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miRNeasy Serum/ Plasma Handbook

miRNeasy Serum/Plasma Kit

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

Sample to Insight

QIAGEN

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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
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.

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 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.

Intended Use

The miRNeasy Serum/Plasma Kit is intended for molecular biology applications. This product is 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	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 approx. 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 the use of a synthetic spike-in control for normalization, such as the miRCURY RNA Spike-In Kit, for RT (cat. no. 339390).

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 to remove most of the residual DNA and proteins from the lysate by organic extraction.

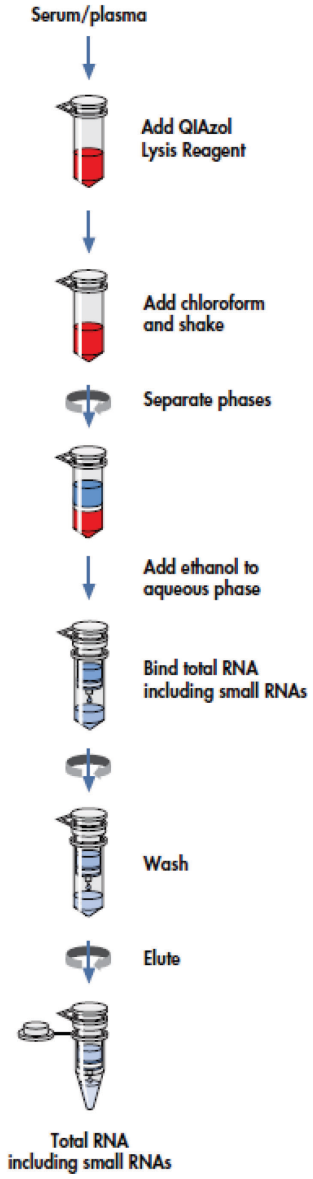
QIAzol Lysis Reagent is added to serum or plasma samples. After addition of chloroform, the lysate is separated into aqueous and organic phases by centrifugation. RNA partitions to the upper, aqueous phase, while DNA partitions to the interphase and proteins to the lower, organic phase or the interphase.

QIAGEN offers a new solution for isolation of total RNA, including miRNA, from animal and human plasma and serum. This new solution is called miRNeasy Serum/Plasma Advanced Kit and allows faster results omitting phenol/chloroform usage by utilizing QIAGENs new QIAzol-free Advanced chemistry.

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



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.

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 Tissue Cells Advanced 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 31).

Real-time RT-PCR detection of miRNAs using the miRCURY® LNA® miRNA PCR systems

In general, real-time RT-PCR is recommended to accurately quantify yields of miRNA. The miRCURY LNA miRNA PCR system allows sensitive and specific quantification and profiling of miRNA expression using SYBR® Green-based or probe-based real-time PCR. Both the SYBR Green detection-based miRCURY LNA miRNA PCR system and the probe-based miRCURY LNA miRNA Probe PCR System comprise all the required components to set up and conduct miRNA quantification and expression profiling, from conversion of RNA into cDNA to real-time PCR detection of miRNAs and straightforward data analysis. The systems both use the same miRCURY LNA RT Kit for generation of cDNA – one cDNA reaction for all miRNAs. Each system then has their own dedicated master mix kit, the miRCURY LNA SYBR Green PCR Kit and the miRCURY LNA miRNA Probe PCR Kit, as well as a broad variety of system-specific LNA-enhanced miRCURY LNA assay and panel products. The RNA Spike-In Kit enables quality control of the RNA isolation, cDNA synthesis, and PCR amplification steps of miRCURY LNA

miRNA qPCR experiments. For both systems, individual assays for mature miRNAs for a variety of different species can be ordered on GeneGlobe® (**www.qiagen.com/GeneGlobe**). Alternatively, for high-throughput and screening experiments, miRCURY LNA PCR Panels and miRCURY LNA Probe PCR Panels enable rapid profiling of the complete miRNome. Other Focus panels like the miRCURY LNA miRNA Serum/Plasma Focus PCR Panel and Serum/Plasma Focus Probe PCR Panel are available for the detection of mature miRNAs. Find out more about the miRCURY LNA miRNA PCR systems at **www.qiagen.com**.

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 miRCURY RNA Spike-In Kit, for RT (cat. no. 339390), must be purchased separately (see "Ordering Information", page 31)
- Equipment and tubes for serum/plasma collection and separation (see Appendix A, page 22):
  - 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 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. We recommend using the miRCURY RNA Spike-In Kit, for RT (cat. no. 339390), 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 22.

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. 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.
- 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 4: chloroform (µl)	Protocol step 6: approx. volume of upper aqueous phase (µl)	Protocol step 7: 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.

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

4. 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.

5. Place the tube containing the lysate on the benchtop at room temperature for 2–3 min.

6. 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.

7. 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.

8. 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 9.

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

9. Repeat step 8 using the remainder of the sample. Discard the flow-through.*

   Reuse the collection tube in step 10.

10. 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 11.

11. 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 12.

12. 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.

13. 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).

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

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

14. 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 4), 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 5 and 6 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.
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.

		Comments and suggestions
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 C, page 27).
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 B: General Remarks on Handling RNA" (page 25) and "Appendix A: Recommendations for Serum and Plasma Collection, Separation, and Storage" (page 22).
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 B: General Remarks on Handling RNA" (page 25). 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 6 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.
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 13).

* 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 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 h.

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 9 ml 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: 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 suitable surface decontamination reagents. 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 26), or rinse with chloroform* if the plasticware is chloroform resistant. To decontaminate electrophoresis tanks, clean with detergent (e.g., 0.5% SDS),* rinse with RNase-free water, rinse with ethanol (if the tanks are ethanol-resistant), and allow to dry.

* 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.

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 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.

* 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.

Appendix C: 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 28), the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity.

* 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.

When measuring RNA samples, be certain that cuvettes are RNase-free, especially if the RNA is to be recovered after spectrophotometry. This can be accomplished by washing cuvettes with 0.1 M NaOH, 1 mM EDTA,* followed by washing with RNase-free water (see "Solutions", page 26). 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.

* 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.

For determination of RNA concentration, however, we recommend dilution of the sample in a buffer with neutral pH since the relationship between absorbance and concentration (A_{260} reading of 1 = 44 µg/ml RNA) is based on an extinction coefficient calculated for RNA at neutral pH (see "Quantification of RNA", page 27).

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, RNase-free Reagents and Buffers	217184
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
RNA Spike-In Kit, For RT	Contains the UniSp2, UniSp4, and UniSp5 RNA Spike-in Template Mix and the cel-miR-39-3p RNA Spike-in Template	339390
miRNeasy Tissue/Cells Advanced Mini Kit (50)	For 50 preps: RNeasy Mini Spin Columns, gDNA Eliminator Spin Columns, Collection Tubes, RNase-Free Water and Buffers	217604
miRNeasy Tissue/Cells Advanced Micro Kit (50)	For 50 preps: RNeasy UCP MinElute spin columns, gDNA Eliminator Spin Columns, Collection Tubes, RNase-Free Water and Buffers	217684
miRNeasy 96 Advanced QIAcube HT Kit	For 480 preps: RNeasy 96 plates, RNase-free water, buffers	217261
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

Product	Contents	Cat. no.
Collection Tubes (2 ml)	1000 Collection Tubes (2 ml)	19201
Related products for quantitative, real-time RT-PCR		
miRCURY LNA miRNA Probe PCR Assay (200)	Complete premixed assays containing LNA-enhanced target-specific foward primer and probe. For 200 reactions	339350
miRCURY LNA miRNA PCR Assays	Contains forward and reverse primers for 200 reactions; for SYBR Green-based detection	339306
miRCURY LNA RT Kit	For 8–64 cDNA synthesis reactions: 5x RT SYBR Green Reaction Buffer, 5x RT Probe Reaction Buffer, 10x RT Enzyme Mix, UniSp6, RNA Spike-in template, RNase-Free Water	339340
QuantiNova LNA PCR Assays (200)	Predesigned mRNA/lncRNA-specific primer mixture in a single tube; for 200 qPCR reactions or 400 dPCR reactions	249990
QuantiNova Reverse Transcription Kit (50)	For 50 x 20 µl reactions: 100 µl 8x gDNA Removal Mix, 50 µl Reverse Transcription Enzyme, 200 µl Reverse Transcription Mix (containing RT primers), 100 µl Internal Control RNA, 1.9 ml RNase-Free Water	205411
QuantiNova® Probe RT-PCR Kit (100)	For 100 x 20 µl reactions: 1 ml QuantiNova Probe RT-PCR Master Mix, 20 µl QuantiNova Probe RT Mix, 20 µl Internal Control RNA, 500 µl Yellow Template Dilution Buffer, 250 µl ROX™ Reference Dye, 1.9 µl RNase-Free Water	208352

Product	Contents	Cat. no.
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
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
QIAseq miRNA NGS 48 Index IL (96)	Sequencing adapters, primers, and indexes compatible with Illumina platforms. Cuttable Ht format (dried primers) with 96 indexes for 96 samples	331565
QIAseq miRNA NGS 12 Index TF (12)	Sequencing adapters, primers, and indices compatible with Thermo Fisher platforms; 12 indices for12 samples	331582
QIAseq miRNA NGS 48 Index TF (96)	Sequencing adapters, primers, and indices compatible with Thermo Fisher platforms; two 48 indices for 96 samples	331585

Product	Contents	Cat. no.
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.
April 2022	Updated the Kit Contents, Introduction, and Ordering Information sections. Removed all mentions of miRNeasy Serum/Plasma Spike-In Control (cat. no. 219610) all throughout the handbook. Removed the "miRNA quantification using the miScript PCR System" subsection under Introduction. Added the "Real-time RT-PCR detection of miRNAs using the miRCURY LNA miRNA PCR systems" section under Introduction. Replaced miRNeasy Serum/Plasma Spike-In Control with miRCURY RNA Spike-In Kit, for RT (cat. no. 339390), in "Equipment and Reagents to Be Supplied by User" section. Removed the "For reconstitution of CE_miR-39_1 miScript Primer Assay" subsection. Removed Appendix B and updated the arrangement of appendices accordingly.

Notes

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

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