August 2017

miRNeasy® Serum/ Plasma Advanced Kit Handbook

For purification of total RNA, including miRNA, from serum and plasma

Sample to Insight

QIAGEN

Contents

Kit Contents

miRNeasy Serum/Plasma Advanced Kit (50)	**(50)**
Catalog no.	**217204**
Number of preps	**50**
RNeasy® UCP MinElute® Spin Columns (each packaged with a 2 ml Collection Tube)	50
Collection Tubes (1.5 ml)	50
Collection Tubes (2 ml)	50
Buffer RPL*	20 ml
Buffer RPP	8 ml
Buffer RWT*†	15 ml
Buffer RPE‡	11 ml
RNase-Free Water	10 ml
Quick-Start Protocol	1

* Contains a guanidine salt. Not compatible with disinfectants containing bleach. See page 5 for safety information.

† 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.

Storage

The miRNeasy Serum/Plasma Advanced Kit (cat. no. 217204) is shipped at ambient temperature. Store the RNeasy UCP MinElute spin columns immediately at 2–8°C. Store the remaining components dry at room temperature (15–25°C). All kit components are stable for at least 9 months under these conditions.

Intended Use

The miRNeasy Serum/Plasma Advanced Kit is 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 these 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 waste containing Buffer RWT or Buffer RPL.

Buffer RWT and Buffer RPL contain guanidine thiocyanate, which can form highly reactive compounds when combined with bleach. If liquid containing these solutions 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 miRNeasy Serum/Plasma Advanced Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

Interest in smaller RNA species, such as miRNA, has increased over the past years as researchers understand the regulatory role of small non-coding RNAs. The miRNeasy Serum/Plasma Advanced Kit is designed for purification of cell-free total RNA – primarily miRNA and other small RNA – from small volumes of serum and plasma.

When working with serum and plasma samples, we recommend the use of a synthetic spike-in control for normalization, such as the miRNeasy Serum/Plasma Spike-In Control, which must be ordered separately (cat. no. 219610).

The miRNeasy Serum/Plasma Advanced Kit offers a phenol-free protocol to isolate high yields of cell-free total RNA including miRNA from only 200 µl of serum or plasma.

Principle and workflow

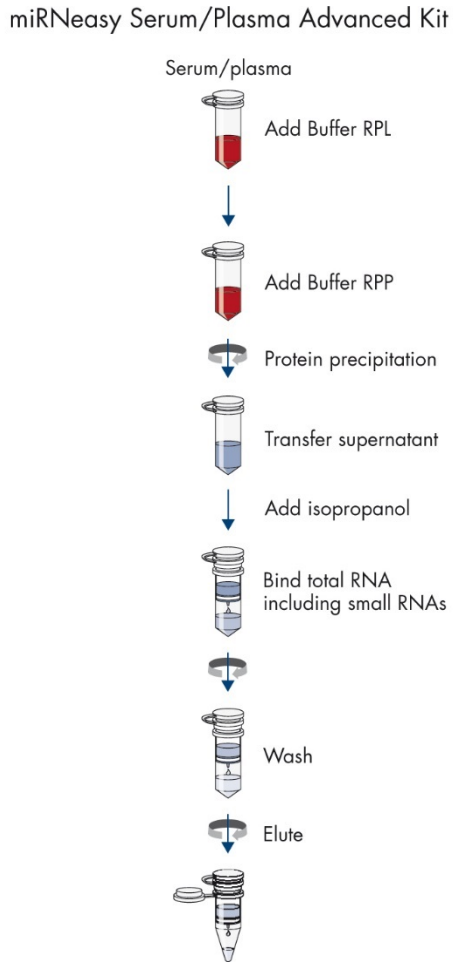
The miRNeasy Serum/Plasma Advanced Kit combines guanidine-based lysis of samples, an inhibitor removal step and silica-membrane–based purification of total RNA. Buffer RPL, included in the kit, contains guanidine thiocyanate as well as detergents that are designed to facilitate lysis and denature protein complexes and RNases. Therefore, RNA in samples lysed in buffer RPL are stable and protected from degradation.

Buffer RPL is added to serum or plasma samples. After thoroughly mixing to ensure a complete lysis, Buffer RPP is added to precipitate inhibitors (mostly proteins that are highly concentrated in serum/plasma samples) by centrifugation.

The supernatant containing the RNA is transferred to a new microcentrifuge tube, and isopropanol is added to provide appropriate binding conditions for all RNA molecules from approximately 18 nucleotides (nt) upwards. The sample is then applied to the RNeasy UCP

MinElute spin column, where the total RNA binds to the membrane and all contaminants are efficiently washed away. High-quality RNA is eluted in a small volume of RNase-free water.

Serum and plasma contain primarily small RNAs, therefore, enrichment of miRNAs and other small RNAs in a separate fraction is usually not required.



Automated sample preparation on the QIAcube®

Purification of total RNA, including miRNA, can be automated on the QIAcube. The innovative QIAcube uses advanced technology to process QIAGEN spin columns, enabling seamless integration of automated, low-throughput sample prep into your laboratory workflow. Sample preparation using the QIAcube follows the same steps as the manual procedure (i.e., lyse, bind, wash and elute), enabling you to continue using the miRNeasy Serum/Plasma Advanced Kit for purification of high-quality RNA.

The QIAcube is 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/MyQIAcube**.

qPCR-based miRNA quantification using the miScript® PCR system

In general, qPCR is recommend to accurately quantify yields of miRNA. 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**.

Description of protocols

This handbook contains one protocol on page 14 for purification of cell-free total RNA, including miRNA, from serum or plasma using the miRNeasy Serum/Plasma Advanced Kit. The appendices contain additional protocols 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.

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.

The following supplies are also required:

- 100% Isopropanol
- 80% Ethanol*
- Sterile, RNase-free pipet tips
- 1.5 ml or 2 ml microcentrifuge tubes
- Microcentrifuge with rotor for 2 ml tubes for centrifugation at room temperature
- Disposable gloves
- The miRNeasy Serum/Plasma Spike-In Control must be purchased separately (see Ordering Information on page 46)
- Equipment and tubes for serum/plasma collection and separation (see Appendix B, page 24):
  - For serum: primary blood collection tube(s) without anticoagulants such as EDTA or citrate
  - For plasma: primary blood collection tube(s) containing EDTA as anticoagulant
  - Conical tube(s)
  - Refrigerated centrifuge with a swinging bucket rotor and fixed-angle rotor

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

Important Notes

Volume of starting material

The preferred volume of starting material is set to 200 µl. This volume is usually sufficient to also detect low-abundance miRNA in serum/plasma samples. When using only 200 µl of starting material, only one loading step onto the RNeasy UCP MinElute column is needed. Using higher amounts of serum/plasma will lead to more handling steps, while only increasing the amount of recovered miRNA by 1–2 cycles, which is not much more than normal sample to sample variation. Furthermore, using more starting material than recommended increases the chance of co-isolating additional PCR inhibitors with the RNA, and this can affect the PCR efficiency and oppose the higher yield.

Yields of total RNA purified with the miRNeasy Serum/Plasma Advanced Kit vary strongly between different plasma samples. However, they are usually too low for quantification by OD measurement. We recommend using the miRNeasy Serum/Plasma Spike-In Control (cat. no. 219610) and the corresponding Ce_miR-39_1 miScript Primer Assay (MS00019789) to monitor miRNA purification and amplification.

Optional: For reconstitution of Ce_miR-39_1 miScript Primer Assay

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

- 1 ml of 1 M Tris/HCl, pH 8.0 (autoclaved)
- 0.2 ml of 0.5 M EDTA, pH 8.0 (autoclaved)
- 98.8 ml of distilled water

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

To reconstitute the 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 to avoid repeated freezing and thawing.

Specifications of RNeasy UCP MinElute spin columns

Table 1. RNeasy UCP MinElute spin column specifications

Description	Specification
Maximum binding capacity	45 µg RNA
Maximum loading volume	700 µl
RNA size distribution	RNA >18 nucleotides approximately
Minimum elution volume	10 µl
Maximum amount of serum or plasma	600 µl

Note: If the recommended sample volume is exceeded, RNA yields will not be consistent and may be reduced, even if the binding capacity of the RNeasy UCP MinElute spin column is not exceeded.

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

This protocol is intended as a guideline for the purification of cell-free total RNA, which primarily includes small RNAs such as miRNAs, from small volumes (up to 600 µl) of serum and plasma using the miRNeasy Serum/Plasma Advanced Kit. Processing of more than 200 µl sample is not recommended, because the amounts of contaminants introduced by larger sample volumes may interfere with the purification process.

For recommendations on collection, preparation and storage of cell-free plasma and serum, see Appendix B on page 24.

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 to –30°C or –65 to –90°C in aliquots is recommended. To process frozen lysates, incubate at 37°C in a water bath until samples are completely thawed and salts are dissolved. Avoid prolonged incubation, which may compromise RNA integrity.
- See Appendix F on page 43 for optional on-column DNase digestion. DNase I digestion is not recommended for plasma or serum samples. Cell-free body fluids typically do not contain significant amounts of DNA. 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 plasma or serum.
- Buffers RPL and RWT may form a precipitate upon storage. If necessary, redissolve by warming and then place at room temperature (15–25°C).
- Equilibrate buffers to room temperature (15–25°C) before starting the protocol.

- Buffers RPL and RWT contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach.
- All steps should be performed at room temperature (15–25°C). Work quickly.
- The procedure is suitable for use with either serum samples or plasma samples containing EDTA. For citrate plasma samples, refer to the protocol in Appendix A on page 20. Plasma samples containing heparin should not be used, because this anticoagulant can interfere with downstream assays, such as RT-PCR.

Things to do before starting

- Buffers RWT and RPE are supplied as concentrates. Before using for the first time, add the required volumes of ethanol (96–100%), as indicated on the bottles, to obtain a working solution.
- Prepare a working solution of miRNeasy Serum/Plasma Spike-In Control as described in Appendix C, page 28.
- Use of carrier RNA (e.g., 1 µg MS2 RNA, Roche®, cat. no. 10 165 948 001) or bacterial ribosomal RNA (Roche, cat. no. 10 206 938 001) may increase recovery in some cases. Do not use polyA RNA.

Procedure

1. Prepare serum or plasma or thaw frozen samples.
2. Transfer 200 µl serum or plasma into a 2 ml microcentrifuge tube.

   Note: It is possible to process up to 600 µl of serum/plasma sample. In this case, adapt the volume of RPL and RPP according to Table 2.

Table 2. Adapting Buffer RPL and RPP volumes for larger starting sample volumes

Serum/plasma	Buffer RPL	Buffer RPP
200 µl	60 µl	20 µl
400µl	120 µl	40 µl
600 µl	180 µl	60 µl

3. Add 60 µl Buffer RPL. Close the tube caps and vortex for >5 s. Leave at room temperature for 3 min.

   Note: If using a volume of serum/plasma other than 200 µl, adapt the volume of Buffer RPL according to Table 2.

4. Add 3.5 µl miRNeasy Serum/Plasma Spike-In Control (1.6 x 10^8 copies/µl working solution) and mix thoroughly.

   For details on making appropriate stocks and working solutions of miRNeasy Serum/Plasma Spike-In Control, see Appendix C on page 28.

5. Add 20 µl Buffer RPP. Close the tube caps and mix vigorously by vortexing for >20 s. Incubate at room temperature for 3 min.

   Thorough mixing is important for subsequent phase separation.

   Note: If using a volume of serum/plasma other than 200 µl, adapt the volume of Buffer RPP according to Table 2.

6. Centrifuge at 12000 *x g* for 3 min at room temperature to pellet the precipitate.

   Note: Supernatant should be clear and colorless.

7. Transfer supernatant (~230 µl for 200 µl serum/plasma) to a new microcentrifuge tube. Add 1 volume of isopropanol. Mix well by vortexing.

8. Transfer the entire sample to an RNeasy UCP MinElute column. Close the lid, and centrifuge for 15 s at ≥8000 *x g*. Discard the flow-through.

9. Pipet 700 µl Buffer RWT onto the RNeasy UCP MinElute spin column. Close the lid, and centrifuge for 15 s at ≥8000 *x g*. Discard the flow-through.

   Reuse the collection tube in the next step.

10. Pipet 500 µl Buffer RPE onto the RNeasy UCP MinElute spin column. Close the lid, and centrifuge for 15 s at ≥8000 *x g*. Discard the flow-through.

    Reuse the collection tube in the next step.

11. Add 500 µl of 80% ethanol to the RNeasy UCP MinElute spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 *x g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.

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

12. Place the RNeasy UCP MinElute spin column in 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 flow-through and the collection tube.

    Note: 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).

    Note: 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.

13. Place the RNeasy UCP MinElute spin column in a new 1.5 ml collection tube (supplied). Add 20 µl RNase-free water directly to the center of the spin column membrane and incubate 1 min. Close the lid, and centrifuge for 1 min at full speed to elute the RNA.

    Note: As little as 10 µl RNase-free water can be used for elution if a higher RNA concentration is required, but the yield might be reduced. 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 UCP MinElute spin column is 2 µl, so elution with 20 µl RNase-free water results in a 18 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
Clogged column	Make sure to follow recommendations in Appendix B (page 24) for removal of residual cellular material. After thawing of frozen samples, remove cryoprecipitates by centrifugation or filtration, if necessary (see Appendix B). Make sure to not transfer any precipitate from step 6 on page 16 when transferring the supernatant to a fresh tube
Centrifugation temperature too low	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 UCP 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 UCP MinElute spin column.
Low miRNA yield or poor performance of miRNA in downstream applications	
a) 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, Volume of starting material).
b) Elution buffer incorrectly dispensed	Add elution buffer to the center of the RNeasy UCP 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 UCP 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	Cell-free RNA in plasma and serum typically consists of small RNA species only and will therefore not resemble intact RNA from cells or tissue. 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 (page 38) and Appendix B: Recommendations for Serum and Plasma Collection, Separation and Storage (page 24).
b) RNase contamination	Cell-free RNA in plasma and serum typically consists of small RNA species only, and will therefore not resemble intact RNA from cells or tissue. Nevertheless, the following precautions are recommended to avoid complications due to RNA degradation. 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 (page 38). 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) Salt carryover during elution	Ensure that Buffer RPE is at 20–30°C.
b) Ethanol carryover	After the final membrane wash, be sure to dry the RNeasy UCP MinElute spin column by centrifugation at full speed with open lids for 5 min (protocol step 12 on page 17).
Contamination by genomic DNA	
Sample inappropriately handled	Invert tubes gently to mix contents after blood collection. Vigorous mixing or shaking can promote hemolysis. Generate plasma as quickly as possible after blood collection. Long delays can promote hemolysis or apoptotic cell death. Perform the second centrifugation or filtration before freezing the plasma, if possible.

Appendix A: Purification of Total RNA, Including miRNA, from Citrate Plasma

This protocol is intended as a guideline for the purification of cell-free total RNA from small volumes (up to 600 µl) of citrate plasma using the miRNeasy Serum/Plasma Advanced Kit. Processing of more than 200 µl sample is not recommended, because the amounts of contaminants introduced by larger sample volumes may interfere with the purification process.

For recommendations on collection, preparation and storage of cell-free plasma and serum, see Appendix B, page 24.

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 to –30°C or –65 to –90°C in aliquots is recommended. To process frozen lysates, incubate at 37°C in a water bath until samples are completely thawed and salts are dissolved. Avoid prolonged incubation, which may compromise RNA integrity.
- See Appendix F on page 43 for optional on-column DNase digestion. DNase I digestion is not recommended for plasma or serum samples. Cell-free body fluids typically do not contain significant amounts of DNA. 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 plasma or serum.
- Buffers RPL and RWT may form a precipitate upon storage. If necessary, redissolve by warming and then place at room temperature (15–25°C).
- Equilibrate buffers to room temperature (15–25°C) before starting the protocol.
- Buffers RPL and RWT contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach.

- All steps should be performed at room temperature (15–25°C). Work quickly.
- The procedure is suitable for use with plasma samples containing citrate. For serum or EDTA plasma samples, refer to the protocol on page 14. Plasma samples containing heparin should not be used, because this anticoagulant can interfere with downstream assays, such as RT-PCR.

Things to do before starting

- Buffers RWT and RPE are supplied as concentrates. Before using for the first time, add the required volumes of ethanol (96–100%), as indicated on the bottles, to obtain a working solution.
- Prepare a working solution of miRNeasy Serum/Plasma Spike-In Control as described in Appendix B, page 24.
- Use of carrier RNA (e.g., 1 µg MS2 RNA, Roche, cat. no. 10 165 948 001) or bacterial ribosomal RNA (Roche, cat. no. 10 206 938 001) may increase recovery in some cases. Do not use polyA RNA.

Procedure

1. Prepare serum or plasma or thaw frozen samples.
2. Transfer 200 µl serum or plasma into a 2 ml microcentrifuge tube.

   Note: It is possible to process up to 600 µl serum/plasma sample. In this case please adapt the volume of RPL and RPP according to Table 3.

Table 3. Adapting Buffer RPL and RPP volumes for larger starting sample volumes

Serum/plasma	Buffer RPL	Buffer RPP
200 µl	60 µl	60 µl
400µl	120 µl	120 µl
600 µl	180 µl	180 µl

3. Add 60 µl Buffer RPL. Close the tube caps and vortex for >5 s. Leave at room temperature for 3 min.

   Note: If using a volume of serum/plasma other than 200 µl, adapt the volume of Buffer RPL according to Table 3.

4. Add 3.5 µl miRNeasy Serum/Plasma Spike-In Control (1.6 x 10^8 copies/µl working solution) and mix thoroughly.

   For details on making appropriate stocks and working solutions of miRNeasy Serum/Plasma Spike-In Control, see Appendix C on page 28.

5. Add 60 µl Buffer RPP. Close the tube caps and mix vigorously by vortexing for >20 s. Incubate at room temperature for 3 min.

   Thorough mixing is important for subsequent phase separation.

   Note: If using a volume of serum/plasma other than 200 µl, adapt the volume of Buffer RPP according to Table 3.

6. Centrifuge at 12000 *x g* for 3 min at room temperature to pellet the precipitate.

   Note: Supernatant should be clear and colorless.

7. Transfer supernatant (~230 µl for 200 µl serum/plasma) to a new microcentrifuge tube. Add 1 volume of isopropanol. Mix well by vortexing.

8. Transfer the entire sample to an RNeasy UCP MinElute column. Close the lid, and centrifuge for 15 s at ≥8000 *x g*. Discard the flow-through.

9. Pipet 700 µl Buffer RWT to the RNeasy UCP MinElute spin column. Close the lid, and centrifuge for 15 s at ≥8000 *x g*. Discard the flow-through.

   Reuse the collection tube in the next step.

10. Pipet 500 µl Buffer RPE onto the RNeasy UCP MinElute spin column. Close the lid, and centrifuge for 15 s at ≥8000 *x g*. Discard the flow-through.

    Reuse the collection tube in the next step.

11. Add 500 µl of 80% ethanol to the RNeasy UCP MinElute spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 *x g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.

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

12. Place the RNeasy UCP MinElute spin column in 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 flow-through and the collection tube.

    Note: 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 counter-clockwise).

    Note: 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.

13. Place the RNeasy UCP MinElute spin column in a new 1.5 ml collection tube (supplied). Add 20 µl RNase-free water directly to the center of the spin column membrane and incubate 1 min. Close the lid, and centrifuge for 1 min at full speed to elute the RNA.

    Note: As little as 10 µl RNase-free water can be used for elution if a higher RNA concentration is required, but the yield might be reduced. 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 UCP MinElute spin column is 2 µl, so elution with 20 µl RNase-free water results in a 18 µl eluate.

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

To isolate circulating, cell-free nucleic acids from whole blood samples, we recommend following these protocols, which include an initial low g-force centrifugation step to separate cells from plasma or serum followed by a high g-force centrifugation or filtration step to remove all remaining cellular debris. The latter centrifugation 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 nucleic acids released *in vitro*. Use of gel barrier tubes generally results in fewer residual cells.

The speed at which the second centrifugation step is performed will influence the recovery of different types of nucleic acid. Centrifugation at medium speed (e.g., 3000 *x g*) will effectively remove cellular material, including thrombocyte fragments and apoptotic bodies. Centrifugation at higher speed (e.g., 16,000 *x g*) may in addition remove intact chromatin from ruptured blood cells, but may also remove larger extracellular vesicles that may contain cell-free nucleic acid (especially mRNA).

Syringe filters with 0.8 µm pore size (e.g., Sartorius® Minisart® NML (cat. no. 16592) or Millipore® Millex®-AA (cat. no. SLAA033SB)) remove remaining cell fragments and debris strictly based on size, irrespective of density. These filters have dead volumes of around 100–200 µl.

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 EDTA as

anticoagulant). 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.

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 of 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, 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 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 recommendations above).

   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 or 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 –65°C to –90°C.
7. 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 3000 *x g* and 4°C or pass through a 0.8 µm filter. Transfer supernatant to a new tube, and begin the 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 (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.

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 9ml 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 acids by gDNA and RNA derived from damaged blood cells.

4. 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 recommendations above). 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 –65°C to –90°C.

7. 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 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 C: 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., Lysis Buffer RPL), prior to addition of Buffer RPP and protein precipitation. 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 an 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. The miRNeasy Serum/Plasma Spike-In Control

stock should be stored at –65 to –90°C. For large volumes, first aliquot into smaller volumes prior to long-term storage at –65 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 4.

Table 4. 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 (protocol on page 15)	Add 2 µl of 4 x 10^{9} copies/µl dilution to 48 µl RNase-free water	1.6 x 10^{8}
Add 2 µl of 4 x 10^{9} copies/µl dilution to 48 µl RNase-free water	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 15).

Important points before starting

- To ensure reproducibility, always use freshly prepared cDNA to generate a standard curve. Perform PCRs for generation of the standard curve and PCRs 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, miScript II RT Kit and the miScript SYBR Green PCR Kit. For more details, consult the *miScript PCR System Handbook* or visit **www.qiagen.com/miRNA**.

Procedure

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

   For details of preparation of miRNeasy Serum/Plasma Spike-In Control working solution, see Table 4 on 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).
2. Prepare the reverse transcription reaction on ice according to Table 5.

Table 5. 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.

3. Gently mix, briefly centrifuge, and then store on ice.
4. Incubate for 60 min at 37°C.
5. Incubate for 5 min at 95°C to inactivate miScript Reverse Transcriptase Mix and place on ice.
6. 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.
7. Use the diluted reverse transcription reaction to prepare cDNA serial dilutions according to Table 6.

Table 6. 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)

8. Using 2 µl from each tube in Table 6, set up separate PCRs according to Table 7.

   We recommend setting up each reaction in triplicate.

Table 7. Reaction setup for real-time PCR

Component	Volume/reaction (384-well)	Volume/reaction (96-well)	Volume*/reaction (Rotor-Disc®)
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.

9. Mix thoroughly and proceed with PCR using the cycling conditions in Table 8.

   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 8. 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.

10. Extract C_T values for miRNeasy Serum/Plasma Spike-In Control from each reaction.
11. 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. 0.

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 29.

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 miRNeasy Serum/Plasma Advanced Kit), miScript II RT Kit, and the miScript SYBR Green PCR Kit. For more information, consult the *miScript PCR System Handbook* or visit **www.qiagen.com/miRNA**.

Procedure

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

   For details of preparation of miRNeasy Serum/Plasma Spike-In Control working solution, see Table 4 on page 29.

2. During RNA purification, add 3.5 µl miRNeasy Serum/Plasma Spike-In Control working solution from step 1 above (1.6 x 10^8 copies/µl) to the sample after lysis with Buffer RPL (see page 16, step 3). 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.

3. Continue with the RNA purification protocol (page 14). After RNA elution in 20 µl RNase-free water (step 13, page 17) 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.

4. Prepare the reverse transcription reaction on ice according to Table 9.

Table 9. 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.

5. Gently mix, briefly centrifuge, and then store on ice.
6. Incubate for 60 min at 37°C.
7. Incubate for 5 min at 95°C to inactivate miScript Reverse Transcriptase Mix and place on ice.
8. 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).
9. Set up PCRs according to Table 10.

   We recommend setting up each reaction in triplicate.

Table 10. Reaction setup for real-time PCR

Component	Volume/reaction (384-well)	Volume/reaction (96-well)	Volume*/reaction (Rotor-Disc)
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	6.5 µl	5 µl
Diluted reverse transcription reaction	2 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.

10. Mix thoroughly and proceed with PCR using the cycling conditions in Table 11.

    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 11. 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.

11. Extract C_T values and determine the mean C_T value for miRNeasy Serum/Plasma Spike-In Control from each reaction.
12. Compare with the miRNeasy Serum/Plasma Spike-In Control standard curve to determine recovery of miRNeasy Serum/Plasma Spike-In Control.

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.

For removal of RNase contamination from bench surfaces, nondisposable plasticware and laboratory equipment (e.g., pipets and electrophoresis tanks), general laboratory reagents can be used. To decontaminate plasticware, rinse with 0.1 M NaOH, 1 mM EDTA* followed by RNase-free water (see "Solutions", page 39), or rinse with chloroform* if the plasticware is chloroform-resistant. To decontaminate electrophoresis tanks, clean with detergent (e.g., 0.5%

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

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 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

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material 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 minutes.

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

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

Storage of DNA/RNA eluates

Purified DNA/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 DNA or RNA is detectable after 1 year.

Quantification of RNA

The concentration of ccfRNA should not be determined by spectrophotometric quantification, because the amounts present in serum and plasma are usually too low for reliable measurements. Small amounts of DNA and RNA can best be quantified using quantitative PCR / RT-PCR. Fluorometric quantification (e.g., using Qubit®) is often unreliable for short nucleic acid fragments.

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 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-based 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

Cell-free RNA from serum or plasma consists mainly of small RNAs of less than 100 nucleotides. Appearance of rRNA bands is usually indicative of contamination by cells or cell debris. Therefore, RNA integrity cannot be analyzed by denaturing agarose gel electrophoresis and ethidium bromide* staining or by using the QIAxcel® system or Agilent® 2100 Bioanalyzer.

* 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

Appendix F: Optional On-column DNase Digestion with the RNase-Free DNase Set

The RNase-Free DNase Set (cat. no. 79254) provides efficient on-column digestion of DNA during RNA purification. The DNase is efficiently removed in subsequent wash steps.

Note: Standard DNase buffers are not compatible with on-column DNase digestion. Use of other buffers may affect the binding of RNA to the RNeasy membrane, reducing RNA yield and integrity.

Lysis and homogenization of the sample and binding of RNA to the RNeasy membrane are performed according to the standard protocol. After washing with a reduced volume of Buffer RWT, the RNA is treated with DNase I while bound to the RNeasy membrane. The DNase I is removed by a second wash with Buffer RWT. Washing with Buffer RPE and elution of RNA are then performed according to the standard protocol.

Important points before starting

- Generally, DNase digestion is not required since DNA levels in plasma/serum samples are very low and RNeasy technologies efficiently remove most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). DNA can also be removed by a DNase digestion following RNA purification. Please note that an additional DNase digest might lead to losses in RNA yield.
- Do not vortex the reconstituted DNase I. DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube.

Things to do before starting

- Prepare DNase I stock solution before using the RNase-Free DNase Set for the first time. Dissolve the lyophilized DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided. To avoid loss of DNase I, do not open the vial. Inject RNase-free water into the vial using an RNase-free needle and syringe. Mix gently by inverting the vial. Do not vortex.
- For long-term storage of DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at –15 to –30°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.
- For processing samples containing <1 µg total RNA approximately, prepare Buffer RWT by adding 45 ml isopropanol to the concentrate (instead of adding 30 ml ethanol as usually recommended). Buffer RWT can be ordered separately for this protocol (cat. no. 1067933).

Procedure

1. Prepare and load samples onto the RNeasy UCP MinElute spin column as indicated in steps 1–8 of the protocol on page 15 or page 21 (Appendix A). Instead of performing step 9 (addition of Buffer RWT), follow steps 2–6 below.
2. Add 350 µl Buffer RWT (prepared with isopropanol; see above "Things to do before starting") to the RNeasy UCP MinElute spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 *x g* (≥10,000 rpm) to wash the membrane. Discard the flow-through.

   Reuse the collection tube in the next step.
3. Add 10 µl DNase I stock solution (see above) to 70 µl Buffer RDD. Mix by gently inverting the tube, and centrifuge briefly to collect residual liquid from the sides of the tube.

   Buffer RDD is supplied with the RNase-Free DNase Set.

Note: DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube. Do not vortex.

4. Add 80µl of the DNase I incubation mix directly to the RNeasy UCP MinElute spin column membrane, and place on the benchtop (20–30°C) for 15 min.

   Note: Be sure to add the DNase I incubation mix directly to the RNeasy UCP MinElute spin column membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.

5. Add 500 µl Buffer RWT (prepared with isopropanol) to the RNeasy UCP MinElute spin column. Close the lid gently, and centrifuge for 15 s at ≥ 8000 *x g* (≥ 10,000 rpm). Save the flow-through for use in step the next step.

6. Place the spin column in a new 2 ml collection tube (supplied). Apply the flow-through from step 5 to the spin column. Centrifuge for 15 s.

   If you were performing the Protocol: Purification of Total RNA, Including miRNA, From Serum and Plasma, continue with step 9 on page 16 at ≥ 8000 *x g* (≥ 10,000 rpm).

   If you were performing the protocol in Appendix A: Purification of Total RNA, Including miRNA, from Citrate Plasma, continue with step 9 on page 22.

Ordering Information

Product	Contents	Cat. no.
miRNeasy Serum/ Plasma Advanced Kit (50)	For 50 total RNA preps: 50 RNeasy UCP MinElute Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	217204
miRNeasy Serum/ Plasma Spike-In Control	10 pmol lyophilized *C. elegans* miR-39 miRNA mimic	219610
Ce_miR-39_1 miScript Primer Assay	miScript Primer assay with *C. elegans* miR-39 specific primers	MS00019789
Related products		
miRNeasy Mini Kit (50)	For 50 total RNA preps: 50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), QIAzol® Lysis Reagent, RNase-free Reagents and Buffers	217004
miRNeasy Micro Kit (50)	For 50 total RNA preps: 50 RNeasy UCP MinElute spin columns, Collection Tubes (1.5 ml and 2 ml), QIAzol Lysis Reagent, RNase-free Reagents and Buffers	217084
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

Product	Contents	Cat. no.
miScript II RT Kit (50)	For 50 cDNA synthesis reactions: miScript Reverse Transcriptase Mix, 10x miScript Nucleics Mix, 5x miScript HiSpec Buffer, 5x miScript HiFlex Buffer, RNase-Free Water	218161
miScript SYBR Green PCR Kit (200)	For 200 reactions: QuantiTect® SYBR Green PCR Master Mix, miScript Universal Primer	218073
miScript SYBR Green PCR Kit (1000)	For 1000 reactions: QuantiTect SYBR Green PCR Master Mix, miScript Universal Primer	218075
miScript Primer Assay (100)	miRNA-specific primer; available via GeneGlobe	Varies*
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
Related products for next-generation sequencing		
QIAseq™ miRNA Library Kit (12)	For 12 sequencing prep reactions: 3′ ligation, 5′ ligation, reverse-transcription, cDNA cleanup, library amplification and library cleanup reagents; quality control primers	331502
QIAseq miRNA NGS 12 Index IL (12)	Sequencing adapters, primers and indexes compatible with Illumina platforms; 12 indexes for 12 samples	331592

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

Product	Contents	Cat. no.
QIAseq miRNA Library Kit (96)	For 96 sequencing prep reactions: 3′ ligation, 5′ ligation, reverse-transcription, cDNA cleanup, library amplification and library cleanup reagents; quality control primers	331505
QIAseq miRNA NGS 48 Index IL (96)	Sequencing adapters, primers and indexes compatible with Illumina platforms; two 48 indexes for 96 samples	331595

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

Notes

Notes

Limited License Agreement for the miRNeasy Serum/Plasma Advanced Kit

Use of this product signifies the agreement of any purchaser or user of the product to the following terms:

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