January 2020

miRNeasy Serum/ Plasma Handbook

miRNeasy Serum/Plasma Kit
For purification of total RNA, including miRNA, from animal and human plasma and serum

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

Sample to Insight

QIAGEN

Contents

Kit Contents

miRNeasy Serum/Plasma Kit	**(50)**
Catalog no.	**217184**
Number of preps	**50**
RNeasy® MinElute® Spin Columns (each packaged with a 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	1

* Contains a guanidine salt. Not compatible with disinfectants containing bleach. See page 6 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.

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

Shipping and Storage

The miRNeasy Serum/Plasma Kit (cat. no. 217184) is 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 under these conditions, if not otherwise stated on the label.

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 12), 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 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 27) or lyophilized.

Intended Use

The miRNeasy Serum/Plasma Kit 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.

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

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

QIAzol Lysis Reagent and Buffer RWT 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 Kit and miRNeasy Serum/Plasma Spike-In Control is tested against predetermined specifications to ensure consistent product quality.

Introduction

To date, the majority of gene expression studies have focused on mRNA levels. However in recent years, interest in smaller RNA species, such as miRNA, has increased. Most commercial RNA purification kits do not recover RNA molecules smaller than ~200 nucleotides. The miRNeasy Serum/Plasma Kit is designed for purification of cell-free total RNA — primarily miRNA and other small RNA — from small volumes of serum and plasma. This kit may also be used for small volumes of other body fluids (e.g., urine).

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

Principle and procedure

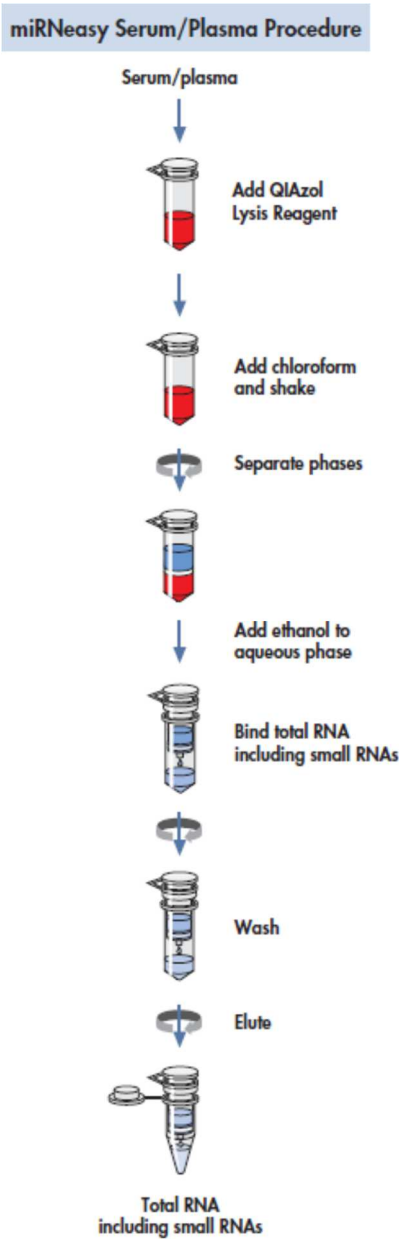
The miRNeasy Serum/Plasma Kit combines phenol/guanidine-based lysis of samples and silica-membrane–based purification of total RNA. QIAzol Lysis Reagent, included in the kit, is a monophasic solution of phenol and guanidine thiocyanate, designed to facilitate lysis, to denature protein complexes and RNases, and also to remove most of the residual DNA and proteins from the lysate by organic extraction.

QIAzol Lysis Reagent is added to serum or plasma samples. After 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 extracted, and ethanol 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 MinElute spin column, where the total RNA binds to the membrane and phenol and other contaminants are efficiently washed away. High-quality RNA is then 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.


miRNeasy Serum/Plasma Procedure
Serum/plasma
Add QIAzol Lysis Reagent
Add chloroform and shake
Separate phases
Add ethanol to aqueous phase
Bind total RNA including small RNAs
Wash
Elute
Total RNA including small RNAs

Description of protocol

This handbook contains one protocol on page 15 for purification of cell-free total RNA, including miRNA, from serum or plasma using the miRNeasy Serum/Plasma Kit. 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 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 and miRNA can also be copurified from formalin-fixed, paraffin-embedded (FFPE) tissue sections using the miRNeasy FFPE Kit (see ordering information, page 44).

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**.

Automated purification of RNA on QIAcube Instruments

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

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

QIAcube Connect.

Equipment and Reagents to Be Supplied by User

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

- Chloroform (without added isoamyl alcohol)
- Ethanol (70%, 80% and 96–100%)*
- Sterile, RNase-free 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 44)
- Equipment and tubes for serum/plasma collection and separation (see Appendix A, 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.

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 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 to avoid repeated freezing and thawing.

Important Notes

Volume of starting material

The volume of starting material is limited primarily by the amount of QIAzol Lysis Reagent required for proper phase separation. Using less than the recommended 5 volumes of QIAzol Lysis Reagent can result in inefficient phase separation, lower yields and/or copurification of inhibitors. It is not recommended to use more than 200 µl of serum or plasma. Higher sample volumes may result in reduced RNA yield and copurification of inhibitors.

Yields of total RNA achieved with the miRNeasy Serum/Plasma Kit 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. 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
Maximum amount of serum or plasma	200 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 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 200 µl) of serum and plasma using the miRNeasy Serum/Plasma Kit. The protocol can also be used for small volumes of other body fluids, such as urine. 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 A, page 24.

This protocol requires 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 –30°C to –15°C or –90 to –65°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.
- DNase I digestion is not recommended for plasma or serum samples. Cell-free body fluids typically do not contain significant amounts of DNA, and the combined QIAzol and RNeasy technologies efficiently remove most of the trace amounts of DNA in plasma and serum. 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.

- 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 7), all protocol and centrifugation steps should be performed at room temperature.
- 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.

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 27.
- 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 poly-A RNA.

Procedure

1. Prepare serum or plasma or thaw frozen samples.
2. Add 5 volumes QIAzol Lysis Reagent (see Table 2 for guidelines). Mix by vortexing or pipetting up and down.

Table 2. QIAzol Lysis Reagent volumes for various serum/plasma volumes

Serum/plasma (µl)	Protocol step 2: QIAzol Lysis Reagent (µl)	Protocol step 5: chloroform (µl)	Protocol step 7: approx. volume of upper aqueous phase (µl)	Protocol step 8: 100% ethanol (µl)
≤50	250	50	150	225
100	500	100	300	450
200	1000	200	600	900

Note: If the volume of plasma or serum is not limited, we recommend using 100–200 µl per RNA preparation.

Note: After addition of QIAzol Lysis Reagent, lysates can be stored at –70°C for several months.

3. Place the tube containing the lysate on the benchtop at room temperature (15–25°C) for 5 min.
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 B, page 27.
5. Add chloroform of an equal volume to the starting sample to the tube containing the lysate and cap it securely (see Table 2 for guidelines). Vortex or shake vigorously for 15 s.

   Thorough mixing is important for subsequent phase separation.
6. Place the tube containing the lysate on the benchtop at room temperature for 2–3 min.
7. Centrifuge for 15 min at 12,000 x *g* at 4°C. After centrifugation, heat the centrifuge up to room temperature if the same centrifuge will be used for the next centrifugation steps.

   After centrifugation, the sample separates into 3 phases: an upper, colorless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase. See Table 2 for the approximate volume of the aqueous phase.

8. Transfer the upper aqueous phase to a new collection tube (not supplied). Avoid transfer of any interphase material. Add 1.5 volumes of 100% ethanol and mix thoroughly by pipetting up and down several times. Do not centrifuge. Continue without delay with step 9.

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

9. Pipet up to 700 µl of the sample, including any precipitate that may have formed, into an RNeasy MinElute spin column in a 2 ml collection tube (supplied). Close the lid gently and centrifuge at ≥8000 x *g* (≥10,000 rpm) for 15 s at room temperature. Discard the flow-through.*

   Reuse the collection tube in step 10.

10. Repeat step 9 using the remainder of the sample. Discard the flow-through.*

    Reuse the collection tube in step 11.

11. 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) to wash the column. Discard the flow-through.*

    Reuse the collection tube in step 12.

12. 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) to wash the column. Discard the flow-through.

    Reuse the collection tube in step 13.

* Flow-through contains QIAzol Lysis Reagent or Buffer RWT and is therefore not compatible with bleach. See page 6 for safety information.

13. Pipet 500 µl of 80% ethanol onto the RNeasy 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 collection tube with the flow-through.

    Note: 80% ethanol should be prepared with ethanol (96–100%) and RNase-free water.

    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.

14. 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.

15. 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, and 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.
b) Lysate not sufficiently mixed before centrifugation	After addition of chloroform (step 5), 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 6 and 7 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 column	
Centrifugation temperature too low	Except for phase separation (step 7), 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.

	Comments and suggestions
Low miRNA yield or poor performance of miRNA in downstream applications	
Incorrect ethanol concentration	Be sure to use the ethanol concentrations specified in the protocol steps.
Low or no recovery of RNA	
a) Too much starting material	In subsequent preparations, reduce the amounts of starting material. It is essential to use the correct amount of starting material (see page 14).
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.
Low A_{260}/A_{280} value	
a) Not enough QIAzol Lysis Reagent used	Reduce the amount of starting material and/or increase the volume of QIAzol Lysis Reagent.
b) Sample not incubated for 5 min after addition of QIAzol Lysis Reagent	Place the sample at room temperature for 5 min after addition of QIAzol Lysis Reagent, as indicated in the protocol (step 3). This step is important to promote dissociation of nucleoprotein complexes.
c) Water used to dilute RNA for A_{260}/A_{280} measurement	Use 10 mM Tris·Cl,* pH 7.5, not RNase-free water, to dilute the sample before measuring purity (see Appendix D, page 40).

* 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.

		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 C: General Remarks on Handling RNA" (page 37) and "Appendix A: 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. 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 37). 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 7 should be performed at 4°C. Make sure that the centrifuge does not heat above 10°C during centrifugation.
b)	Interphase contamination of aqueous phase	Contamination of the aqueous phase with the interphase can result in carryover of contaminants into the RNA eluate. Make sure to transfer the aqueous phase without interphase contamination.
c)	Salt carryover during elution	Ensure that Buffer RPE is at 20–30°C.

		Comments and suggestions
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 14).

Appendix A: 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 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. Therefore, an equivalent step to remove cellular debris is also recommended for other body fluids, such as urine.

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 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.

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 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, the following high-speed centrifugation will remove additional cellular debris and minimize contamination of cell-free nucleic acids by gDNA and RNA derived from damaged blood cells.

A4. Centrifuge plasma samples in conical tubes for 10 min at 16,000 x *g* and 4°C in a fixed-angle rotor.

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 plasma will be used for nucleic acid purification on the same day. For longer storage, keep plasma frozen in aliquots at –90°C to –65°C.

A7. Before using frozen plasma for nucleic acid purification, thaw at room temperature.

Optional: To remove cryoprecipitates, centrifuge thawed plasma samples for 5 min at 16,000 x *g* and 4°C (in a fixed angle rotor). Transfer supernatant to a new tube, and begin nucleic acid purification protocol.

Procedure: serum separation and storage

A1. Collect whole blood in Sarstedt S-Monovette® Serum-Gel 9 ml tubes (cat. no. 02.1388) containing clot activator (or any other primary blood collection tube without clot activator and without anticoagulants, such as EDTA or citrate). 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 intermediate buffy coat layer, facilitating recovery of serum.

A3. Carefully transfer the upper (yellow) serum phase to a new tube (with conical bottom) without disturbing the intermediate buffy coat layer (containing white blood cells and platelets). Normally up to 3–5 ml serum 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 serum.

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

A4. Centrifuge serum samples in conical tubes for 10 min at 16,000 x *g* and 4°C in a fixed-angle rotor.

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 –90°C to –65°C.

A7. Before using frozen serum for nucleic acid purification, thaw at room temperature.

Optional: To remove cryoprecipitates, centrifuge thawed serum samples for 5 min at 16,000 x *g* and 4°C (in a fixed angle rotor). 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 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. miRNeasy Serum/Plasma Spike-In Control stock

should be stored at –90°C to –65°C. For large volumes, first aliquot into smaller volumes prior to long-term storage at –90°C to –65°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 15)	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 29)	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 (provided in the miRNeasy Serum/Plasma Kit), miScript II RT Kit, 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 yet thoroughly.

For details of preparation of miRNeasy Serum/Plasma Spike-In Control working solution, see Table 3, page 28.

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 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	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 29.

Important point 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 Kit), miScript II RT Kit, 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 yet thoroughly.

For details of preparation of miRNeasy Serum/Plasma Spike-In Control working solution, see Table 3, page 28.

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 4, page 16). 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 16). After RNA elution in 14 µl RNase-free water (step 15, 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.

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

Table 8. Reverse transcription reaction components

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 x 10^5 copies/µl (assuming 100% recovery during RNA purification and reverse transcription).

B9. Set up PCRs 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. 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 38), or rinse with chloroform* if the plasticware is chloroform-resistant. To

* 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.

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.

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

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of the purine residues has 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.

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

Storage of RNA

Purified RNA may be stored at –30°C to –15°C or –90°C to –65°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 (see "Spectrophotometric quantification of RNA" below). For small amounts of RNA, however, it may be difficult to determine amounts photometrically. Small amounts of RNA can be accurately 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 41), 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

* 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.

"Solutions", page 38). 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:

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

* 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.

(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 40).

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

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 Agilent 2100 Bioanalyzer also provides an RNA Integrity Number (RIN) as a useful measure of RNA integrity. Ideally, the 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.

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.

* 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.

Ordering Information

Product	Contents	Cat. no.
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
miRNeasy Serum/Plasma Spike-In Control	10 pmol lyophilized *C. elegans* miR-39 miRNA mimic	219610
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 MinElute Spin Columns, Collection Tubes (1.5 ml and 2 ml), QIAzol Lysis Reagent, RNase-free Reagents and Buffers	217084
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

Product	Contents	Cat. no.
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
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
QIAcube Connect — for fully automated nucleic acid extraction with QIAGEN spin-column kits		
QIAcube Connect†	Instrument, connectivity package, 1-year warranty on parts and labor	Inquire
Starter Pack, QIAcube	Filter-tips, 200 µl (1024), 1000 µl filter-tips (1024), 30 ml reagent bottles (12), rotor adapters (240), elution tubes (240), rotor adapter holder	990395

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

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

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.

Document Revision History

Date	Changes
January 2020	Updated text, ordering information and intended use for QIAcube Connect.

Limited License Agreement for miRNeasy Serum/Plasma Kit and miRNeasy Serum/Plasma Spike-In Control

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