation have been time-consuming and inconsistent due to the use of ultracentrifugation.

The exoRNeasy Kits are designed for rapid purification of total vesicular RNA — including noncoding RNA, mRNA, miRNA, and other small RNA — from serum or plasma (up to 1 ml with the midi format, and up to 4 ml with the maxi format), cerebrospinal fluid (CSF; up to 2/8 ml), cell culture supernatant (up to 32 ml using the Maxi format), or urine (up to 4/16 ml). Other cell-free biofluids have not been tested thoroughly, but may also be compatible.

The exoRNeasy Serum/Plasma Starter Kit provides both ExoEasy Midi and Maxi columns, enabling analysis at varied volumes.

When working with cell-free biofluid samples, we recommend use of a synthetic spike-in control for normalization between multiple samples, such as the miRNeasy Serum/Plasma Spike-In Control (cat. no. 219610) or the miRCURY RNA Spike-In Kit, for RT (cat. no. 339390). These spike-in controls must be ordered separately.

Principle and procedure

The exoEasy Maxi Kit uses a membrane-based affinity binding step to isolate exosomes and other EVs from cell-free biofluids. The method does not distinguish EVs by size or cellular origin, and it is not dependent on the presence of a particular epitope. Instead, it makes use of a generic biochemical feature of vesicles to recover the entire spectrum of EVs present in a sample.

For analysis of RNA specifically from EVs, it is essential to remove residual cells, cell fragments, and apoptotic bodies by filtration or centrifugation beforehand (see recommendations in Appendix A). Otherwise, the amount of residual cellular RNA far exceeds the cell-free RNA, thereby making its analysis virtually impossible.

Particulate matter other than vesicles – such as larger protein complexes that are especially abundant in plasma and serum – is largely removed during the binding step and the ensuing wash step.

A phenol/guanidine-based combined lysis and elution step recovers vesicular RNA from the exoEasy spin columns, which is followed by silica-membrane–based purification of total RNA. QIAzol Lysis Reagent, included in the kit, is a monophasic solution of phenol and guanidine thiocyanate, designed to facilitate lysis. This reagent denatures protein complexes and RNases, and also removes most of the residual DNA and proteins from the lysate by organic extraction.

After the lysis and elution step and addition of chloroform, the lysate is separated into aqueous and organic phases by centrifugation. RNA partitions to the upper, aqueous phase, while DNA partitions to the interphase and proteins to the lower, organic phase or the interphase.

The upper, aqueous phase is transferred to a new tube, and ethanol is added to provide appropriate binding conditions for all RNA molecules, including miRNA and other small RNA. The sample is then applied to the RNeasy MinElute spin column, where the RNA binds to the membrane. Phenol and other contaminants are efficiently washed away. High-quality RNA is then eluted in a small volume of RNase-free water.

RNA from EVs has a different size distribution compared to cellular RNA. Even though full-length mRNA and also rRNA are present and efficiently recovered by exoRNeasy, the proportion of small RNA is much higher. Enrichment of small RNA in a separate fraction is usually not required; but for some applications for analysis of mRNA and other long RNAs, removal of short RNAs might be beneficial (see "Appendix B: Isolation of Long Exosomal RNA Only").

Note: For brevity, this handbook uses the terms *exosomal RNA* or *vesicular RNA* to refer to total RNA from all exosomes and other extracellular vesicles.

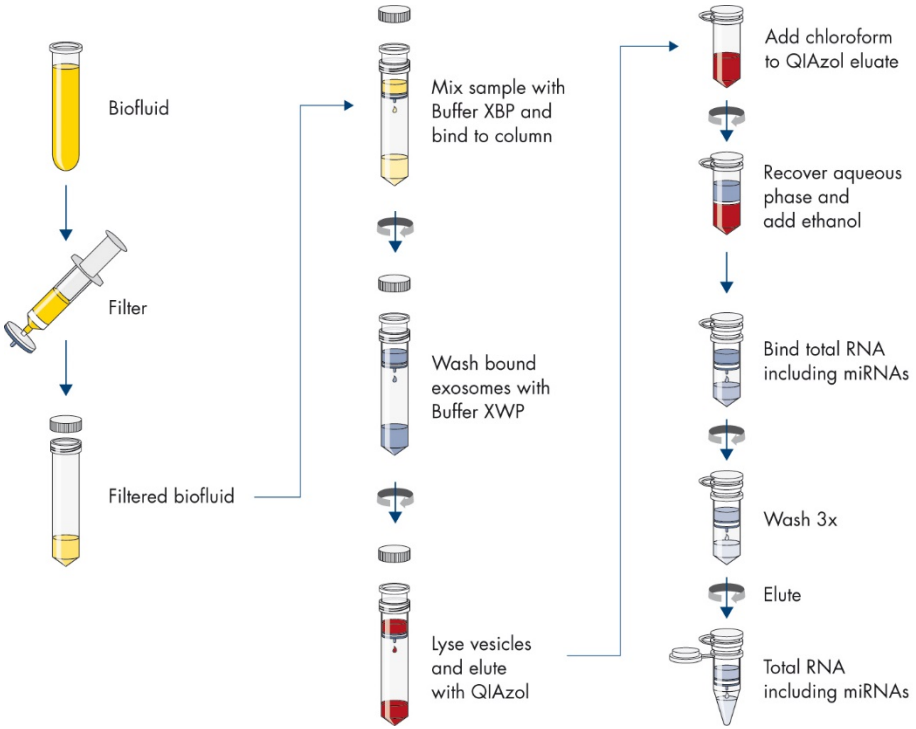

Figure 1. The exoRNeasy Midi/Maxi Kit – exosome purification and total RNA isolation in less than 1 hour. Starting with filtered biofluid, the exoRNeasy Midi/Maxi Kits provide isolation of EVs in just 20 min. A subsequent 35 min isolation procedure yields total RNA, including miRNA.

Descriptions of protocols

This handbook contains protocols for RNA purification from exosomes and other EVs from plasma, serum, urine, CSF, and cell culture supernatant samples, using the exoRNeasy Midi and Maxi Kits. For recommended maximum sample input volumes, see Table 1, page 18.

For certain applications (e.g., certain NGS library preparation methods), excluding short RNA may be beneficial or recommended. In such cases, the exoRNeasy protocols can be modified to exclude RNA shorter than approximately 200 nt. For details please refer to Appendix B.

Protocol: Purification of Total Exosomal RNA, Including miRNA, from Serum and Plasma

This protocol is for isolating total RNA from exosomes and other EVs, including mRNA, miRNA, and other noncoding RNAs, from 100 µl – 1 ml serum and plasma using the exoRNeasy Midi Kit or from 1–4 ml serum and plasma using the exoRNeasy Serum/Plasma Maxi Kit.

Protocol: Purification of Exosomal RNA, Including miRNA, from Cerebrospinal Fluid

This protocol is intended as a guideline for the purification of total RNA, including mRNA, miRNA, and other noncoding RNAs, from exosomes and other EVs in CSF. Use 100 µl – 2 ml CSF with the exoRNeasy Midi Kit or 2–8 ml CSF with the exoRNeasy Maxi Kit. Processing more than 2 ml of sample using the Midi column or 8 ml of sample using the Maxi column is not recommended, as the number of EVs in larger sample volumes may exceed the binding capacity of the exoEasy spin column.

Protocol: Purification of Exosomal RNA, Including miRNA, from Cell Culture Supernatants

This protocol is intended as a guideline for the purification of total RNA, including mRNA, miRNA, and other noncoding RNAs, from exosomes and other EVs in cell culture supernatant, using the exoRNeasy Maxi Kit. 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.

Purification of exosomal RNA from urine samples

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 (cat. no. 77900). 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. Another option is 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 much more concentrated than urine collected later in the day.

Protocol: Purification of Long Exosomal RNA from Urine

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

Binding long RNA only (at lower alcohol concentration) greatly reduces the risk of copurifying 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.

Protocol: Purification of Short Exosomal RNA, Including miRNA, from Urine (Requires Reagent UI)

This protocol is for isolating short RNA from up to 4 ml (Midi) or up to 16 ml (Maxi) of prefiltered urine. Reagent UI (sold separately) is added to the QIAzol lysate prior to phase separation, to prevent carryover of inhibitors into the aqueous phase. 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 avoid any potential issues with inhibition of enzymatic assays, it is not recommended to use this protocol for mRNA or other long RNA analyses, especially from morning urine.

Protocol: Purification of Long Exosomal RNA and Short RNA (Including miRNA) in Separate Fractions from Urine (Requires Reagent UI)

This protocol is intended for the purification of long and short RNA from up to 4 ml (Midi) or up to 16 ml (Maxi) of prefiltered urine in separate fractions. Binding long RNA only (at lower alcohol concentration) greatly reduces the risk of copurifying metabolites that may act as enzymatic inhibitors. 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.

Automated purification of nucleic acids on QIAcube Instruments

Purification of nucleic acids can be fully automated on QIAcube Connect or the classic QIAcube. The innovative QIAcube instruments use advanced technology to process QIAGEN spin columns, enabling seamless integration of automated, low-throughput sample prep into your laboratory workflow. Sample preparation using QIAcube instruments follows the same steps as the manual procedure (i.e., lyse, bind, wash, and elute), enabling you to automate the RNA purification part of exoRNeasy protocols.

QIAcube instruments are preinstalled with protocols for purification of plasmid DNA, genomic DNA, RNA, viral nucleic acids, and proteins, plus DNA and RNA cleanup. The range of protocols available is continually expanding, and additional QIAGEN protocols can be downloaded free of charge at **www.qiagen.com/qiacubeprotocols**.

Figure 2. QIAcube Connect.

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 safety data sheets (SDSs), available from the product supplier.

- Chloroform (without added isoamyl alcohol)
- Ethanol (70%, 80%, and 96–100%)*
- Sterile, RNase-free pipette tips
- 1.5 ml or 2 ml microcentrifuge tubes
- Microcentrifuges with rotor for 2 ml tubes, for centrifugation at 4°C and at room temperature
- Disposable gloves
- The miRNeasy Serum/Plasma Spike-In Control or miRCURY RNA Spike-In Kit must be purchased separately (see "Ordering Information", page 66)
- Reagent UI for processing of urine for miRNA and other short RNA isolation
- Equipment and tubes for serum/plasma collection and separation (see Appendix A):
  - For serum: primary blood collection tubes without anticoagulants such as EDTA or citrate
  - For plasma: primary blood collection tubes containing an anticoagulant such as EDTA or citrate
  - Conical tubes
  - Refrigerated centrifuge with a swinging bucket rotor and fixed-angle rotor
  - Syringe filters excluding particles larger than 0.8 µm (e.g., using Sartorius® Minisart® NML [Sartorius cat. no. 16592] or Millipore® Millex®-AA [Merck cat. no. SLAA033SB]).
- The RNA purification part of the protocol (from step 7 onward) is compatible with QIAGEN MaXtract™ High Density Tubes (cat. no. 129056).

* Do not use denatured alcohol, which contains other substances such as methanol and methylethylketone.

Important Notes

Volume of starting material

The volume of starting material is limited by the binding capacity of the exoEasy spin column. It is not recommended to use more than 4 ml serum or plasma for the maxi column, or more than 1 ml serum or plasma for the midi column. Higher sample volumes may result in reduced RNA yield and copurification of inhibitors. The exoRNeasy Midi kit has been verified to work with sample volumes down to 100 µl, but at such low sample volumes, the low RNA content may only allow the most abundant transcripts to be robustly quantified (e.g., some housekeeping mRNAs and miRNAs). It is recommended to only use prefiltered biofluids, excluding particles larger than 0.8 µm.

Yields of total RNA achieved with the exoRNeasy Midi/Maxi Kits vary strongly between samples from different individuals. However, they are usually too low for quantification by OD measurement. Use of miRNeasy Serum/Plasma Spike-In Control or miRCURY RNA Spike-In Kit and corresponding miRCURY LNA Primer Assays is recommended to monitor miRNA purification and amplification.

Table 1. exoEasy Midi/Maxi spin column specifications

	exoEasy Midi spin column	exoEasy Maxi spin column	Comment
Max. loading volume	4 ml	16 ml	
Max. volume of serum or plasma	1 ml	4 ml	
Max. volume of urine	4 ml	16 ml	Use of larger volumes may result in inhibitor carryover, especially in short RNA isolation
Max. volume of CSF	2 ml	8 ml	
Max. volume of cell culture supernatant	8 ml	32 ml	Strongly dependent on cell type and culture conditions
For binding of	Exosomes and other EVs	Exosomes and other EVs	Elution and lysis using QIAzol (exoRNeasy workflow) or elution of intact vesicles using Buffer XE (exoEasy workflow)

Table 2. RNeasy MinElute spin column specifications

Maximum binding capacity	45 µg RNA
Maximum loading volume	700 µl
RNA size distribution	RNA >18 nucleotides (approximately)*
Minimum elution volume	10 µl

* Transcripts <18 nt not tested.

Note: If the recommended sample volume is exceeded, RNA yields will not be consistent and may be reduced.

Protocol: Purification of Total Exosomal RNA, Including miRNA, from Serum and Plasma

This protocol is intended as a guideline for the purification of total RNA from exosomes and other EVs, including mRNA, miRNA, and other noncoding RNAs, from 100 µl – 1 ml serum and plasma using the exoRNeasy Midi Kit or from 1–4 ml serum and plasma using the exoRNeasy Maxi Kit. Processing of more than 1 ml (Midi) or 4 ml (Maxi) sample is not recommended, because the number of EVs introduced by larger sample volumes will exceed the binding capacity of the exoEasy Midi/Maxi spin columns.

For recommendations on collection, preparation, and storage of cell-free plasma and serum, see Appendix A.

We recommend using this protocol with the miRNeasy Serum/Plasma Spike-In Control or the miRCURY RNA Spike-In Kit, which must be ordered separately.

Important points before starting

- After collection and centrifugation, plasma or serum can be stored at 2–8°C for up to 6 h or used directly in the procedure. For long-term storage, freezing in aliquots is recommended, at –30 to –15°C for up to 1 month or at –90 to –65°C if longer than a month. To process frozen samples, incubate either in a 37°C water bath or at room temperature until samples are completely thawed. When using the water bath, make sure to not leave the samples at 37°C for longer than necessary. Prolonged incubation may compromise RNA yield and integrity.
- DNase I digestion is not recommended for plasma or serum samples, because 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.
- 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 Midi/Maxi 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 procedure is suitable for use with either serum samples or plasma samples containing citrate or EDTA. Plasma samples containing heparin should not be used because this anticoagulant can interfere with downstream assays, such as RT-PCR.
- The RNA purification part of the protocol (from step 7 onward) is compatible with QIAGEN MaXtract High Density Tubes.

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.

Procedure

1. It is recommended to only use prefiltered plasma, excluding particles larger than 0.8 µm (e.g., using Sartorius Minisart NML or Millipore Millex-AA syringe filters).

   Note: Small volumes of sample can be diluted with PBS prior to filtration to minimize loss of material during filtration. Alternatively, an additional centrifugation step can be performed to eliminate residual cellular material. See Appendix A 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 the sample/XBP mix onto the exoEasy spin column and spin the device for 1 min at 500 x *g*. Discard the flow-through and place the column back into the same collection tube.

   Note: In case any 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 Buffer XWP to the exoEasy Midi spin column or 10 ml Buffer XWP to the exoEasy Maxi spin column, 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 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. 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 for 5 min. This step promotes dissociation of nucleoprotein complexes.

   Note: If any spike-in controls are used, they should be added to the lysate at this point.
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 up 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 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. Continue without delay to step 12.

    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* (≥10,000 rpm) for 15 s at room temperature. Discard the flow-through.*

    Reuse the collection tube in the next step.

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

    Reuse the collection tube in the next step.

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* (≥10,000 rpm). Discard the flow-through.*

    Reuse the collection tube in the next step.

15. Pipet 500 µl Buffer RPE onto 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.

    Reuse the collection tube in the next step.

16. Pipet 500 µl Buffer RPE onto the RNeasy MinElute spin column. Close the lid, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm). 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. Otherwise, carryover of ethanol will occur.

* Flow-through contains QIAzol Lysis Reagent or Buffer RWT 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 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, because 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, because 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.

Protocol: Purification of Exosomal RNA, Including miRNA, from Cerebrospinal Fluid

This protocol is intended as a guideline for the purification of total RNA including mRNA, miRNA and other noncoding RNAs from exosomes and other EVs in CSF. Use 100 µl – 2 ml CSF with the exoRNeasy Midi Kit or 2–8 ml CSF with the exoRNeasy Maxi Kit. Processing more than 2 ml of sample using the Midi column or 8 ml of sample using the Maxi column is not recommended, as the number of EVs in larger sample volumes may exceed the binding capacity of the exoEasy spin column. We recommend using this protocol with the miRNeasy Serum/Plasma Spike-In Control or the miRCURY RNA Spike-In Kit, which must be ordered separately.

Important points before starting

- After collection and either centrifugation or filtration, CSF can be stored for up to 6 h at 2–8°C or it can be used directly in the procedure. For long-term storage, freeze in aliquots at –20°C or at –90 to –65°C. To process frozen samples, incubate either in a 37°C water bath or at room temperature until samples are completely thawed. When using the water bath, make sure to not leave the samples at 37°C for longer than necessary. Prolonged incubation may compromise RNA yield and integrity.
- DNase I digestion is not recommended for CSF samples, because the combined QIAzol and RNeasy technologies efficiently remove 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 (protocol step 10), 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 part of the protocol (from step 7 onward) is compatible with QIAGEN MaXtract High Density Tubes.

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.

Procedure

1. It is recommended to only use prefiltered CSF. CSF should be filtered to exclude particles larger than 0.8 µm (e.g., using Sartorius Minisart NML or Millipore Millex-AA 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).
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 or 4 ml using the Maxi or Midi Kit, respectively) 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.

   Optional: To remove residual liquid from the membrane, centrifuge at 5000 x *g* for 1 min.

4. Add 10 ml XWP (3.5 ml for the Midi column) 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 for 5 min.

   This step promotes the dissociation of nucleoprotein complexes.

   Note: If any spike-in controls are used, they should be added to the lysate at this point.

8. 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.
9. Incubate at room temperature for 2–3 min.
10. Centrifuge at 12,000 x *g* for 15 min at 4°C. After centrifugation, heat the centrifuge to room temperature 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.

11. 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 12.

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

12. Pipet a maximum of 700 µl sample, including any precipitate that may have formed, into 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. Discard the flow-through.*

    Reuse the collection tube in the next step.

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

    Reuse the collection tube in the next step.

14. 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 the next step.

15. 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 the next step.

16. Pipet 500 µl Buffer RPE into 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.

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 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).

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

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.

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. Gently close the lid, let the column stand for 1 min, 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, because 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.

Protocol: Purification of Exosomal RNA, Including miRNA, from Cell Culture Supernatants

This protocol is intended as a guideline for the purification of total RNA including mRNA, miRNA and other noncoding RNAs from exosomes and other EVs in cell culture supernatant, using the exoRNeasy Maxi Kit. 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 or the miRCURY RNA Spike-In Kit, 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 either centrifugation or filtration, cell culture supernatants can be stored for up to 6 h at 2–8°C, or they can be used directly in the procedure. For long-term storage, freeze in aliquots at –20°C or at –90 to –65°C. To process frozen samples, incubate either in a 37°C water bath or at room temperature until samples are completely thawed. When using the water bath, make sure to not leave the samples at 37°C for longer than necessary. Prolonged incubation may compromise RNA yield and integrity.
- DNase I digestion is not recommended for cell culture supernatants, because the combined QIAzol and RNeasy technologies efficiently remove 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.
- QIAzol Lysis Reagent and Buffer RWT contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach.
- Except for phase separation (protocol step 10), 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 part of the protocol (from step 7 onward) is compatible with QIAGEN MaXtract High Density Tubes.

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.

Procedure

1. It is recommended to only use prefiltered cell culture supernatants. Supernatants should be filtered to exclude particles larger than 0.8 µm (e.g., using Sartorius Minisart NML or Millipore Millex-AA 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).
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 for 5 min.

   This step promotes the dissociation of nucleoprotein complexes.

   Note: If any spike-in controls are used, they should be added to the lysate at this point.

8. 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.
9. Incubate at room temperature for 2–3 min.
10. Centrifuge at 12,000 x *g* for 15 min at 4°C. After centrifugation, heat the centrifuge to room temperature 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.

11. 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 12.

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

12. Pipet a maximum of 700 µl sample, including any precipitate that may have formed, into 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. Discard the flow-through.*

    Reuse the collection tube in the next step.

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

    Reuse the collection tube in the next step.

14. 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 the next step.

15. 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 the next step.

16. Pipet 500 µl Buffer RPE into 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.

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 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

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

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.

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. Gently close the lid, let the column stand for 1 min, 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, because 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.

Protocol: Purification of Long Exosomal RNA from Urine

This protocol is intended as a guideline for the purification of long RNA (approximately >200 nt) from exosomes and other EVs, from urine volumes of up to 4 ml (Midi) or up to 16 ml (Maxi) (2 exoEasy column loadings) using the exoRNeasy Midi/Maxi Kit. Processing of more than 4 ml (Midi) or 16 ml (Maxi) sample is not recommended, because 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 "Protocol: Purification of Long Exosomal RNA and Short RNA (Including miRNA) in Separate Fractions from Urine (Requires Reagent UI)". To isolate both long and short RNA in one fraction, follow "Protocol: Purification of Short Exosomal RNA, Including miRNA, from Urine (Requires Reagent UI)".

For recommendations on collection, preparation, and storage of cell-free plasma and serum, see "Appendix A: Recommendations for Sample Collection, Separation, and Storage".

Important points before starting

- Although meaningful results have been produced using urine that has been stored at 2–8°C for 1 week after collection, we highly recommend using urine directly after collection, or freezing urine in aliquots at –30 to –15°C if to be stored for up to a month or at –90 to –65°C for longer-term storage. To process frozen samples, incubate either in a 37°C water bath or at room temperature until samples are completely thawed. When using the water bath, make sure to not leave the samples at 37°C for longer than necessary. Prolonged incubation may compromise RNA yield and integrity.
- DNase I digestion is not recommended for urine samples, because 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.
- 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 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 (from step 7 onward) is compatible with QIAGEN MaXtract High Density Tubes.

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 prefiltered urine, excluding particles larger than 0.8 µm by using syringe filters (e.g., Sartorius Minisart NML or Millipore Millex-AA).

   Note: Alternatively, an additional centrifugation step can be performed to eliminate residual cellular material. See Appendix A 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 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. 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 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 to step 12.

    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 the next step.

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

    Reuse the collection tube in the next step.

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 the next step.

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.

    Reuse the collection tube in the next step.

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 come into contact with 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.

Protocol: Purification of Short Exosomal RNA, Including miRNA, from Urine (Requires Reagent UI)

This protocol is intended as a guideline for the purification of short RNA from exosomes and other EVs, from urine volumes up to 4 ml (Midi) or up to 16 ml (Maxi) (2 exoEasy column loadings) 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, because 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 Long Exosomal RNA and Short RNA (Including miRNA) in Separate Fractions from Urine (Requires Reagent UI)". To isolate long RNA only, follow "Protocol: Purification of Long Exosomal RNA from Urine".

For recommendations on collection, preparation, and storage of cell-free plasma and serum, see Appendix A.

We recommend using this protocol with the miRNeasy Serum/Plasma Spike-In Control or the miRCURY RNA Spike-In Kit, which must be ordered separately.

Important points before starting

- Although meaningful results have been produced using urine that has been stored at 2–8°C for 1 week after collection, we highly recommend using urine directly after collection, or freezing urine in aliquots at –30 to –15°C if to be stored for up to a month or at –90 to –65°C for longer-term storage. To process frozen samples, incubate either in a 37°C water bath or at room temperature until samples are completely thawed. When using the water bath, make sure to not leave the samples at 37°C for longer than necessary. Prolonged incubation may compromise RNA yield and integrity.
- DNase I digestion is not recommended for urine samples, because 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.
- 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 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 (from step 7 onward) is compatible with QIAGEN MaXtract High Density Tubes.

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 use only prefiltered urine, excluding particles larger than 0.8 µm by using syringe filters (e.g., using Sartorius Minisart NML or Millipore Millex-AA).

   Note: Alternatively, an additional centrifugation step can be performed to eliminate residual cellular material. See Appendix A 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 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. 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 for 5 min.

   This step promotes dissociation of nucleoprotein complexes.

   Note: If any spike-in controls are used, they should be added to the lysate at this point.
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 by vortexing. Do not centrifuge. Continue without delay to step 12.

    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 the next step.

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

    Reuse the collection tube in the next step.

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 the next step.

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.

    Reuse the collection tube in the next step.

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 come into contact with 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 prevent 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, because residual

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

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, because 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.

Protocol: Purification of Long Exosomal RNA and Short RNA (Including miRNA) in Separate Fractions from Urine (Requires Reagent UI)

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 and additional Buffer RWT are required.

We recommend using this protocol with the miRNeasy Serum/Plasma Spike-In Control or the miRCURY RNA Spike-In Kit, which must be ordered separately.

Processing of more than 4 ml (Midi) or 16 ml (Maxi) sample is not recommended, because 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 “Protocol: Purification of Short Exosomal RNA, Including miRNA, from Urine (Requires Reagent UI)”. To isolate long RNA only, follow “Protocol: Purification of Long Exosomal RNA from Urine”.

For recommendations on collection, preparation and storage of cell-free plasma and serum, see Appendix A.

Important points before starting

- Although meaningful results have been produced using urine that has been stored at 2–8°C for 1 week after collection, we highly recommend using urine directly after collection, or freezing urine in aliquots at –30 to –15°C if to be stored for up to a month or at –90 to –65°C for longer-term storage. To process frozen samples, incubate either in a 37°C water bath or at room temperature until samples are completely thawed. When using the water bath, make sure to not leave the samples at 37°C for longer than necessary. Prolonged incubation may compromise RNA yield and integrity.
- DNase I digestion is not recommended for urine samples, because 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.
- 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 (from step 7 onward) is compatible with QIAGEN MaXtract High Density Tubes.

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 prefiltered urine, excluding particles larger than 0.8 µm by using syringe filters (e.g., using Sartorius Minisart NML or Millipore Millex-AA).

   Note: Alternatively, an additional centrifugation step can be performed to eliminate residual cellular material. See Appendix A 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 for 5 min at 5000 x *g* to wash the column and remove residual buffer. Discard the flow-through 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 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. 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 for 5 min.

   This step promotes dissociation of nucleoprotein complexes.

   Note: If any spike-in controls are used, they should be added to the lysate at this point.

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 to step 12.

    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 the next step.

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

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

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 the next step.

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.

    Reuse the collection tube in the next step.

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 come into contact with 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, because 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, because 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)

19. 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 then mix thoroughly by vortexing. Do not centrifuge. Proceed immediately to the next step.
20. 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 the next step.
21. Repeat step 21 using the remainder of the sample. Discard the flow-through.*

    Reuse the collection tube in the next step.
22. 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.*
23. 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.
24. Pipet 500 µl Buffer RPE into the RNeasy MinElute Spin Column. Close the lid, and then 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 come into contact with the flow-through. Otherwise, carryover of ethanol will occur.

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

25. 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, because residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.

26. 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, because 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.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: **www.qiagen.com/FAQ/FAQList.aspx**. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit **www.qiagen.com**).

		Comments and suggestions
Phases do not separate completely		
a)	No chloroform added or chloroform not pure	Make sure to add chloroform that does not contain isoamyl alcohol or other additives (low concentrations of ethanol or amylene typically used for stabilization of chloroform are not critical).
b)	Lysate not sufficiently mixed before centrifugation	After addition of chloroform, the lysate must be vigorously shaken. If the phases are not well separated, shake the tube vigorously for at least 15 s and repeat the incubation and centrifugation in steps 9 and 10 of the corresponding protocol.
c)	Organic solvents present in samples used for RNA purification	Make sure that the starting sample does not contain organic solvents (e.g., ethanol, DMSO), strong buffers, or alkaline reagents. These can interfere with the phase separation.
Clogged exoEasy column		
Sample still contains cellular material or coagulated protein		Make sure to follow recommendations in "Appendix A: Recommendations for Sample Collection, Separation, and Storage" for removal of residual cellular material. After thawing frozen samples, remove cryoprecipitates by centrifugation or filtration if necessary (see Appendix A).
Clogged RNeasy column		
Centrifugation temperature too low		Except for phase separation (step 10), all centrifugation steps should be performed at room temperature. Some centrifuges may cool to below 20°C even when set at 20°C. This can cause precipitates to form that can clog the RNeasy MinElute spin column and reduce RNA yield. If this happens, set the centrifugation temperature to 25°C. Warm the ethanol-containing lysate to 37°C before transferring to the RNeasy MinElute spin column.

		Comments and suggestions
Low miRNA yield or poor performance of miRNA in downstream applications		
	Incorrect ethanol concentration	Be sure to use the ethanol concentrations specified in the protocol steps.
Low or no recovery of RNA		
a)	Too much starting material	In subsequent preparations, reduce the amounts of starting material. It is essential to use the correct amount of starting material (see page 17).
b)	Elution buffer incorrectly dispensed	Add elution buffer to the center of the RNeasy MinElute spin column membrane to ensure that the buffer completely covers the membrane.
c)	RNA still bound to the membrane	Repeat the elution step of the protocol, but incubate the RNeasy MinElute spin column on the bench for 10 min after adding RNase-free water and before centrifugation.
RNA degraded		
a)	Sample inappropriately handled	EVs typically contain high amounts of small RNA species and much lower amounts of ribosomal RNA compared to cells, and will therefore not resemble intact RNA from cells or tissue on electropherograms. Nevertheless, the following precautions are recommended to avoid complications due to RNA degradation: Perform the protocol quickly, especially the first few steps. See "Appendix D: General Remarks on Handling RNA" and "Appendix A: Recommendations for Sample Collection, Separation, and Storage.
b)	RNase contamination	EVs typically contain high amounts of small RNA species, and will therefore not resemble intact RNA from cells or tissue on electropherograms. Nevertheless, the following precautions are recommended to avoid complications due to RNA degradation: Although all buffers have been tested and are guaranteed RNase-free, RNases can be introduced during use. Make sure not to introduce any RNases during the procedure or later handling. See "Appendix D: General Remarks on Handling RNA". Do not put RNA samples into a vacuum dryer that has been used in DNA preparations where RNases may have been used.
RNA does not perform well in downstream experiments		
a)	Phase separation performed at too high a temperature	The phase separation in step 10 should be performed at 4°C. Make sure that the centrifuge does not heat above 10°C during centrifugation.
b)	Interphase contamination of aqueous phase	Contamination of the aqueous phase with the interphase can result in carryover of contaminants into the RNA eluate. Make sure to transfer the aqueous phase without interphase contamination. The use of MaXtract High Density Tubes can help prevent interphase carryover.

		Comments and suggestions
c)	Salt carryover during elution	Ensure that Buffer RPE is at 20–30°C.
d)	Ethanol carryover	After the final membrane wash, be sure to dry the RNeasy MinElute spin column by centrifugation at full speed with open lids for 5 min.

Appendix A: Recommendations for Sample Collection, Separation, and Storage

In order to specifically isolate vesicular RNA from biofluids, we recommend following these protocols. To generate serum or plasma, an initial low *g*-force centrifugation step removes blood cells. Afterward, and for all other biofluids, either a high *g*-force centrifugation step or filtration has to be performed to remove all remaining cells, cellular debris, apoptotic bodies, etc. The latter centrifugation/filtration step significantly reduces the amount of cellular or genomic DNA and RNA in the sample. Because of the much higher abundance of RNA in cells, even small amounts of cellular debris can have a very significant effect on RNA profiling of cell-free fluids. The sooner after blood collection this removal of cellular materials is performed, the lower the risk of additional background from blood cell-derived vesicles generated in vitro. Use of gel barrier tubes for generation of plasma or serum generally results in fewer residual cells after the first centrifugation.

Because binding of vesicles to the exoEasy membrane is not selective for a specific subpopulation or size range of EVs, the filtration or centrifugation step performed at this stage can also influence the size range of vesicles from which RNA will be isolated. The recommended 0.8 µm filter pore size (or centrifugation at 3,000 x *g*) will effectively exclude cellular material, including thrombocyte fragments, but still retain the vast majority of EVs, whereas use of a 0.2 or 0.45 µm filter (or centrifugation at, e.g., 16,000 x *g*) will remove some of the larger vesicles.

The recommended syringe filters (e.g. Sartorius Minisart NML or Millipore Millex-AA) have dead volumes of around 100–200 µl. Small volumes of sample (<1 ml) can be diluted with PBS prior to filtration to reduce loss of material.

Procedure: plasma separation and storage

1. Collect whole blood in BD Vacutainer® Venous Blood Collection Tubes (cat. no. 367525) containing EDTA (or any other primary blood collection tube containing an anticoagulant such as EDTA or citrate. Also compatible are PAXgene® Blood ccfDNA Tubes [cat. no. 768115] and Cell-Free DNA BCT® – but not RNA BCT – tubes from Streck). Store tubes at room temperature or 4°C and process within 1 hour.

   Note: Do not use heparin-containing blood collection tubes as this anticoagulant can interfere with downstream assays, such as RT-PCR.

2. Centrifuge blood samples in primary blood collection tubes for 10 min at 1900 x *g* (3000 rpm) and 4°C using a swinging bucket rotor.

3. Carefully transfer the upper (yellow) plasma phase to a new tube (with conical bottom) without disturbing the intermediate buffy coat layer (containing white blood cells and platelets). Normally up to 4–5 ml plasma can be obtained from 10 ml whole blood.

   Note: Carryover of white blood cells and platelets from the buffy coat layer is the most likely source of cellular miRNA/RNA contamination in plasma.

   Note: Plasma can be used for cell-free nucleic acid purification at this stage. However, the following high-speed centrifugation will remove additional cellular debris and minimize contamination of cell-free nucleic acids by gDNA and RNA derived from damaged blood cells.

4. Centrifuge plasma samples in conical tubes for 15 min at 3000 x *g* (or 10 min at 16,000 x *g* – see above) and 4°C or pass through a 0.8 µm filter (see "Equipment and Reagents to Be Supplied by User".)

   This will remove additional cellular nucleic acids attached to cell debris.

5. Carefully transfer cleared supernatant to a new tube without disturbing the pellet (which forms a smear along the outer side/bottom of the centrifugation tube).

6. Store at 2–8°C until further processing, if plasma will be used for nucleic acid purification on the same day. For longer storage, keep plasma frozen in aliquots at –90 to –65°C.

7. Before using frozen plasma for nucleic acid purification, incubate either in a 37°C water bath or at room temperature until the plasma is completely thawed. When using the water bath, make sure to not leave the plasma at 37°C for longer than necessary.

   Optional: To remove cryoprecipitates, centrifuge thawed plasma samples for 5 min at 3000 x *g* and 4°C or pass through a 0.8 µm filter. Transfer supernatant to a new tube, and begin nucleic acid purification protocol.

Procedure: serum separation and storage

1. Collect whole blood in a primary blood collection tube with or without clot activator, but without anticoagulants such as EDTA or citrate (e.g., Sarstedt S-Monovette® Serum-Gel 9 ml tubes, cat. no. 02.1388). For complete clotting, leave tubes at room temperature for 10 min to 1 h.

   Note: Tubes with clot activator can be processed after 10 min clotting time, while tubes without clot activator should be stored for at least 30 min at room temperature to allow clotting to take place.

2. Centrifuge tubes for 10 min at 1900 x *g* (3000 rpm) and 4°C using a swinging bucket rotor.

   Note: If using Sarstedt S-Monovette Serum-Gel 9 ml tubes, a gel bed will form between the upper serum phase and the lower cellular phase, facilitating recovery of serum.

3. Carefully transfer the upper (yellow) serum phase to a new tube (with conical bottom) without disturbing the pellet containing cellular material. Normally up to 3–5 ml serum can be obtained from 10 ml of whole blood.

   Note: Prevent transfer of cellular material from the lower phase.

   Note: Serum can be used for cell-free nucleic acid purification at this stage. However, an additional filtration or centrifugation will remove additional cellular debris and minimize contamination of cell-free nucleic acids by gDNA and RNA derived from damaged blood cells.

4. Centrifuge serum samples in conical tubes for 15 min at 3000 x *g* (or 10 min at 16,000 x *g* – see above) and 4°C or pass through a 0.8 µm filter (see "Equipment and Reagents to Be Supplied by User").

   This will remove additional cellular nucleic acids attached to cell debris.

5. Carefully transfer cleared supernatant to a new tube without disturbing the pellet (which forms a smear along the outer side of the centrifugation tube).

6. Store at 2–8°C until further processing, if serum will be used for nucleic acid purification on the same day. For longer storage, keep serum frozen in aliquots at –90 to –65°C.

7. Before using frozen serum for nucleic acid purification, incubate either in a 37°C water bath or at room temperature until the serum is completely thawed. When using the water bath, make sure to not leave the serum at 37°C for longer than necessary..

   Optional: To remove cryoprecipitates, centrifuge thawed serum samples for 5 min at 3000 x *g* and 4°C or pass through a 0.8 µm filter. Transfer supernatant to a new tube, and begin nucleic acid purification protocol.

Appendix B: Isolation of Long Exosomal RNA Only

For certain applications (e.g., certain NGS library preparation methods) excluding short RNA may be beneficial or recommended. In such cases, the exoRNeasy protocols can be modified to exclude RNA shorter than approximately 200 nt.

The protocols for urine already contain an option to isolate long RNA (page 34) and short RNA (page 39) separately.

In the protocols for serum and plasma (page 19), CSF (page 24) and cell culture supernatant (page 29), follow the procedure normally until step 10.

In step 11, instead of using 2 volumes 100% ethanol to adjust the binding conditions, use 1 volume 70% ethanol.

Follow the remaining steps normally.

Appendix C: Isolation of Long Exosomal RNA and Short RNA, Including miRNA, in Separate Fractions

In some cases, it may be desirable to isolate long and short RNA in separate fractions (e.g., to prepare separate NGS libraries for miRNA and mRNA analysis).

The protocols for urine already contain an option to isolate long and short RNA in separate fractions. In the protocols for serum and plasma (page 19), CSF (page 24) and cell culture supernatant (page 29), follow the procedure normally until step 10.

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

In step 11, instead of using 2 volumes of 100% ethanol to adjust the binding conditions, use 1 volume of 70% ethanol.

Pipet the flow-through of steps 12 and 13, 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.

Follow the remaining steps normally to isolate RNA larger than approximately 200 nt.

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

1. To the combined flow-through from steps 12 and 13, add 0.7 volumes of 100% isopropanol, and then mix thoroughly by vortexing. Do not centrifuge. Proceed immediately to step 2.

2. 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 the next step.

3. Repeat step 21 using the remainder of the sample. Discard the flow-through.*

   Reuse the collection tube in the next step.

4. 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 the next step.

5. 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.

   Reuse the collection tube in the next step.

6. Pipet 500 µl Buffer RPE into the RNeasy MinElute Spin Column. Close the lid, and then 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 come into contact with the flow-through. Otherwise, carryover of ethanol will occur.

7. 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, because 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.

8. 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.
9. 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, because the spin column membrane will not be sufficiently hydrated.
10. 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.

Appendix D: General Remarks on Handling RNA

Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to degrade RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the purification procedure. In order to create and maintain an RNase-free environment, the following precautions must be taken during pretreatment and use of disposable and nondisposable vessels and solutions while working with RNA.

General handling

Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible. Keep purified RNA on ice when aliquots are pipetted for downstream applications.

To remove RNase contamination from bench surfaces, nondisposable plasticware, and laboratory equipment (e.g., pipettes and electrophoresis tanks), the use of a decontamination solution is recommended. RNase contamination can alternatively be removed using general laboratory reagents. To decontaminate plasticware, rinse with 0.1 M NaOH, 1 mM EDTA* followed by RNase-free water (see "Solutions", page 64), or rinse with chloroform* if the plasticware is chloroform-resistant. To decontaminate electrophoresis tanks, clean with

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

detergent (e.g., 0.5% SDS),* rinse with RNase-free water, rinse with ethanol (if the tanks are ethanol-resistant), and allow to dry.

Disposable plasticware

The use of sterile, disposable polypropylene tubes is recommended throughout the procedure. These tubes are generally RNase-free and do not require pretreatment to inactivate RNases.

Glassware

Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent,* thoroughly rinsed, and oven-baked at 240°C for 4 h or more (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware can be treated with DEPC* (diethyl pyrocarbonate), as described in "Solutions" below.

Solutions

Solutions (water and other solutions)* should be treated with 0.1% DEPC. DEPC is a strong – but not absolute – inhibitor of RNases. It is commonly used at a concentration of 0.1% to inactivate RNases on glass or plasticware or to create RNase-free solutions and water. DEPC inactivates RNases by covalent modification. Add 0.1 ml DEPC to 100 ml of the solution to be treated and shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 h at 37°C. Autoclave for 15 min to remove any trace of DEPC. DEPC will react with primary amines and cannot be used directly to treat Tris* buffers. DEPC is highly unstable in the presence of Tris buffers and decomposes rapidly into ethanol and CO_2. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris to make the appropriate buffer. Trace amounts of DEPC will modify purine residues in RNA by carbethoxylation. Carbethoxylated RNA is translated with very low efficiency in cell-free systems. However, its ability to form DNA:RNA or RNA:RNA hybrids is not seriously affected unless a large fraction

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

of the purine residues have been modified. Residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 min.

Note: RNeasy buffers are guaranteed RNase-free without using DEPC treatment and are therefore free of any DEPC contamination.

Appendix E: Storage, Quantification, and Determination of RNA Quality

Storage of RNA

Purified RNA may be stored at –30 to –15°C for up to a month or at –90 to –65°C if longer, in RNase-free water. Under these conditions, no degradation of RNA is detectable after 1 year.

Quantification of RNA

The concentration of RNA should be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer, e.g., the QIAxpert (see "Spectrophotometric quantification of RNA" below). However, for small amounts of RNA, which are typically obtained from EVs, it may be difficult to determine amounts photometrically. Small amounts of RNA can be quantified using an Agilent® 2100 Bioanalyzer, quantitative RT-PCR, or fluorometric quantification. When purifying RNA from particularly small samples (e.g., laser-microdissected samples, or from plasma or serum), quantitative, real-time RT-PCR should be used for quantification.

DNA contamination

No currently available purification method can guarantee that RNA is completely free of DNA. While exoRNeasy Kits will remove the vast majority of cellular DNA, trace amounts may still remain, depending on the amount and nature of the sample. However, serum, plasma, and other cell-free body fluids contain very little DNA.

For analysis of very low-abundance targets, any interference by residual DNA contamination can be detected by performing real-time RT-PCR control experiments in which no reverse transcriptase is added prior to the PCR step.

To prevent any interference by DNA in gene expression analysis real-time RT-PCR applications, we recommend designing primers that anneal at intron splice junctions so that genomic DNA will not be amplified. QuantiNova® LNA PCR Assays from QIAGEN are designed for SYBR® Green or Probe real-time RT-PCR analysis of RNA sequences (without detection of genomic DNA) where possible (the assays can be ordered online at **www.qiagen.com/GeneGlobe**). For real-time RT-PCR assays where amplification of genomic DNA cannot be avoided, we recommend using the QuantiNova Reverse Transcription Kit for reverse transcription. The kit integrates fast cDNA synthesis with rapid removal of genomic DNA contamination.

miScript® Primer Assays or miRCURY LNA miRNA PCR Assays for miRNA quantification do not detect genomic DNA.

Integrity of RNA

The integrity and size distribution of total RNA purified with miRNeasy Kits can be checked by denaturing agarose gel electrophoresis and ethidium bromide* staining or by using the QIAxcel® system or Agilent 2100 Bioanalyzer. The respective ribosomal RNAs should appear as sharp bands or peaks. The apparent ratio of 28S rRNA to 18S rRNA should be approximately 2:1. If the ribosomal bands or peaks of a specific sample are not sharp, but appear as a smear toward smaller-sized RNAs, it is likely that the sample suffered major degradation either before or during RNA purification. The QIAxcel and Agilent 2100 Bioanalyzer also provide an RNA Integrity Score (RIS) or RNA Integrity Number (RIN) as useful measures of RNA integrity. Ideally, the RIS/RIN should be close to 10, but in many cases (particularly with tissue samples), RNA quality is greatly influenced by how well the original sample was preserved.

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

Cell-free RNA from serum or plasma contains high proportions of small RNAs of less than 100 nucleotides. Even though full-length mRNA and intact ribosomal RNA can be isolated from EVs, the rRNA peaks are not always visible on the Bioanalyzer. RIS or RIN are therefore not useful as indicators of RNA integrity for cell-free RNA from EVs (or from total plasma or serum).

Ordering Information

Product	Contents	Cat. no.
exoRNeasy 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, RNase-free Reagents and Buffers	77164
exoRNeasy 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, RNase-free Reagents and Buffers	77144
exoRNeasy Serum/ Plasma Starter Kit	For 20 RNA preps: 10 exoEasy Maxi, 10 exoEasy Midi, and 20 RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml, 2 ml, and 50 ml), QIAzol Lysis Reagent, RNase-free Reagents and Buffers	77023
Related products		
miRNeasy Serum/Plasma Spike-In Control	10 pmol lyophilized C. elegans miR-39 miRNA mimic	219610
miRCURY RNA Spike-In Kit, for RT	UniSp2, UniSp4, and UniSp5 RNA Spike-in Template Mix and the cel-miR-39-3p RNA Spike-in Template	339390
MaXtract High Density (200 x 2 ml)	200 x 2 ml MaXtract High Density Tubes	129056
miRNeasy Serum/Plasma Advanced Kit (50)	For 50 total RNA preps: 50 RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml and 2 ml), QIAzol Lysis Reagent, RNase-free Reagents and Buffers	217204

Product	Contents	Cat. no.
miRNeasy 96 Advanced QIAcube HT Kit (5)	For 480 preps: RNeasy 96 plates, RNase-free water, buffers	217261
Collection Tubes (2 ml)	1000 Collection Tubes (2 ml)	19201
Related products for automation		
QIAcube Connect*	Instrument, connectivity package, 1-year warranty on parts and labor	Inquire
Starter Pack, QIAcube	Reagent bottle racks (3), 200 µl filter-tips (1024), 1000 µl filter-tips (1024), 30 ml reagent bottles (12), rotor adapters (240), rotor adapter holder	990395

* All QIAcube Connect instruments are provided with a region-specific connectivity package, including tablet and equipment necessary to connect to the local network. Further, QIAGEN offers comprehensive instrument service products, including service agreements, installation, introductory training and preventive subscription. Contact your local sales representative to learn about your options.

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Document Revision History

Date	Changes
08/2019	Updated text and ordering information for QIAcube Connect. Clarified instructions for thawing samples.

Limited License Agreement for exoRNeasy Midi/Maxi Kit

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