January 2015

exoRNeasy Serum/Plasma Handbook

exoRNeasy Serum/Plasma Maxi Kit
exoRNeasy Serum/Plasma Midi Kit
exoRNeasy Serum/Plasma Starter Kit
For purification of total RNA, including miRNA, from exosomes and other extracellular vesicles (EVs) in animal and human plasma and serum

miRNeasy Serum/Plasma Spike-In Control
For normalization of miRNA purification from serum or plasma

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Contents

Kit Contents

exoRNeasy Serum/Plasma Maxi Kit	**(50)**
Catalog no.	**77064**
Number of preps	**50**
exoEasy Maxi Spin Columns (in 50 ml Collection Tube)	50
Collection Tubes (50 ml)	50
Buffer XBP	4 x 55 ml
Buffer XWP	3 x 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
Ce_miR-39_1 miScript® Primer Assay	(100)§
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.

§ Sufficient primer provided for 100 x 50 µl assays following reconstitution.

exoRNeasy Serum/Plasma Midi Kit	**(50)**
Catalog no.	**77044**
Number of preps	**50**
exoEasy Midi Spin Columns (in 15 ml Collection Tube)	50
Collection Tubes (15 ml)	50
Buffer XBP	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
Ce_miR-39_1 miScript® Primer Assay	(100)§
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.

§ Sufficient primer provided for 100 x 50 µl assays following reconstitution.

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
Ce_miR-39_1 miScript® Primer Assay	(100)§
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.

§ Sufficient primer provided for 100 x 50 µl assays following reconstitution.

miRNeasy Serum/Plasma Spike-In Control	**(10 pmol)**
Catalog no.	**219610**
Lyophilized *C. elegans* miR-39 miRNA mimic	10 pmol

Shipping and Storage

The exoRNeasy Serum/Plasma Kits (cat. no. 77064, 77044, 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 Ce_miR-39_1 miScript Primer Assay at –15°C to –30°C, either lyophilized or reconstituted (see next paragraph). Store the remaining components dry at room temperature. All kit components are stable for at least 9 months upon arrival under these conditions.

To reconstitute Ce_miR-39_1 miScript Primer Assay, briefly centrifuge the vial, add 550 µl TE, pH 8.0 (see "Equipment and Reagents to Be Supplied by User", page 8), and mix by vortexing the vial 4–6 times. This will provide sufficient primer for 100 x 50 µl reactions. We recommend freezing the reconstituted primers in aliquots in order to avoid repeated freezing and thawing.

The miRNeasy Serum/Plasma Spike-In Control is shipped at ambient temperature. Store at –15°C to –30°C, either reconstituted (see page 30) or lyophilized.

Intended Use

The exoRNeasy Serum/Plasma Kits and the miRNeasy Serum/Plasma Spike-In Control 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.

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.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of exoRNeasy Serum/Plasma 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 Serum/Plasma Kits are designed for rapid purification of total vesicular RNA — including non-coding 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. The exoRNeasy Serum/Plasma Starter Kit provides both ExoEasy Midi and Maxi columns, enabling analysis at varied volumes of prefiltered plasma or serum.

When working with serum and plasma samples, we recommend use of a synthetic spike-in control for normalization between multiple samples, such as the miRNeasy Serum/Plasma Spike-In Control. The miRNeasy Serum/Plasma Spike-In Control must be ordered separately (cat. no. 219610). The exoRNeasy Serum/Plasma Kits include a miScript Primer Assay that detects the miRNeasy Serum/Plasma Spike-In Control using real-time PCR.

Principle and procedure

The exoRNeasy Serum/Plasma Kits use a membrane-based affinity binding step to isolate exosomes and other EVs from serum and plasma.

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.

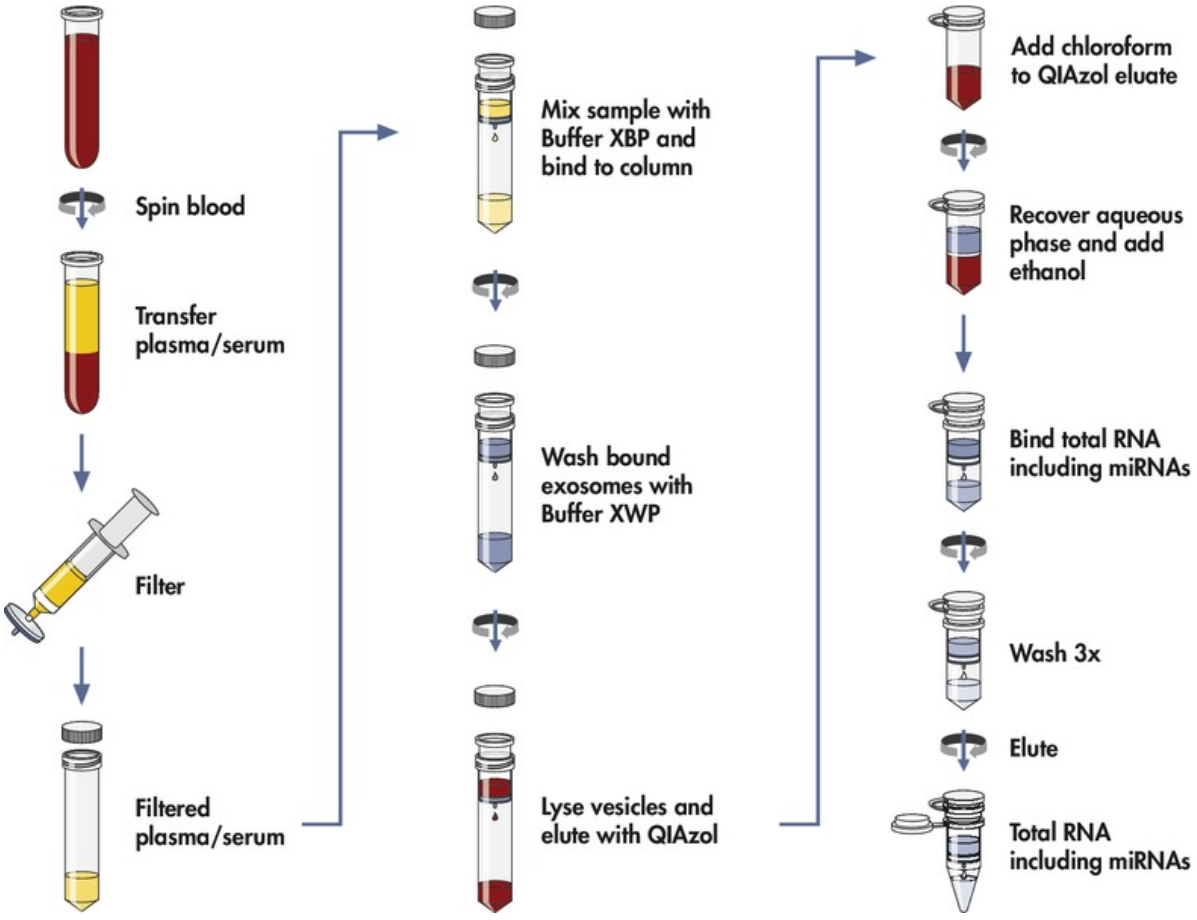

Figure 1. The exoRNeasy Serum/Plasma Kit – exosome purification and total RNA isolation in less than 1 hour. Starting with filtered plasma, the exoRNeasy Serum/Plasma Kits provide isolation of EVs in just 20 minutes. A subsequent 35-minute isolation procedure yields total RNA, including miRNA.

Description of protocols

This handbook contains two protocols on pages 14 and 18 for purification of total RNA, including miRNA, from exosomes and other EVs in plasma and serum using the exoRNeasy Serum/Plasma Maxi Kit or the exoRNeasy Serum/Plasma Midi Kit. The Midi Kit is designed for the purification of up to 1 ml prefiltered serum or plasma, and the Maxi Kit for up to 4 ml prefiltered serum or plasma. The exoRNeasy Serum/Plasma Starter Kit contains materials for 10 preparations each, in midi and maxi format, to allow the analysis of varied volumes of prefiltered plasma or serum. In addition, protocols are provided in the appendices for collection, preparation, and storage of samples, and for use of a spike-in control in serum/plasma miRNA profiling using the miScript PCR System.

miRNA purification from cells and tissue, in 96 wells, and from FFPE tissues

A range of miRNeasy Kits is available for various sample types. The miRNeasy Serum/Plasma Kit is designed for purification of all cell-free total RNA from small volumes of serum and plasma. The miRNeasy Mini and Micro Kits enable low-throughput RNA purification from cells or tissues using spin columns. For high-throughput purification in a 96-well format, the miRNeasy 96 Kit is recommended. Total RNA including miRNA can also be copurified from formalin-fixed, paraffin-embedded (FFPE) tissue sections using the miRNeasy FFPE Kit (see ordering information, page 46).

miRNA quantification using the miScript PCR System

The miScript PCR System allows sensitive and specific quantification and profiling of miRNA expression using SYBR® Green-based real-time PCR. The robust miScript PCR System comprises the miScript II RT Kit, the miScript SYBR Green PCR Kit, miScript Assays, and miScript miRNA PCR Arrays. It covers all the steps of miRNA quantification, from conversion of RNA into cDNA to real-time PCR detection of miRNAs and straightforward data analysis.

Individual assays for mature miRNAs, precursor miRNAs, and other small noncoding RNAs can be ordered at the GeneGlobe® Web portal (www.qiagen.com/GeneGlobe). Alternatively, for high-throughput experiments, miScript miRNA PCR Arrays enable rapid profiling of the complete miRNome or pathway-focused panels of mature miRNAs for a variety of species. Find out more about the miScript PCR System at www.qiagen.com/miRNA.

Equipment and Reagents to Be Supplied by User

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

- Chloroform (without added isoamyl alcohol)
- Ethanol (70%, 80%, and 96–100%)*
- Sterile, RNase-free pipet tips
- 1.5 ml or 2 ml microcentrifuge tubes
- Microcentrifuge(s) (with rotor for 2 ml tubes) for centrifugation at 4°C and at room temperature (15–25°C)
- Disposable gloves
- The miRNeasy Serum/Plasma Spike-In Control must be purchased separately (see ordering information, page 46)
- Equipment and tubes for serum/plasma collection and separation (see Appendix A, page 27):
    - For serum: primary blood collection tube(s) without anticoagulants such as EDTA or citrate
    - For plasma: primary blood collection tube(s) containing an anticoagulant such as EDTA or citrate
    - Conical tube(s)
    - 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 (cat. no. 16592) or Millipore® Millex®-AA (cat. no. SLAA033SB)).
- The RNA purification part of the protocol (following step 7) is compatible with QIAGEN MaXtract High Density Tubes (cat. no. 129056).

For reconstitution of Ce_miR-39_1 miScript Primer Assay

TE, pH 8.0 contains 10 mM Tris·Cl and 1 mM EDTA. To prepare 100 ml TE, pH 8.0, mix the following stock solutions:

- 1 ml 1 M Tris·Cl, pH 8.0 (autoclaved)

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

- 0.2 ml 0.5 M EDTA, pH 8.0 (autoclaved)
- 98.8 ml distilled water

Alternatively, ready-made TE can be purchased from chemicals suppliers.

To reconstitute Ce_miR-39_1 miScript Primer, briefly centrifuge the vial, add 550 µl TE, pH 8.0, and mix by vortexing the vial 4–6 times. We recommend freezing the reconstituted primers in aliquots in order to avoid repeated freezing and thawing.

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 Serum/Plasma 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 pre-filtered plasma, excluding particles larger than 0.8 µm.

Yields of total RNA achieved with the exoRNeasy Serum/Plasma 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 (cat. no. 219610) and corresponding Ce_miR-39_1 miScript Primer Assay (included in the kit) is recommended to monitor miRNA purification and amplification.

Table 1. exoEasy Maxi spin column specifications

Maximum volume of serum or plasma	4 ml
Maximum loading volume	15 ml
Allows recovery of	Exosomes and other extracellular vesicles (EVs)

Table 1. exoEasy Midi spin column specifications

Maximum volume of serum or plasma	1 ml
Maximum loading volume	4 ml
Allows recovery of	Exosomes and other extracellular vesicles (EVs)

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 <18nt not tested.

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

Protocol: Purification of Total RNA, including miRNA, from Serum and Plasma using the exoRNeasy Serum/Plasma Maxi Kit

This protocol is intended as a guideline for the purification of total RNA from exosomes and other extracellular vesicles (EVs), including mRNA, miRNA, and other non-coding RNAs, from 1 to 4 ml serum and plasma using the exoRNeasy Serum/Plasma Maxi Kit. Processing of more than 4 ml sample is not recommended, because the number of EVs introduced by larger sample volumes will exceed the binding capacity of the exoEasy Maxi spin column. Processing of smaller volumes of sample (down to 100 µl) is possible using the exoRNeasy Midi Kit (catalog no. 77044).

For recommendations on collection, preparation, and storage of cell-free plasma and serum, see Appendix A, page 27.

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

Important points before starting

- After collection and centrifugation, plasma or serum can be stored at 2–8°C for up to 6 hours or used directly in the procedure. For long-term storage, freezing at –15°C to –30°C or –65°C to –90°C in aliquots is recommended. To process frozen samples, incubate at 37°C in a water bath until samples are completely thawed. Avoid prolonged incubation, which may compromise RNA yield and integrity.
- DNase I digestion is not recommended for plasma or serum samples since the combined QIAzol and RNeasy technologies efficiently remove any DNA present in EVs. In addition, miScript Primer Assays and most other assays for mature miRNA are not affected by the presence of small amounts of genomic DNA. On-column DNase treatment may reduce recovery of small RNA from EVs.
- Buffer RWT may form a precipitate upon storage. If necessary, redissolve by warming and then place at room temperature (15–25°C).
- QIAzol Lysis Reagent and Buffer RWT contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach.
- Except for phase separation (step 11), all protocol and centrifugation steps should be performed at room temperature.
- Centrifugation of the exoEasy 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 (following step 7) is compatible with QIAGEN MaXtract High Density Tubes (cat. no. 129056).

Things to do before starting

- Buffers RWT and RPE are supplied as concentrates. Before using for the first time, add the required volumes of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.
- Prepare a working solution of miRNeasy Serum/Plasma Spike-In Control as described in Appendix B, page 30.

Procedure

1. **It is recommended to only use pre-filtered plasma, excluding particles larger than 0.8 µm (e.g. using Sartorius Minisart NML (cat. no. 16592) or Millipore Millex-AA (cat. no. SLAA033SB) syringe filters).**
   Note: Alternatively, an additional centrifugation step can be performed to eliminate residual cellular material. See Appendix A (page 27) 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 10 ml XWP and spin 5 min at 5000 x *g* to wash the column and remove residual buffer. Discard the flow-through together with the collection tube.**

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

5. **Transfer the spin column to a fresh collection tube.**
6. **Add 700 µl QIAzol to the membrane. Spin for 5 min at 5000 x *g* to collect the lysate and transfer completely to a 2 ml tube (not supplied).**
7. **Briefly vortex the tube containing the lysate and incubate at room temperature (15–25°C) for 5 min.**
   This step promotes dissociation of nucleoprotein complexes.
8. **Optional: Add 3.5 µl miRNeasy Serum/Plasma Spike-In Control (1.6 x 10^8 *copies*/µl working solution).**
   For details on making appropriate stocks and working solutions of miRNeasy Serum/Plasma Spike-In Control, see Appendix B, page 30.
9. **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.
10. **Incubate at room temperature (15–25°C) for 2–3 min.**
11. **Centrifuge for 15 min at 12,000 x *g* at 4°C. After centrifugation, heat the centrifuge up to room temperature (15–25°C) if the same centrifuge will be used for the next centrifugation steps.**
    After centrifugation, the sample separates into 3 phases: an upper, colorless aqueous phase containing RNA; a thin, white interphase; and a lower, red organic phase. The volume of the aqueous phase should be approximately 400 µl.
12. **Transfer the upper aqueous phase to a new collection tube (not supplied). Avoid transfer of any interphase material. Add 2 volumes of 100% ethanol (e.g., for 400 µl aqueous phase, add 800 µl ethanol) and mix thoroughly by pipetting up and down several times. Do not centrifuge. Continue without delay to step 13.**
    A precipitate may form after addition of ethanol, but this will not affect the procedure.
13. **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 (15–25°C). Discard the flow-through.***
    Reuse the collection tube in step 14.
14. **Repeat step 13 using the remainder of the sample. Discard the flow-through.***

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

Reuse the collection tube in step 15.

15. **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 step 16.

16. **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 step 17.

17. **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 contact the flow-through. Otherwise, carryover of ethanol will occur.

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

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

    It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.

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

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

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

Protocol: Purification of Total RNA, including miRNA, from Serum and Plasma using the exoRNeasy Serum/Plasma Midi Kit

This protocol is intended as a guideline for the purification of total RNA from exosomes and other extracellular vesicles (EVs), including mRNA, miRNA, and other non-coding RNAs, from 100 µl up to 1 ml serum and plasma using the exoRNeasy Serum/Plasma Midi Kit. Processing of more than 1 ml sample is not recommended, because the number of EVs introduced by larger sample volumes will exceed the binding capacity of the exoEasy Midi spin column.

For recommendations on collection, preparation, and storage of cell-free plasma and serum, see Appendix A, page 27.

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

Important points before starting

- After collection and centrifugation, plasma or serum can be stored at 2–8°C for up to 6 hours or used directly in the procedure. For long-term storage, freezing at –15°C to –30°C or –65°C to –90°C in aliquots is recommended. To process frozen samples, incubate at 37°C in a water bath until samples are completely thawed. Avoid prolonged incubation, which may compromise RNA yield and integrity.
- DNase I digestion is not recommended for plasma or serum samples since the combined QIAzol and RNeasy technologies efficiently remove any DNA present in EVs. In addition, miScript Primer Assays and most other assays for mature miRNA are not affected by the presence of small amounts of genomic DNA. On-column DNase treatment may reduce recovery of small RNA from EVs.
- Buffer RWT may form a precipitate upon storage. If necessary, redissolve by warming and then place at room temperature (15–25°C).
- QIAzol Lysis Reagent and Buffer RWT contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach.
- Except for phase separation (step 11), all protocol and centrifugation steps should be performed at room temperature.
- Centrifugation of the exoEasy Midi 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 (following step 7) is compatible with QIAGEN MaXtract High Density Tubes (cat. no. 129056).

Things to do before starting

- Buffers RWT and RPE are supplied as concentrates. Before using for the first time, add the required volumes of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.
- Prepare a working solution of miRNeasy Serum/Plasma Spike-In Control as described in Appendix B, page 30.

Procedure

1. **It is recommended to only use pre-filtered plasma, excluding particles larger than 0.8 µm (e.g. using Sartorius Minisart NML (cat. no. 16592) or Millipore Millex-AA (cat. no. SLAA033SB) syringe filters).**
   Note: 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 (page 27) for detailed recommendations).
2. **Add 1 volume buffer XBP to 1 volume of sample. Mix well immediately by gently inverting the tube 5 times.**
   Note: For samples diluted with PBS, the volume of XBP should be equal to that of the diluted sample.
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 and spin 5 min at 5000 x *g* to wash the column and remove residual buffer. Discard the flow-through together with the collection tube.**

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

5. **Transfer the spin column to a fresh collection tube.**
6. **Add 700 µl QIAzol to the membrane. Spin for 5 min at 5000 x *g* to collect the lysate and transfer completely to a 2 ml tube (not supplied).**
7. **Briefly vortex the tube containing the lysate and incubate at room temperature (15–25°C) for 5 min.**
   This step promotes dissociation of nucleoprotein complexes.
8. **Optional: Add 3.5 µl miRNeasy Serum/Plasma Spike-In Control (1.6 x 10^8 *copies*/µl working solution).**
   For details on making appropriate stocks and working solutions of miRNeasy Serum/Plasma Spike-In Control, see Appendix B, page 30.
9. **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.
10. **Incubate at room temperature (15–25°C) for 2–3 min.**
11. **Centrifuge for 15 min at 12,000 x *g* at 4°C. After centrifugation, heat the centrifuge up to room temperature (15–25°C) if the same centrifuge will be used for the next centrifugation steps.**
    After centrifugation, the sample separates into 3 phases: an upper, colorless aqueous phase containing RNA; a thin, white interphase; and a lower, red organic phase. The volume of the aqueous phase should be approximately 400 µl.
12. **Transfer the upper aqueous phase to a new collection tube (not supplied). Avoid transfer of any interphase material. Add 2 volumes of 100% ethanol (e.g., for 400 µl aqueous phase, add 800 µl ethanol) and mix thoroughly by pipetting up and down several times. Do not centrifuge. Continue without delay to step 13.**
    A precipitate may form after addition of ethanol, but this will not affect the procedure.
13. **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 (15–25°C). Discard the flow-through.***

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

Reuse the collection tube in step 14.

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

    Reuse the collection tube in step 15.

15. **Add 700 µl Buffer RWT to the RNeasy MinElute spin column. 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 step 16.

16. **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 step 17.

17. **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 contact the flow-through. Otherwise, carryover of ethanol will occur.

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

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

    It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.

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

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

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

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 (step 9), 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 10 and 11 of the 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 Appeendix A (page 27) for removal of residual cellular material. After thawing of frozen samples, remove cryoprecipitates by centrifugation or filtration, if necessary (see Appendix A).
Clogged RNeasy column	

	Comments and suggestions
Centrifugation temperature too low	Except for the phase separation (step 11), all centrifugation steps should be performed at room temperature (15–25°C). 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.
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 12).
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.

	Comments and suggestions
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 C: General Remarks on Handling RNA" (page 40) and "Appendix A: Recommendations for Serum and Plasma Collection, Separation, and Storage" (page 27).
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 C: General Remarks on Handling RNA" (page 40). 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 11 should be performed at 4°C. Make sure that the centrifuge does not heat above 10°C during centrifugation.

	Comments and suggestions
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. Use of MaXtract (cat. no. 129056) can help to avoid interphase carryover.
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 (protocol step 18).

References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc.

For a complete list of references, contact QIAGEN Technical Services or your local distributor.

Appendix A: Recommendations for Serum and Plasma Collection, Separation, and Storage

In order to specifically isolate vesicular RNA from serum and plasma, we recommend following these protocols, which include an initial low *g*-force centrifugation step to separate cells from plasma or serum followed by a higher *g*-force centrifugation or filtration step to remove all remaining cellular debris. 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 (several orders of magnitude), 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 generally results in fewer residual cells.

Because binding of vesicles to the exoEasy membrane is not selective for a specific sub-population 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*), for example, will remove some of the larger vesicles.

The recommended syringe filters (e.g. Sartorius® Minisart® NML (cat. no. 16592) or Millipore® Millex®-AA (cat. no. SLAA033SB)) have dead volumes of around 100–200 µl. Small volumes of sample (<1ml) can be diluted with PBS prior to filtration to reduce loss of material.

Procedure: plasma separation and storage

A1. 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). Store tubes at room temperature (15–25°C) 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.

A2. 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.

A3. 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, vesicular 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.

A4. Centrifuge plasma samples in conical tubes for 15 min at 3,000 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.

A5. 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).

A6. 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 –65°C to –90°C.

A7. Before using frozen plasma for nucleic acid purification, thaw at room temperature (15–25°C). Optional: To remove cryoprecipitates, centrifuge thawed plasma samples for 5 min at 3,000 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

A1. 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 (15–25°C) 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.

A2. 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 9ml tubes, a gel bed will form between the upper serum phase and the lower cellular phase, facilitating recovery of serum.

A3. 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, vesicular 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.

A4. Centrifuge serum samples in conical tubes for 15 min at 3,000 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.

A5. Carefully transfer cleared supernatant to a new tube without disturbing the pellet (which forms a smear along the outer side of the centrifugation tube).

A6. 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 –65°C to –90°C.

A7. Before using frozen serum for nucleic acid purification, thaw at room temperature (15–25°C). Optional: To remove cryoprecipitates, centrifuge thawed serum samples for 5 min at 3,000 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: Use of the miRNeasy Serum/Plasma Spike-In Control in Serum/Plasma miRNA Profiling

There is currently no clear consensus in the research community on what should be used as a normalization control for miRNA expression profiling in a serum or plasma sample. Many researchers choose to spike a synthetic miRNA into their RNA prep to monitor RNA recovery and reverse transcription efficiency. This RNA is added to samples after the addition of denaturant (e.g., QIAzol Lysis Reagent), prior to addition of chloroform and phase separation. After real-time RT-PCR, the C_T value obtained with the assay targeting the synthetic miRNA permits normalization between samples, which can control for varying RNA purification yields and amplification efficiency. In addition, RNA recovery can be assessed by comparing the C_T value to a standard curve of the synthetic miRNA generated independently of the RNA purification procedure. QIAGEN recommends the miRNeasy Serum/Plasma Spike-In Control (cat. no. 219610) for use as an internal control for miRNA expression profiling in serum or plasma. This appendix includes details of preparation of miRNeasy Serum/Plasma Spike-In Control stock and working solution, a protocol for generating a miRNeasy Serum/Plasma Spike-In Control standard curve, and a protocol for assessing the recovery of miRNeasy Serum/Plasma Spike-In Control after RNA purification.

Note: Use of the miRNeasy Serum/Plasma Spike-In Control is not recommended for cell or tissue samples.

Preparation of miRNeasy Serum/Plasma Spike-In Control

The miRNeasy Serum/Plasma Spike-In Control is a *C. elegans* miR-39 miRNA mimic and is supplied lyophilized at 10 pmol per tube. Reconstitute by adding 300 µl RNase-free water per tube, resulting in a 2 x 10^{10} copies/µl stock. Reconstituted miRNeasy Serum/Plasma Spike-In Control stock should be stored at –65°C to –90°C. For large volumes, first aliquot into smaller volumes prior to long-term storage at –65°C to –90°C.

When working with miRNeasy Serum/Plasma Spike-In Control, first add 4 µl of 2 x 10^{10} copies/µl miRNeasy Serum/Plasma Spike-In Control stock to 16 µl RNase-free water, resulting in a 4 x 10^9 copies/µl dilution. If performing purification of RNA from serum and plasma, add 2 µl of the 4 x 10^9 copies/µl dilution to 48 µl RNase-free water to provide a 1.6 x 10^8 copies/µl working solution. If generating a standard curve, add 2 µl of the 4 x 10^9 copies/µl dilution to 78 µl RNase-free water that contains carrier RNA (e.g., 10 ng/µl MS2 [Roche, cat. no. 10 165 948 001] or bacterial ribosomal RNA [Roche,

cat. no. 10 206 938 001]) to provide a 1 x 10^8 copies/µl working solution. These dilutions are summarized in Table 3.

Table 3. miScript Serum/Plasma Spike-In Control dilutions

Purpose	Dilution	Concentration (copies/µl)
Stock	Add 300 µl RNase-free water to lyophilized miScript Serum/Plasma Spike-In Control (10 pmol)	2 x 10^{10}
Dilution	Add 4 µl stock (2 x 10^{10} copies/µl) to 16 µl RNase-free water	4 x 10^9
Working solution for RNA purification (page 14)	Add 2 µl of 4 x 10^9 copies/µl dilution to 48 µl RNase-free water	1.6 x 10^8
Working solution for generation of standard curve (page 31)	Add 2 µl of 4 x 10^9 copies/µl dilution to 78 µl RNase-free water containing 10 ng/µl MS2 (Roche, cat. no. 10 165 948 001) or bacterial ribosomal RNA (Roche, cat. no. 10 206 938 001)	1 x 10^8

Protocol: Generation of miRNeasy Serum/Plasma Spike-In Control Standard Curve

This protocol is for generating a real-time PCR standard curve of miRNeasy Serum/Plasma Spike-In Control that is independent of a serum/plasma sample and RNA purification procedure. The standard curve allows estimation of the recovery of miRNeasy Serum/Plasma Spike-In Control when it is added to a serum/plasma sample that is subsequently used for RNA purification (see protocol on page 14).

Important points before starting

- To ensure reproducibility, always use freshly prepared cDNA to generate a standard curve. Perform PCR for generation of the standard curve and PCR on RNA from the serum/plasma samples of interest in the same run. Do not store cDNA dilutions for later use.

■ This protocol uses the following components of the miScript PCR System: Ce_miR-39_1 miScript Primer Assay (provided in the exoRNeasy Serum/Plasma Maxi Kit), miScript II RT Kit, and miScript SYBR Green PCR Kit. For more information, consult the *miScript PCR System Handbook* or visit www.qiagen.com/miRNA.

Procedure

B1. Prepare a 1 x 10^8 copies/µl working solution of miRNeasy Serum/Plasma Spike-In Control. Mix gently but thoroughly.

For details of preparation of miRNeasy Serum/Plasma Spike-In Control working solution, see Table 3, page 29.

For dilution of the control, we recommend RNase-free water containing 10 ng/µl MS2 (Roche, cat. no. 10 165 948 001) or bacterial ribosomal RNA (Roche, cat. no. 10 206 938 001).

B2. Prepare the reverse transcription reaction on ice according to Table 4.

Table 4. Reverse transcription reaction components

Component	Volume
miRNeasy Serum/Plasma Spike-In Control from step 1 (1 x 10^8 copies/µl)	2.2 µl (2.2 x 10^8 copies/µl)
Total RNA sample*	2 µl (~100 ng)
5x miScript HiSpec Buffer or 5x miScript HiFlex Buffer†	4 µl
10x miScript Nucleics Mix	2 µl
RNase-free water	7.8 µl
miScript Reverse Transcriptase Mix	2 µl
Total volume	**20 µl**

* Any total RNA sample can be used here to provide a complex RNA background.

† The correct buffer to use depends on the subsequent PCR application. Consult the *miScript PCR System Handbook* for more details.

B3. Gently mix, briefly centrifuge, and then store on ice.

B4. Incubate for 60 min at 37°C.

B5. Incubate for 5 min at 95°C to inactivate miScript Reverse Transcriptase Mix and place on ice.

B6. Add 200 µl RNase-free water to the reverse transcription reaction.

This results in a miRNeasy Serum/Plasma Spike-In Control concentration of 1 x 10^6 copies/µl.

B7. Use the diluted reverse transcription reaction to prepare cDNA serial dilutions according to Table 5.

Table 5. cDNA serial dilutions

Tube	cDNA	Water	Concentration spike-in control	Use in PCR
1	20 µl diluted cDNA	20 µl	5 x 10^5 copies/µl	2 µl (1 x 10^6 copies)
2	5 µl from tube 1	45 µl	5 x 10^4 copies/µl	2 µl (1 x 10^5 copies)
3	5 µl from tube 2	45 µl	5 x 10^3 copies/µl	2 µl (1 x 10^4 copies)
4	5 µl from tube 3	45 µl	5 x 10^2 copies/µl	2 µl (1 x 10^3 copies)

B8. Using 2 µl from each tube in Table 5, set up separate PCRs according to Table 6.

We recommend setting up each reaction in triplicate.

Table 6. Reaction setup for real-time PCR

Component	Volume/ reaction (384-well)	Volume/ reaction (96-well)	Volume/ reaction (Rotor-Disc® 100)*
2x QuantiTect® SYBR Green PCR Master Mix	5 µl	12.5 µl	10 µl
10x miScript Universal Primer	1 µl	2.5 µl	2 µl
10x Ce_miR-39_1 miScript Primer Assay	1 µl	2.5 µl	2 µl
RNase-free water	1 µl	5.5 µl	4 µl
Template cDNA from Table 5	2 µl	2 µl	2 µl
Total volume	**10 µl**	**25 µl**	**20 µl**

* These volumes can also be used for reactions set up in Strip Tubes for use with the Rotor-Gene® Q 72-Well Rotor.

B9. Mix thoroughly and proceed with PCR using the cycling conditions in Table 7.

Note: Perform dissociation curve analysis of the PCR product(s) to verify their specificity and identity. Dissociation curve analysis is an analysis step built into the software of real-time cyclers. Follow the instructions provided by the supplier.

Table 7. Cycling conditions for real-time PCR

Step	Time	Temperature	Additional comments
PCR initial activation step	**15 min**	**95°C**	HotStarTaq DNA Polymerase is activated by this heating step.
3-step cycling:*†‡			
Denaturation	15 s	94°C	
Annealing	30 s	55°C	
Extension§	30 s	70°C	Perform fluorescence data collection.
Cycle number	40 cycles¶		Cycle number depends on the amount of template cDNA and abundance of the target.

* For Bio-Rad® models CFX96™ and CFX384™: adjust the ramp rate to 1°C/s.

† For Eppendorf® Mastercycler® ep realplex models 2, 2S, 4, and 4S: for the Silver Thermoblock, adjust the ramp rate to 26%; for the Aluminum Thermoblock, adjust the ramp rate to 35%.

‡ If using a Roche® LightCycler® 480, adjust the ramp rate to 1°C/s.

§ Due to software requirements, the fluorescence detection step must be at least 30 s with the ABI PRISM® 7000 or 34 s with the Applied Biosystems® 7300 and 7500.

¶ If using a Roche LightCycler 480, use 45 cycles.

B10. Extract C_T values for miRNeasy Serum/Plasma Spike-In Control from each reaction.

B11. Generate a standard curve by plotting the log copy number miRNeasy Serum/Plasma Spike-In Control used in each PCR against the mean C_T value.

Protocol: Assessment of Recovery of miRNeasy Serum/Plasma Spike-In Control after miRNA Purification

This protocol is a guideline for the addition of miRNeasy Serum/Plasma Spike-In Control to a serum/plasma sample during RNA purification, followed by determination of recovery of miRNeasy Serum/Plasma Spike-In Control by real-time RT-PCR using the standard curve generated in the protocol on page 31.

Important points before starting

- This protocol uses the following components of the miScript PCR System: Ce_miR-39_1 miScript Primer Assay (provided in the exoRNeasy Serum/Plasma Maxi Kit), miScript II RT Kit, and miScript SYBR Green PCR Kit. For more information, consult the *miScript PCR System Handbook* or visit www.qiagen.com/miRNA.

Procedure

B1. Prepare a 1.6 x 10^8 copies/µl working solution of miRNeasy Serum/Plasma Spike-In Control. Mix gently but thoroughly.

For details on preparation of miRNeasy Serum/Plasma Spike-In Control working solution, see Table 3, page 29.

B2. During RNA purification, add 3.5 µl miRNeasy Serum/Plasma Spike-In Control working solution from step B1 (1.6 x 10^8 copies/µl) to the sample after lysis with QIAzol Lysis Reagent (see step 7, page 15). Mix thoroughly.

We recommend addition of miRNeasy Serum/Plasma Spike-In Control after lysis to avoid degradation by endogenous RNases in the sample. This can be modified if desired.

B3. Continue with RNA purification (page 15). After RNA elution in 14 µl RNase-free water (step 19, page 14), miRNeasy Serum/Plasma Spike-In Control is present in the eluate at 4 x 10^7 copies/µl.

If a different elution volume is used, calculate the miRNeasy Serum/Plasma Spike-In Control concentration accordingly.

B4. Prepare the reverse transcription reaction on ice according to Table 8.

Table 8. Reverse transcription reaction components

Component	Volume
Purified RNA (containing miRNeasy Serum/Plasma Spike-In Control)	1.5 µl
5x miScript HiSpec Buffer or 5x miScript HiFlex Buffer*	4 µl
10x miScript Nucleics Mix	2 µl
RNase-free water	10.5 µl
miScript Reverse Transcriptase Mix	2 µl
Total volume	**20 µl**

* The correct buffer to use depends on the subsequent PCR application. Consult the *miScript PCR System Handbook* for more details.

B5. Gently mix, briefly centrifuge, and then store on ice.

B6. Incubate for 60 min at 37°C.

B7. Incubate for 5 min at 95°C to inactivate miScript Reverse Transcriptase Mix and place on ice.

B8. Add 200 µl RNase-free water to the reverse transcription reaction.

This results in a miRNeasy Serum/Plasma Spike-In Control concentration of 2.7×10^5 copies/µl (assuming 100% recovery during RNA purification and reverse transcription).

B9. Set up PCR reactions according to Table 9.

We recommend setting up each reaction in triplicate.

Table 9. Reaction set up for real-time PCR

Component	Volume/ reaction (384-well)	Volume/ reaction (96-well)	Volume/ reaction (Rotor-Disc 100)*
2x QuantiTect SYBR Green PCR Master Mix	5 µl	12.5 µl	10 µl
10x miScript Universal Primer	1 µl	2.5 µl	2 µl
10x Ce_miR-39_1 miScript Primer Assay	1 µl	2.5 µl	2 µl
RNase-free water	2 µl	6.5 µl	5 µl
Diluted reverse transcription reaction	1 µl	1 µl	1 µl
Total volume	**10 µl**	**25 µl**	**20 µl**

* These volumes can also be used for reactions set up in Strip Tubes for use with the Rotor-Gene Q 72-Well Rotor.

B10. Mix thoroughly and proceed with PCR using the cycling conditions in Table 10.

Note: Perform dissociation curve analysis of the PCR product(s) to verify their specificity and identity. Dissociation curve analysis is an analysis step built into the software of real-time cyclers. Follow the instructions provided by the supplier.

Table 10. Cycling conditions for real-time PCR

Step	Time	Temperature	Additional comments
PCR initial activation step	**15 min**	**95°C**	HotStarTaq DNA Polymerase is activated by this heating step.
3-step cycling:*†‡			
Denaturation	15 s	94°C	
Annealing	30 s	55°C	
Extension§	30 s	70°C	Perform fluorescence data collection.
Cycle number	40 cycles¶		Cycle number depends on the amount of template cDNA and abundance of the target.

* For Bio-Rad models CFX96 and CFX384: adjust the ramp rate to 1°C/s.

† For Eppendorf Mastercyler ep realplex models 2, 2S, 4, and 4S: for the Silver Thermoblock, adjust the ramp rate to 26%; for the Aluminum Thermoblock, adjust the ramp rate to 35%.

‡ If using a Roche LightCycler 480, adjust the ramp rate to 1°C/s.

§ Due to software requirements, the fluorescence detection step must be at least 30 s with the ABI PRISM 7000 or 34 s with the Applied Biosystems 7300 and 7500.

¶ If using a Roche LightCycler 480, use 45 cycles.

B11. Extract C_T values and determine the mean C_T value for miRNeasy Serum/Plasma Spike-In Control from each reaction.

B12. Compare with the miRNeasy Serum/Plasma Spike-In Control standard curve to determine recovery of miRNeasy Serum/Plasma Spike-In Control.

Appendix C: 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., pipets and electrophoresis tanks), use of RNaseKiller (cat. no. 2500080) from 5 PRIME (www.5prime.com) 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 41), or rinse with chloroform* if the plasticware is chloroform-resistant. To decontaminate electrophoresis tanks, clean with 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.

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

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 hours 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 hours at 37°C. Autoclave for 15 minutes 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 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 minutes.

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

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

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

Storage of RNA

Purified RNA may be stored at –15°C to –30°C or –65°C to –90°C 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). For small amounts of RNA, however, which are typically obtained from EVs it is usually not possible 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.

Spectrophotometric quantification of RNA

To ensure significance, A_{260} readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 44 µg of RNA per ml (A_{260}=1→ 44 µg/ml). This relation is valid only for measurements at a neutral pH. Therefore, if it is necessary to dilute the RNA sample, this should be done in a buffer with neutral pH.* As discussed below (see "Purity of RNA", page 43), the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity.

When measuring RNA samples, be certain that cuvettes are RNase-free, especially if the RNA is to be recovered after spectrophotometry. This can be accomplished by washing cuvettes with 0.1 M NaOH, 1 mM EDTA,* followed by washing with RNase-free water (see "Solutions", page 41). Use the buffer in which the RNA is diluted to zero the spectrophotometer. An example of the calculation involved in RNA quantification is shown below:

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

Volume of RNA sample = 100 µl

Dilution = 10 µl of RNA sample + 490 µl of 10 mM Tris·Cl,* pH 7.0 (1/50 dilution)

Measure absorbance of diluted sample in a 1 ml cuvette (RNase-free)

A_{260} = 0.2

Concentration of RNA sample = 44 µg/ml x A_{260} x dilution factor

= 44 µg/ml x 0.2 x 50

= 440 µg/ml

Total amount = concentration x volume in milliliters

= 440 µg/ml x 0.1 ml

= 44 µg of RNA

Purity of RNA

The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV spectrum, such as protein. However, the A_{260}/A_{280} ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting A_{260}/A_{280} ratio can vary greatly. Lower pH results in a lower A_{260}/A_{280} ratio and reduced sensitivity to protein contamination.* For accurate values, we recommend measuring absorbance in 10 mM Tris·Cl, pH 7.5. Pure RNA has an A_{260}/A_{280} ratio of 1.9–2.1† in 10 mM Tris·Cl, pH 7.5. Always be sure to calibrate the spectrophotometer with the same solution used for dilution.

For determination of RNA concentration, however, we recommend dilution of the sample in a buffer with neutral pH since the relationship between absorbance and concentration (A_{260} reading of 1 = 44 µg/ml RNA) is based on an extinction coefficient calculated for RNA at neutral pH (see "Quantification of RNA", page 42).

DNA contamination

No currently available purification method can guarantee that RNA is completely free of DNA, even when it is not visible on an agarose gel. While miRNeasy Kits will remove the vast majority of cellular DNA, trace amounts may

* Wilfinger, W.W., Mackey, M., and Chomczynski, P. (1997) Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. BioTechniques **22**, 474.

† Values up to 2.3 are routinely obtained for pure RNA (in 10 mM Tris·Cl, pH 7.5) with some spectrophotometers.

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, such as with ABI PRISM and LightCycler instruments, we recommend designing primers that anneal at intron splice junctions so that genomic DNA will not be amplified. QuantiTect Primer Assays from QIAGEN are designed for SYBR Green 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 QuantiTect Reverse Transcription Kit for reverse transcription. The kit integrates fast cDNA synthesis with rapid removal of genomic DNA contamination.

Alternatively, gene expression analysis can be performed using QuantiFast® Probe Assays and the QuantiFast Probe RT-PCR Plus Kit, which includes an integrated genomic DNA removal step.

miScript Primer Assays, used with the miScript PCR System 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 towards 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 material safety data sheets (MSDSs), 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

Products	Contents	Cat. no.
exoRNeasy Serum/Plasma Maxi Kit	For 50 RNA preps: 50 exoEasy Maxi and RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml, 2 ml, and 50 ml), QIAzol Lysis Reagent, Ce_miR-39_1 miScript Primer Assay, RNase-free Reagents and Buffers	77064
exoRNeasy Serum/Plasma Midi Kit	For 50 RNA preps: 50 exoEasy Midi and RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml, 2 ml, and 50 ml), QIAzol Lysis Reagent, Ce_miR-39_1 miScript Primer Assay, RNase-free Reagents and Buffers	77044
exoRNeasy Serum/Plasma Starter Kit	For 20 RNA preps: 10 exoEasy Maxi, 10 exoEasy Midi, and 20 RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml, 2 ml, and 50 ml), QIAzol Lysis Reagent, Ce_miR-39_1 miScript Primer Assay, RNase-free Reagents and Buffers	77023
Related products		
miRNeasy Serum/Plasma Spike-In Control	10 pmol lyophilized *C. elegans* miR-39 miRNA mimic	219610
MaXtract High Density (200 x 2 ml)	200 x 2 ml MaXtract High Density Tubes	129056
miRNeasy Serum/Plasma Kit (50)	For 50 total RNA preps: 50 RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml and 2 ml), QIAzol Lysis Reagent, Ce_miR-39_1 miScript Primer Assay, RNase-free Reagents and Buffers	217184

Products	Contents	Cat. no.
miRNeasy 96 Kit (4)	For 4 x 96 total RNA preps: 4 RNeasy 96 plates, Collection Microtubes (racked), Elution Microtubes CL, Caps, S-Blocks, AirPore Tape Sheets, QIAzol Lysis Reagent, RNase-Free Reagents and Buffers	217061
miRNeasy FFPE Kit (50)	For 50 total RNA preps: 50 RNeasy MinElute Spin Columns, 50 gDNA Eliminator Spin Columns, Collection Tubes, Proteinase K, and RNase-Free Reagents and Buffers.	217504
Collection Tubes (2 ml)	1000 Collection Tubes (2 ml)	19201
Related products for quantitative, real-time RT-PCR		
miScript II RT Kit (12)	For 12 cDNA synthesis reactions: miScript Reverse Transcriptase Mix, 10x miScript Nucleics Mix, 5x miScript HiSpec Buffer, 5x miScript HiFlex Buffer, RNase-Free Water	218160
miScript II RT Kit (50)	For 50 cDNA synthesis reactions: miScript Reverse Transcriptase Mix, 10x miScript Nucleics Mix, 5x miScript HiSpec Buffer, 5x miScript HiFlex Buffer, RNase-Free Water	218161
miScript SYBR Green PCR Kit (200)	For 200 reactions: QuantiTect SYBR Green PCR Master Mix, miScript Universal Primer	218073
miScript SYBR Green PCR Kit (1000)	For 1000 reactions: QuantiTect SYBR Green PCR Master Mix, miScript Universal Primer	218075

Products	Contents	Cat. no.
miScript Primer Assay (100)	miRNA-specific primer; available via GeneGlobe	Varies*
miScript PreAMP PCR Kit (12)	For 12 preamplification reactions: 5x miScript PreAMP Buffer, HotStarTaq DNA Polymerase (2 U/µl), miScript PreAMP Universal Primer, miR-16 miScript Primer Assay, SNORD95 miScript Primer Assay, miRNA reverse transcription control (miRTC), *C. elegans* miR-39 miScript Primer Assay, RNase-Free Water	331451
miScript PreAMP PCR Kit (60)	For 60 preamplification reactions: 5x miScript PreAMP Buffer, HotStarTaq DNA Polymerase (2 U/µl), miScript PreAMP Universal Primer, miR-16 miScript Primer Assay, SNORD95 miScript Primer Assay, miRNA reverse transcription control (miRTC), *C. elegans* miR-39 miScript Primer Assay, RNase-Free Water	331452
Pathway-Focused miScript miRNA PCR Array	Array of assays for a pathway, disease, or gene family for human, mouse, rat, dog, or rhesus macaque miRNAs; available in 96-well, 384-well, or Rotor-Disc 100 format	Varies

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

Products	Contents	Cat. no.
miRNome miScript miRNA PCR Array	Array of assays for the complete human, mouse, rat, dog, or rhesus macaque miRNome; available in 96-well, 384-well, or Rotor-Disc 100 format	Varies
Related products for automation		
QIAcube (110V)	Robotic workstation for automated purification of DNA, RNA, or proteins using QIAGEN spin-column kits, 1-year warranty on parts and labor	9001292
QIAcube (230 V)	Robotic workstation for automated purification of DNA, RNA, or proteins using QIAGEN spin-column kits, 1-year warranty on parts and labor	9001293

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