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QIAamp® ccfDNA/RNA Handbook

For purification of total circulating cell-free DNA and RNA from 1–4 ml human plasma and serum

Sample to Insight

QIAGEN

Contents

Kit Contents 4

Storage 4

Intended Use 5

Safety Information 6

Quality Control 6

Introduction 7

- Principle and workflow 8
- Automated purification of nucleic acids on QIAcube Instruments 10

Equipment and Reagents to Be Supplied by User 11

Important Notes 12

- Volume of starting material 12

Protocol: Purification of Cell-Free DNA and RNA, Including miRNA, from Serum and Plasma 13

- Important points before starting 13
- Things to do before starting 14
- Procedure 14
- Cleanup (automatable on QIAcube Connect) 16

Troubleshooting Guide 18

Appendix A: Recommendations for Serum and Plasma Collection, Separation and Storage 20

- Procedure: plasma separation and storage 21
- Procedure: serum separation and storage 22

Appendix B: Analysis of only ccfRNA or ccfDNA 24
Analysis of ccfRNA only 24
Analysis of ccfDNA only 24
Appendix C: General Remarks on Handling RNA 25
Appendix D: Storage, Quantification and Determination of DNA and RNA Quality 28
Ordering Information 29
Document Revision History 32

Kit Contents

QIAamp ccfDNA/RNA Kit (50)	
Catalog no.	**55184**
Number of preps	**50**
RNeasy® Midi Spin Columns (in 15 ml Collection Tube)	50
Collection Tubes (15 ml)	50
Buffer RPL*	4 x 20 ml
Buffer RPP	3 x 8 ml
RNeasy MinElute® Spin Columns (in 2 ml Collection Tube)	50
Collection Tubes (1.5 ml)	50
Collection Tubes (2 ml)	50
Buffer RWT*†	80 ml
Buffer RPE‡	65 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.

Storage

The QIAamp ccfDNA/RNA Kit (cat. no. 55184) is shipped at ambient temperature. Store the RNeasy 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 upon arrival under these conditions, if not otherwise stated on the label.

Intended Use

The QIAamp ccfDNA/RNA Kit is intended for molecular biology applications. The 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 product. 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.**

Buffers RWT and 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 QIAamp ccfDNA/RNA Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

Interest in circulating, cell-free DNA (ccfDNA) has increased over the past years as research into liquid biopsy and its applications in oncology, NIPT and other areas has grown. Circulating, cell-free RNA (ccfRNA) has also gained relevance among researchers, especially in the context of gene fusions, alternative splicing, etc.

ccfDNA usually occurs in fragments corresponding to 1 or 2 nucleosomes (~170 bp or multiples thereof). Contaminating DNAs due to cell death after a blood draw are generally longer and consist of higher multiples of 170 bp (apoptotic ladder). However, longer DNA fragments may also be released from tumor cells. Besides free ccfDNA, several publications have also described ccfDNA in extracellular vesicles (EVs). Cell-free miRNA may occur in EVs or can be associated with free Ago2 (RISC complexes) or other protein complexes. Distribution of the different species can depend on sample handling (e.g., time between blood clotting and plasma generation). mRNA in EVs is almost fully intact, while non-vesicular, long RNA rarely exists – if at all – presumably due to lack of protective proteins, with the exception of fragments of particularly abundant mRNA, like globin mRNA.

One ml of serum or plasma contains approximately the following amounts of ccfDNA and ccfRNA:

- Around 5 ng/ml ccfDNA in healthy donors; up to ~50 ng/ml from cancer patients
- Around 5–10 ng/ml ccfRNA in healthy donors; higher in cancer patients
- miRNA is present at a relatively high copy number, but the concentration is still very low (>100 times lower molecular weight).

The QIAamp ccfDNA/RNA Kit is designed to isolate all of these types of cell-free nucleic acids with high efficiency.

Principle and workflow

Plasma or serum used for isolating ccfDNA/RNA need to be handled properly to minimize contamination by cellular nucleic acids that are more abundant by several orders of magnitude. To remove residual cells, cell fragments, apoptotic bodies, etc., a second centrifugation at higher speed or filtration should be performed after the initial plasma generation. See Appendix A: Recommendations for Serum and Plasma Collection, Separation and Storage on page 20 for details.

The QIAamp ccfDNA/RNA Kit uses a chaotropic lysis buffer, followed by rapid and efficient precipitation of proteins, leaving all nucleic acids in solution. Addition of isopropanol facilitates binding of DNA and RNA to the RNeasy Midi Spin Column for initial purification. Elution from the RNeasy Midi Spin Column is followed by cleanup and concentration on RNeasy MinElute Spin Columns, allowing elution of all ccfDNA/RNA in as little as 14 µl. The procedure is free of phenol and chloroform and requires no processing on vacuum stations or prolonged proteolytic digestion steps.

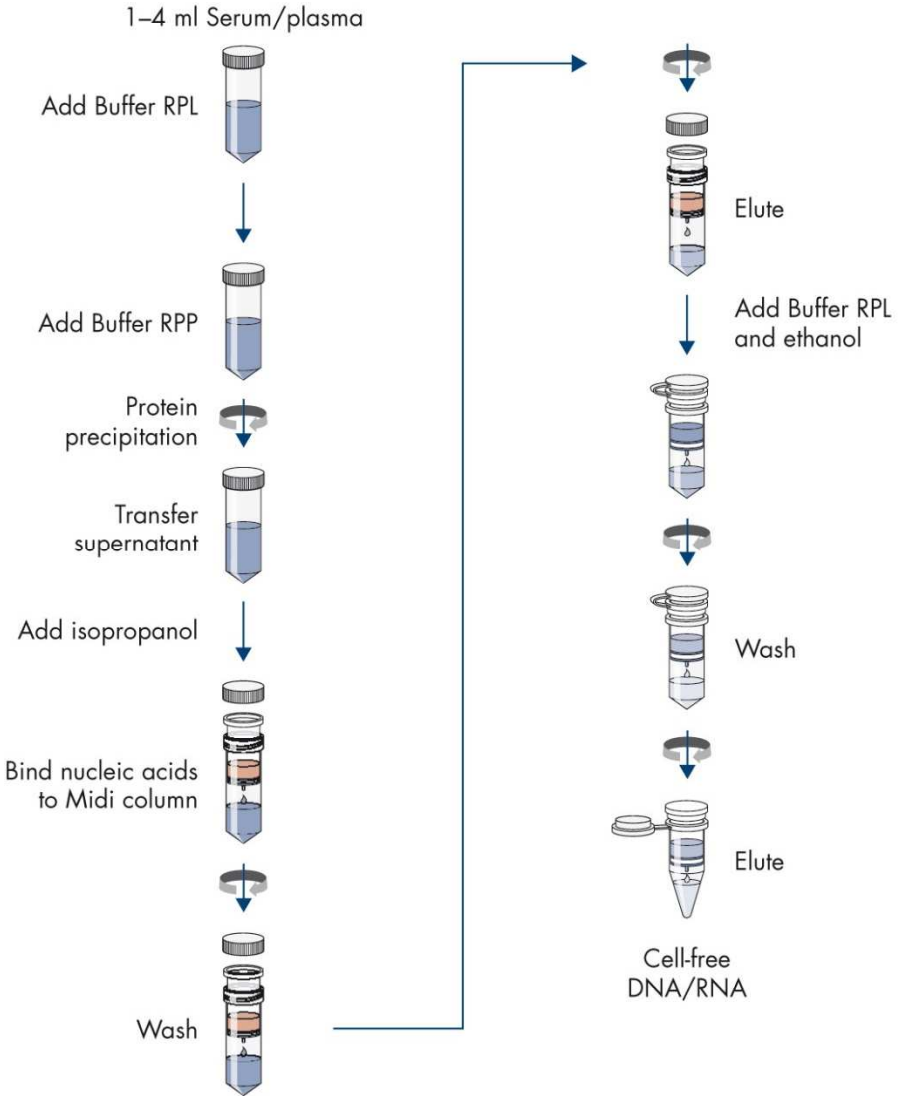

Figure 1. The QIAamp ccfDNA/RNA Kit provides total ccfDNA and RNA isolation in less than 45 minutes. Starting with plasma or serum depleted of residual cells and cell fragments, the QIAamp ccfDNA/RNA Kit uses precipitation-based removal of abundant plasma proteins, isolation of all cell-free nucleic acids on RNeasy Midi Spin Columns, followed by cleanup and concentration on RNeasy MinElute Columns.

Automated purification of nucleic acids on QIAcube Instruments

Purification of nucleic acids, including ccfDNA and RNA, 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 QIAamp ccfDNA/RNA Kit for purification of high-quality ccfDNA and RNA.

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.

The following supplies are also required:

- Ethanol (80%, 96–100%)*
- Isopropanol
- 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 (15–25°C)
- Centrifuge for 15 ml tubes for centrifugation at 4°C and at room temperature, swinging bucket rotor for up to 5000 *x g* and (ideally) a fixed-angle rotor for up to 12,000 *x g*
- Disposable gloves
- Equipment and tubes for serum/plasma collection and separation (see Appendix A, page 20):
  - 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 by the capacity of the RNeasy Midi and MinElute spin columns. It is not recommended to use more than 4 ml serum or plasma. Higher sample volumes will require additional column loading steps and may result in reduced RNA yield and/or copurification of inhibitors. The QIAamp ccfDNA/RNA Kit has been verified to work with sample volumes down to 1 ml. At smaller volumes, the low amounts of ccfDNA and mRNA may severely limit sensitivity for detection of mutations and for quantitation.

Yields of ccfDNA and RNA obtained with the QIAamp ccfDNA/RNA Kit vary strongly between samples from different individuals. However, they are generally too low for quantification by OD measurement. Thus, quantification by qPCR is recommended. See Appendix D: Storage, Quantification and Determination of DNA and RNA Quality on page 28 for more details.

Protocol: Purification of Cell-Free DNA and RNA, Including miRNA, from Serum and Plasma

This protocol is intended as a guideline for the purification of total circulating, cell-free DNA and RNA (including miRNA and other short RNAs) from 1–4 ml serum or plasma using the QIAamp ccfDNA/RNA Kit.

For recommendations on collection, preparation and storage of cell-free plasma and serum, see Appendix A on page 20.

In case, spike-in controls are used with the protocol, they should be added after step 2, before the addition of buffer RPP (or added to assays to assess possible assay inhibition only).

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°C to –65°C in aliquots is recommended. To process frozen samples, incubate at 37°C in a water bath until samples are completely thawed. Avoid prolonged incubation, which may compromise RNA integrity.
- Buffers RPL and RWT may form a precipitate upon storage. If necessary, redissolve by warming and then place at room temperature (15–25°C).
- Make sure buffer RPL is equilibrated back to room temperature before starting the protocol.
- Buffer RPL and RWT contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach.
- Except for isopropanol addition (step 7), all protocol and centrifugation steps should be performed at room temperature.

- Centrifugation of the RNeasy Midi spin columns is performed in a standard laboratory centrifuge with a swinging bucket rotor, preferably capable of up to 5000 *x g*. It is possible to reduce the steps performed at 5000 *x g* down to a minimum force of 3000 *x g* without loss in performance (make sure all liquid has passed through the membrane completely in each step).
- For protein precipitation, centrifugation at 12,000 *x g* for 3 min is recommended. If no centrifuge with rotor suitable for spinning at 12,000 *x g* is available, centrifugation can also be performed at 3000 *x g* for 10 min.
- The procedure has been optimized for use with either serum samples or plasma samples containing EDTA. Plasma samples containing heparin should not be used, because this anticoagulant can interfere with downstream assays, such as RT-PCR.
- Refer to Appendix B: Analysis of only ccfRNA or ccfDNA on page 24 if you intend to analyze only ccfDNA or ccfRNA in downstream assays.

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.

Procedure

1. Prepare serum or plasma or thaw frozen samples. It is recommended to perform an additional centrifugation or filtration step after obtaining the plasma or serum.

   Note: See Appendix A on page 20 for detailed recommendations.
2. Transfer between 1–4 ml serum or plasma into a 15 ml collection tube.
3. Add 300 µl Buffer RPL for each 1 ml of plasma or serum. Close the tube cap and vortex for 5 s. Leave at room temperature (15–25°C) for 3 min.

   Note: In this step, proteins are completely denatured and EVs are lysed.

4. Add 100 µl Buffer RPP for each 1 ml of plasma or serum. Close the tube cap and immediately mix vigorously by vortexing for >20 s. Incubate on ice for 3 min.

   A large protein pellet will form at this step. Vortexing is important to avoid nucleic acid becoming trapped in the pellet.

5. Centrifuge at 12,000 *x g* for 3 min to pellet the precipitate. If no centrifuge with rotor suitable for spinning at 12,000 *x g* is available, centrifugation can also be performed at 3000 *x g* for 10 min.

   Note: Supernatant should be clear and colorless.

6. Transfer supernatant (about 1 ml per ml of serum or plasma) to a new tube. Keep on ice.
7. Add 1 volume ice-cold isopropanol to each tube. Thoroughly mix by vortexing.
8. Pipet up to 4 ml sample, including any precipitate, onto an RNeasy Midi spin column in a 15 ml collection tube. Close the lid and centrifuge at ≥5000 *x g* for 1 min at room temperature in a swinging bucket rotor. Discard the flow-through.

   Reuse the collection tube in the next step.

9. Repeat step 8 using the remainder of the sample (if any remains).

   Note: Make sure all liquid has passed through the column membrane completely, before proceeding to the next step. Centrifuge again at ≥5000 *x g* for 1 min, if necessary.

10. Add 4 ml Buffer RWT to the RNeasy Midi spin column. Close the lid, and centrifuge for 1 min at ≥5000 *x g*. Discard the flow-through.

    Reuse the collection tube in the next step.

11. Pipet 2.5 ml Buffer RPE onto the RNeasy Midi spin column. Close the lid, and centrifuge for 5 min at ≥5000 *x g*. Discard the flow-through.
12. Place the RNeasy Midi spin column into a new 15 ml collection tube (supplied). Add 200 µl RNase-free water directly to the center of the spin column membrane. Close the lid gently, incubate for 1 min, then centrifuge for 1 min at full speed to elute the DNA/RNA.

Cleanup (automatable on QIAcube Connect)

1. Add 200 µl Buffer RPL to 200 µl eluate.
2. Add 800 µl ethanol (96–100%) and mix by pipetting or vortexing.
3. Pipet 700 µl sample, including any precipitate that may have formed, onto an RNeasy MinElute spin column in a 2 ml collection tube (supplied). Close the lid gently and centrifuge at ≥8000 *x g* (≥10,000 rpm) for 15 s at room temperature (15–25°C). Discard the flow-through.

   Reuse the collection tube in the next step.
4. Repeat step 3 using the remainder of the sample.

   Make sure all liquid has passed through the column membrane completely, before proceeding to the next step.

   Reuse the collection tube the next step.
5. Pipet 500 µl Buffer RPE onto the RNeasy MinElute spin column. Close the lid, and centrifuge for 15 s at ≥8000 *x g* (≥10,000 rpm). Discard the flow-through.

   Reuse the collection tube in the next step.
6. Add 500 µl of 80% ethanol to the RNeasy MinElute spin column. Close the lid and centrifuge for 15 s at ≥8000 *x g*. Discard the flow-through and the collection tube.
7. Place the RNeasy 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.

8. Place the RNeasy MinElute spin column in a new 1.5 ml collection tube (supplied). Add 14–20 µl RNase-free water directly to the center of the spin-column membrane. Close the lid gently, incubate for 1 min and centrifuge for 1 min at full speed to elute the DNA/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 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.

   Note: The dead volume of the RNeasy MinElute spin column is 2 µl, so 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
Clogged RNeasy Midi column	
Sample still contains cellular material or coagulated protein	Make sure to follow recommendations in Appendix A (page 20) for removal of residual cellular material. After thawing of frozen samples, remove cryoprecipitates by centrifugation or filtration, if necessary (see Appendix A). Make sure to not transfer any precipitate from step 5 on page 15 when transferring the supernatant to a fresh tube.
Clogged RNeasy MinElute column	
Centrifugation temperature too low	Except for the isopropanol addition (step 7 on page 15), 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.
Low ccfDNA/RNA yield or poor performance in downstream applications	
a) Incorrect isopropanol or ethanol concentration	Be sure to use the isopropanol and ethanol concentrations specified in the protocol steps.
b) Isopropanol too warm	Make sure both isopropanol and supernatant of protein precipitation are cold before adding it (in step 7 on page 15). This avoids carryover of precipitation reagent into eluates.
Low or no recovery of ccfDNA/RNA	
a) Too much starting material	In subsequent preparations, reduce the amounts of starting material. It is essential to use the correct amount of starting material (see page 12, Volume of starting material).

		Comments and suggestions
b)	Lysate too warm	Make sure both the sample and buffer RPL are at room temperature when starting the protocol (after thawing of samples or dissolving precipitates in RPL)
c)	Insufficient mixing after RPP addition	Make sure to thoroughly mix to avoid trapping nucleic acids in the precipitate.
d)	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.
e)	ccfDNA/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.
RNA degraded		
a)	Sample inappropriately handled	ccfRNA typically consists of high amounts of small RNA species and much lower amounts of ribosomal RNA compared to cells, and will therefore not resemble intact RNA from cells or tissue on electropherograms. Nevertheless, the following precautions are recommended to avoid complications due to RNA degradation. Perform the protocol quickly, especially the first few steps. See "Appendix C: General Remarks on Handling RNA" (page 25) and "Appendix A: Recommendations for Serum and Plasma Collection, Separation and Storage" (page 20).
b)	RNase contamination	ccfRNA typically consists of high amounts of small RNA species, and will therefore not resemble intact RNA from cells or tissue on electropherograms. Nevertheless, the following precautions are recommended to avoid complications due to RNA degradation. 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 25). Do not put RNA samples into a vacuum dryer that has been used in DNA preparations where RNases may have been used.
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: Recommendations for Serum and Plasma Collection, Separation and Storage

To specifically isolate circulating cell-free DNA and RNA from serum and plasma, we recommend following these protocols, which include an initial low g-force centrifugation step to separate cells from plasma or serum followed by a higher g-force centrifugation or filtration step to remove all remaining cellular debris. The latter centrifugation/filtration step significantly reduces the amount of cellular or genomic DNA and RNA in the sample. Because of the much higher abundance of DNA and RNA in cells (several orders of magnitude), even small amounts of cellular debris can have a very significant effect on analysis of cell-free nucleic acid. The sooner after blood collection this removal of cellular materials is performed, the lower the risk of additional background from blood cell-derived nucleic acid 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 other anticoagulant, e.g., citrate). Store tubes at room temperature (15–25°C) or 4°C and process within 1 hour.

   Note: Use of citrate-containing tubes may result in reduced recovery of miRNA. 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 0.8µm filter. Transfer supernatant to a new tube, and begin nucleic acid purification protocol.

Appendix B: Analysis of only ccfRNA or ccfDNA

Analysis of ccfRNA only

To avoid any influence from co-purified ccfDNA on the analysis of ccfRNA, specifically by qRT-PCR, for example, we recommend using a cDNA synthesis method that includes a DNA removal step, such as the QuantiNova® Reverse Transcription Kit (cat. nos. 205410, 205411, 205413). Plasma and serum contain extremely small amounts of DNA that is very effectively removed using these methods, which are faster and easier to perform than on-column DNase treatment.

Analysis of ccfDNA only

Small amounts of ccfRNA present in plasma or serum do not significantly influence analysis of ccfDNA by methods, such as qPCR and NGS or quantitation by fluorometric assays.

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 26), or rinse with chloroform* if the plasticware is chloroform-resistant. To decontaminate electrophoresis tanks, clean with

* 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.

detergent (e.g., 0.5% SDS),* rinse with RNase-free water, rinse with ethanol (if the tanks are ethanol-resistant) and allow to dry.

Disposable plasticware

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

Glassware

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

Solutions

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

* 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.

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 RNase-free without using DEPC treatment and are therefore free of any DEPC contamination.

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

Storage of DNA/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 DNA or RNA is detectable after 1 year.

Quantification of ccfDNA and ccfRNA

The concentration of ccfDNA and 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.

Size distribution of ccfDNA/RNA

The size distribution of circulating nucleic acids purified using this procedure can be checked using an Agilent® Bioanalyzer, TapeStation or similar device. These methods can also provide a rough concentration estimate.

Ordering Information

Product	Contents	Cat. no.
QIAamp ccfDNA/RNA Kit (50)	For 50 preps: RNeasy Midi and RNeasy MinElute Spin Columns, Collection Tubes (50 ml), Elution Tubes (1.5 ml and 2 ml), RNase-free reagents and buffers	55184
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® Serum/ Plasma Advanced Kit (50)	For 50 total RNA preps: RNeasy UCP MinElute Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free reagents and buffers	217204
exoRNeasy Serum/ Plasma Maxi Kit (50)	For 50 preps: exoEasy Maxi Spin Columns, RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml and 2 ml), QIAzol® Lysis Reagent, RNase-free reagents and buffers	77064
QIAamp MinElute ccfDNA Mini Kit (50)	For 50 preps (1 or 2 ml sample input volume each): QIAamp UCP MinElute Columns, QIAGEN Proteinase K, Magnetic Bead Suspension, Buffers, Bead Elution Tubes, Collection Tubes (1.5 ml and 2 ml)	55204

Product	Contents	Cat. no.
Related products for quantitative, real-time RT-PCR		
miScript® II RT Kit (12)	For 12 cDNA synthesis reactions: miScript Reverse Transcriptase Mix, 10x miScript Nucleics Mix, 5x miScript HiSpec Buffer, 5x miScript HiFlex Buffer, RNase-Free Water	218160
miScript SYBR® Green PCR Kit (200)	For 200 reactions: QuantiTect® SYBR Green PCR Master Mix, miScript Universal Primer	218073
miScript Primer Assay (100)	miRNA-specific primer; available via GeneGlobe®	Varies†
Pathway-Focused miScript miRNA PCR Array	Array of assays for a pathway, disease or gene family for human, mouse, rat, dog or rhesus macaque miRNAs; available in 96-well, 384-well or Rotor-Disc® 100 format	Varies
ROX™ (100)	For 100 x 20 µl reactions: 1 ml QuantiNova SYBR Green RT-PCR Master Mix, 20 µl QuantiNova SYBR Green RT Mix, 20 µl Internal Control RNA, 500 µl Yellow Template Dilution Buffer, 250 µl ROX™ Reference Dye, 1.9 µl RNase-Free Water	208152
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	2082352

Product	Contents	Cat. no.
QuantiNova SYBR Green PCR Kit (100)	For 100 x 20 µl reactions: 1 ml 2x QuantiNova SYBR Green PCR Master Mix, 500 µl QuantiNova Yellow Template Dilution Buffer, 250 µl QN ROX Reference Dye, 1.9 ml Water	208052
QuantiNova Probe PCR Kit (100)	For 100 x 20 µl reactions: 1 ml 2x QuantiNova Probe PCR Master Mix , 250 µl QN ROX Reference Dye, 500 µl QuantiNova Yellow Template Dilution Buffer, 1.9 ml Water	208252
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 Targeted DNA Panels	Digital DNA sequencing to confidently detect low-frequency variants	Varies
QIAseq Targeted RNA Panels	Digital RNA sequencing for gene expression profiling	Varies

* 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

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Document Revision History

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

Notes

Notes

Limited License Agreement for the QIAamp ccfDNA/RNA Kit

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