April 2021

miRNeasy Serum/ Plasma Advanced Kit Handbook

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

Sample to Insight

QIAGEN

Contents

Kit Contents

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

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

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

‡ Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.

Shipping and Storage

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

Intended Use

The miRNeasy Serum/Plasma Advanced Kit is intended for molecular biology applications. These products are not intended for the diagnosis, prevention or treatment of a disease.

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 these products. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments or to other applicable guidelines.

Safety Information

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

CAUTION	CAUTION: DO NOT add bleach or acidic solutions directly to waste containing Buffer RWT or Buffer RPL.

Buffer RWT and Buffer RPL contain guanidine thiocyanate, which can form highly reactive compounds when combined with bleach. If liquid containing these solutions is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

Quality Control

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

Introduction

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

When working with serum and plasma samples, we recommend the use of a synthetic spike-in control for normalization, such as the miRCURY RNA Spike-In Kit, for RT (cat. no. 339390).

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

Principle and workflow

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

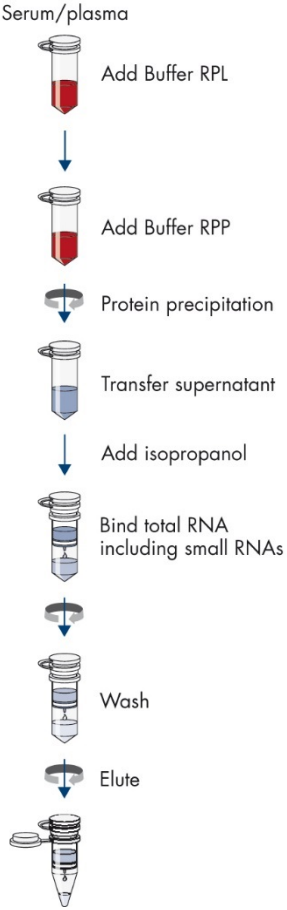
Buffer RPL is added to serum or plasma samples. After thoroughly mixing to ensure a complete lysis, Buffer RPP is added to precipitate inhibitors (mostly proteins that are highly concentrated in serum/plasma samples) by centrifugation.

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

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

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

miRNeasy Serum/Plasma Advanced Kit



Automated purification of miRNA on QIAcube Instruments

Purification of 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 Advanced 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.

Description of protocols

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

Equipment and Reagents to Be Supplied by User

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

The following supplies are also required:

- 100% Isopropanol
- 80% Ethanol*
- Sterile, RNase-free pipet tips
- 1.5 ml or 2 ml microcentrifuge tubes
- Microcentrifuge with rotor for 2 ml tubes for centrifugation at room temperature
- Disposable gloves
- The miRCURY RNA Spike-In Kit, for RT (cat. no. 339390) must be purchased separately (see "Ordering Information", page 37)
- Equipment and tubes for serum/plasma collection and separation (see Appendix B: Recommendations for Serum and Plasma Collection, Separation and Storage, page 25):
  - 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 (for tubes containing citrate instead, see Appendix A, page 21)
  - Conical tube(s)
  - Refrigerated centrifuge with a swinging bucket rotor and fixed-angle rotor

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

Important Notes

Volume of starting material

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

Yields of total RNA purified with the miRNeasy Serum/Plasma Advanced Kit vary strongly between different plasma samples. However, they are usually too low for quantification by OD measurement. We recommend using the miRCURY RNA Spike-In Kit, for RT (cat. no. 339390) to monitor miRNA purification and amplification.

Specifications of RNeasy UCP MinElute spin columns

Table 1. RNeasy UCP MinElute spin column specifications

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

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

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

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

For recommendations on collection, preparation and storage of cell-free plasma and serum, see Appendix B: Recommendations for Serum and Plasma Collection, Separation and Storage on page 25.

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 –15°C or –90°C 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.
- See Appendix E: Optional On-column DNase Digestion with the RNase-Free DNase Set on page 34 for optional on-column DNase digestion. DNase I digestion is not recommended for plasma or serum samples. Cell-free body fluids typically do not contain significant amounts of DNA. In addition, miRCURY LNA miRNA PCR Assays and most other assays for mature miRNA are not affected by the presence of small amounts of genomic DNA. On-column DNase treatment may reduce recovery of small RNA from plasma or serum.
- Buffers RPL and RWT may form a precipitate upon storage. If necessary, redissolve by warming and then place at room temperature (15–25°C).

- Equilibrate buffers to room temperature before starting the protocol.
- Buffers RPL and RWT contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach.
- All steps should be performed at room temperature. Work quickly.
- The procedure is suitable for use with either serum samples or plasma samples containing EDTA. For citrate plasma samples, refer to the protocol in Appendix A: Purification of Total RNA, Including miRNA, from Citrate Plasma on page 21. Plasma samples containing heparin should not be used, because this anticoagulant can interfere with downstream assays, such as RT-PCR.

Things to do before starting

- Buffers RWT and RPE are supplied as concentrates. Before using for the first time, add the required volumes of ethanol (96–100%), as indicated on the bottles, to obtain a working solution.
- 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. Transfer 200 µl serum or plasma into a 2 ml microcentrifuge tube.

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

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

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

3. Add 60 µl Buffer RPL. Close the tube caps and vortex for >5 s. Leave at room temperature (15–25°C) for 3 min.

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

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

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

   Thorough mixing is important for subsequent phase separation.

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

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

   Note: Supernatant should be clear and colorless.

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

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

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

   Reuse the collection tube in the next step.

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

   Reuse the collection tube in the next step.

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

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

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

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

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

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

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

    The dead volume of the RNeasy UCP MinElute spin column is 2 µl, so elution with 20 µl RNase-free water results in a 18 µl eluate.

Troubleshooting Guide

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

	Comments and suggestions
Clogged column	Make sure to follow recommendations in Appendix B: Recommendations for Serum and Plasma Collection, Separation and Storage (page 25) for removal of residual cellular material. After thawing of frozen samples, remove cryoprecipitates by centrifugation or filtration, if necessary (see Appendix B: Recommendations for Serum and Plasma Collection, Separation and Storage). Make sure to not transfer any precipitate from step 5 on page 16 when transferring the supernatant to a fresh tube
Centrifugation temperature too low	All centrifugation steps should be performed at room temperature (15–25°C). Some centrifuges may cool to below 20°C even when set at 20°C. This can cause precipitates to form that can clog the RNeasy UCP MinElute spin column and reduce RNA yield. If this happens, set the centrifugation temperature to 25°C. Warm the ethanol-containing lysate to 37°C before transferring to the RNeasy UCP MinElute spin column.
Low miRNA yield or poor performance of miRNA in downstream applications	
a) Incorrect ethanol concentration	Be sure to use the ethanol concentrations specified in the protocol steps.

	Comments and suggestions
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 13, Volume of starting material).
b) Elution buffer incorrectly dispensed	Add elution buffer to the center of the RNeasy UCP MinElute spin column membrane to ensure that the buffer completely covers the membrane.
c) RNA still bound to the membrane	Repeat the elution step of the protocol, but incubate the RNeasy UCP MinElute spin column on the bench for 10 min after adding RNase-free water and before centrifugation.
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 29) and Appendix B: Recommendations for Serum and Plasma Collection, Separation and Storage (page 25).

	Comments and suggestions
b) RNase contamination	Cell-free RNA in plasma and serum typically consists of small RNA species only, and will therefore not resemble intact RNA from cells or tissue. Nevertheless, the following precautions are recommended to avoid complications due to RNA degradation. RNases can be introduced during use. Make sure not to introduce any RNases during the procedure or later handling. See Appendix C: General Remarks on Handling RNA (page 29). Do not put RNA samples into a vacuum dryer that has been used in DNA preparations where RNases may have been used.
RNA does not perform well in downstream experiments	
a) Salt carryover during elution	Ensure that Buffer RPE is at 20–30°C.
b) Ethanol carryover	After the final membrane wash, be sure to dry the RNeasy UCP MinElute spin column by centrifugation at full speed with open lids for 5 min (protocol step 11 on page 17).
Contamination by genomic DNA	
Sample inappropriately handled	Invert tubes gently to mix contents after blood collection. Vigorous mixing or shaking can promote hemolysis. Generate plasma as quickly as possible after blood collection. Long delays can promote hemolysis or apoptotic cell death. Perform the second centrifugation or filtration before freezing the plasma, if possible.

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

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

For recommendations on collection, preparation and storage of cell-free plasma and serum, see Appendix B: Recommendations for Serum and Plasma Collection, Separation and Storage, page 25.

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 –15°C or –90°C 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.
- See Appendix E: Optional On-column DNase Digestion with the RNase-Free DNase Set on page 34 for optional on-column DNase digestion. DNase I digestion is not recommended for plasma or serum samples. Cell-free body fluids typically do not contain significant amounts of DNA. In addition, miRCURY LNA miRNA PCR Assays and most other assays for mature miRNA are not affected by the presence of small amounts of genomic DNA. On-column DNase treatment may reduce recovery of small RNA from plasma or serum.
- Buffers RPL and RWT may form a precipitate upon storage. If necessary, redissolve by warming and then place at room temperature (15–25°C).
- Equilibrate buffers to room temperature before starting the protocol.

- Buffers RPL and RWT contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach.
- All steps should be performed at room temperature. Work quickly.
- The procedure is suitable for use with plasma samples containing citrate. For serum or EDTA plasma samples, refer to the protocol on page 14. Plasma samples containing heparin should not be used, because this anticoagulant can interfere with downstream assays, such as RT-PCR.

Things to do before starting

- Buffers RWT and RPE are supplied as concentrates. Before using for the first time, add the required volumes of ethanol (96–100%), as indicated on the bottles, to obtain a working solution.
- 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 plasma or thaw frozen samples.
2. Transfer 200 µl plasma into a 2 ml microcentrifuge tube.

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

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

Plasma	Buffer RPL	Buffer RPP
200 µl	60 µl	60 µl
400µl	120 µl	120 µl
600 µl	180 µl	180 µl

3. Add 60 µl Buffer RPL. Close the tube caps and vortex for >5 s. Leave at room temperature (15–25°C) for 3 min.

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

4. **Note**: If any spike-in controls are used, they should be added to the lysate at this point. Add 60 µl Buffer RPP. Close the tube caps and mix vigorously by vortexing for >20 s. Incubate at room temperature for 3 min.

   Thorough mixing is important for subsequent phase separation.

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

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

   Note: Supernatant should be clear and colorless.

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

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

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

   Reuse the collection tube in the next step.

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

   Reuse the collection tube in the next step.

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

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

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

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

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

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

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

    The dead volume of the RNeasy UCP MinElute spin column is 2 µl, so elution with 20 µl RNase-free water results in a 18 µl eluate.

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

To isolate circulating, cell-free nucleic acids from whole blood samples, we recommend following these protocols, which include an initial low *g*-force centrifugation step to separate cells from plasma or serum followed by a high *g*-force centrifugation or filtration step to remove all remaining cellular debris. The latter centrifugation step significantly reduces the amount of cellular or genomic DNA and RNA in the sample. Because of the much higher abundance of RNA in cells, even small amounts of cellular debris can have a very significant effect on RNA profiling of cell-free fluids. The sooner after blood collection this removal of cellular materials is performed, the lower the risk of additional background from blood cell-derived nucleic acids released *in vitro*. Use of gel barrier tubes generally results in fewer residual cells.

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

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

Procedure: plasma separation and storage

1. Collect whole blood in BD Vacutainer® Venous Blood Collection Tubes (cat. no. 367525) containing EDTA (or any other primary blood collection tube containing EDTA as anticoagulant). Store tubes at room temperature (15–25°C) or 4°C and process within 1 hour.

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

2. Centrifuge blood samples in primary blood collection tubes for 10 min at 1900 *x g* (3000 rpm) and 4°C using a swinging bucket rotor.
3. Carefully transfer the upper (yellow) plasma phase to a new tube (with conical bottom) without disturbing the intermediate buffy coat layer (containing white blood cells and platelets). Normally up to 4–5 ml plasma can be obtained from 10 ml of whole blood.

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

   Note: Plasma can be used for cell-free nucleic acid purification at this stage. However, an additional filtration or centrifugation will remove additional cellular debris and minimize contamination of cell-free nucleic acids by gDNA and RNA derived from damaged blood cells.
4. Centrifuge plasma samples in conical tubes for 15 min at 3,000 *x g* (or 10 min at 16,000 *x g* – see above) and 4°C or pass through a 0.8 µm filter (see recommendations above).

   This will remove additional cellular nucleic acids attached to cell debris.
5. Carefully transfer cleared supernatant to a new tube without disturbing the pellet, which forms a smear along the outer side or bottom of the centrifugation tube.
6. Store at 2–8°C until further processing, if plasma will be used for nucleic acid purification on the same day. For longer storage, keep plasma frozen in aliquots at –90°C to –65°C.
7. Before using frozen plasma for nucleic acid purification, thaw at room temperature.

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

Procedure: serum separation and storage

1. Collect whole blood in a primary blood collection tube with or without clot activator, but without anticoagulants, such as EDTA or citrate (e.g., Sarstedt S-Monovette® Serum-Gel 9 ml tubes, cat. no. 02.1388). For complete clotting, leave tubes at room temperature (15–25°C) for 10 min to 1 h.

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

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

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

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

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

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

4. Centrifuge serum samples in conical tubes for 15 min at 3,000 *x g* (or 10 min at 16,000 *x g* – see above) and 4°C or pass through a 0.8 µm filter (see recommendations above). This will remove additional cellular nucleic acids attached to cell debris.

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

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

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

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

Appendix C: 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.

For removal of RNase contamination from bench surfaces, nondisposable plasticware and laboratory equipment (e.g., pipets and electrophoresis tanks), general laboratory reagents can be used. To decontaminate plasticware, rinse with 0.1 M NaOH, 1 mM EDTA* followed by RNase-free water (see "Solutions", page 30), or rinse with chloroform* if the plasticware is chloroform-resistant. To decontaminate electrophoresis tanks, clean with detergent (e.g., 0.5%

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

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

Disposable plasticware

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

Glassware

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

Solutions

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

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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 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 eluates

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

DNA contamination

No currently available purification method can guarantee that RNA is completely free of DNA, even when it is not visible on an agarose gel. Since serum, plasma, and other cell-free body fluids contain very little DNA, and commonly used assays for miRNA detection, such as the miRCURY LNA miRNA PCR Assays and the QIAseq miRNA Library Kit do not detect genomic DNA, no further removal of DNA is integrated in the protocol.

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

To prevent any interference by DNA in gene expression analysis real-time RT-PCR applications, we recommend designing primers that anneal at intron splice junctions so that genomic DNA will not be amplified. QuantiNova® LNA PCR Assays from QIAGEN are designed for SYBR®

Green-based or Probe real-time RT-PCR analysis of RNA sequences (without detection of genomic DNA) where possible (the assays can be ordered online at **www.qiagen.com/GeneGlobe**). For real-time RT-PCR assays where amplification of genomic DNA cannot be avoided, we recommend using the QuantiNova Reverse Transcription Kit for reverse transcription. The kit integrates fast cDNA synthesis with rapid removal of genomic DNA contamination.

Alternatively, gene expression analysis can be performed using QuantiNova Probe RT-PCR Kit, which includes an integrated genomic DNA removal step.

miRCURY LNA miRNA PCR Assays, used with the miRCURY LNA miRNA PCR System for miRNA quantification, do not detect genomic DNA.

Integrity of RNA

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

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

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

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

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

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

Important points before starting

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

Things to do before starting

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

Procedure

1. Prepare and load samples onto the RNeasy UCP MinElute spin column as indicated in steps 1–7 of the protocol on page 15 or page 22 (Appendix A: Purification of Total RNA, Including miRNA, from Citrate Plasma). Instead of performing step 8 (addition of Buffer RWT), follow steps 2–6 below.
2. Add 350 µl Buffer RWT (prepared with isopropanol; see above "Things to do before starting") to the RNeasy UCP MinElute spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 *x g* (≥10,000 rpm) to wash the membrane. Discard the flow-through.

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

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

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

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

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

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

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

   If you were performing the Protocol: Purification of Total RNA, Including miRNA, From Serum and Plasma, continue with step 8 on page 16 at ≥ 8000 *x g* (≥ 10,000 rpm).

   If you were performing the protocol in Appendix A: Purification of Total RNA, Including miRNA, from Citrate Plasma, continue with step 8 on page 23.

Ordering Information

Product	Contents	Cat. no.
miRNeasy Serum/ Plasma Advanced Kit (50)	For 50 total RNA preps: 50 RNeasy UCP MinElute Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	217204
miRCURY RNA Spike-In Kit, for RT	UniSp2, UniSp4, and UniSp5 RNA Spike-in Template Mix and the cel-miR-39-3p RNA Spike-in Template	339390
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
Related products		
miRNeasy 96 Advanced QIAcube HT Kit	For 480 preps: RNeasy 96 plates, RNase-free water, buffers	217261
miRNeasy FFPE Kit (50)	50 RNeasy MinElute Spin Columns, Collection Tubes, Proteinase K, RNase-Free DNase I, DNase Booster Buffer, RNase-Free Buffers, RNase-Free Water	217504

Product	Contents	Cat. no.
exoRNeasy Maxi Kit	For 50 RNA preps: 50 exoEasy Maxi and RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml, 2 ml, and 50 ml), QIAzol Lysis Reagent, RNase-free Reagents and Buffers	77164
exoRNeasy Midi Kit	For 50 RNA preps: 50 exoEasy Midi and RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml, 2 ml, and 50 ml), QIAzol Lysis Reagent, RNase-free Reagents, and Buffers	77144
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

Product	Contents	Cat. no.
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
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

Product	Contents	Cat. no.
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

* 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.

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

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
01/2020	Updated text, ordering information and intended use for QIAcube Connect.
04/2021	Updated the Shipping and Storage section. Revised the handbook to remove the miScript products due to discontinuation and to replace the miScript system with the miRCURY system. Replaced occurrences of product "miRNeasy Serum/Plasma Spike-In Control" with "miRCURY RNA Spike-In Kit, for RT". Removed subsections "PCR-based miRNA quantification using the miScript PCR system" and "Optional: For reconstitution of Ce_miR-39_1 miScript Primer Assay". Replaced occurrences of "miScript Primer Assays" with "miRCURY LNA miRNA PCR Assays". Added a note for spike-in controls in the procedure of the protocol. Updated the Ordering Information section for the replacement of miScript products with the miRCURY System.

Notes

Limited License Agreement for the miRNeasy Serum/Plasma Advanced Kit

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