September 2019

QIAamp® DSP Circulating NA Kit Instructions for Use (Handbook)

Version 1

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REF 61504

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1118364

Sample to Insight

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Intended Use

The QIAamp DSP Circulating NA Kit is a system that uses silica-membrane technology (QIAamp technology) for isolation and purification of circulating cell-free DNA and RNA from human blood plasma samples.

The product is intended to be used by professional users, such as technicians and physicians, who are trained in molecular biological techniques.

The QIAamp DSP Circulating NA Kit is intended for in vitro diagnostic use.

Summary and Explanation

Free-circulating nucleic acids are present in human plasma usually as short fragments, <1000 bp (DNA), <1000 nt (RNA), or as small as 20 nt (miRNAs). The concentration of free-circulating nucleic acids in human blood plasma is usually low and varies considerably between individuals ranging from 1–100 ng/ml in human samples (1–5).

The QIAamp DSP Circulating NA Kit enables efficient purification of circulating nucleic acids from human plasma. Samples can be either fresh or frozen. Extension tubes and vacuum processing on the QIAvac 24 Plus enable starting sample volumes of up to 5 ml, and flexible elution volumes between 20–150 µl allow concentration of nucleic acid species that are present in low concentrations.

Eluted free-circulating genomic DNA or RNA is ready for use in downstream applications or suitable for storage. The QIAamp DSP Circulating NA Kit provides efficient removal of proteins, nucleases, and other impurities.

Principles of the Procedure

The QIAamp DSP Circulating NA procedure comprises 4 steps (lyse, bind, wash, and elute) and is carried out using QIAamp Mini columns on the QIAvac system. The robust procedure helps to minimize sample-to-sample cross-contamination and increases user safety when handling potentially infectious samples.

The simple procedure is suitable for simultaneous processing of up to 24 samples in less than 2 hours.

Sample volumes

QIAamp Mini columns bind fragmented nucleic acids that are as short as 20 nt, but the yield depends on the sample volume and the concentration of circulating nucleic acids in the sample (typically 1–100 ng/ml in plasma). The QIAamp DSP Circulating NA procedure has been optimized for sample volumes of up to 5 ml.

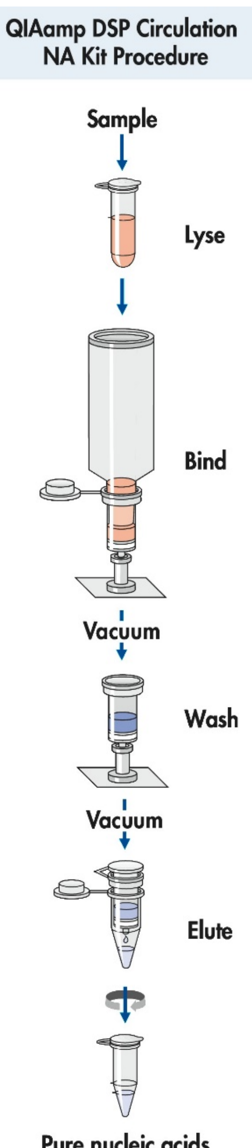


Figure 1. Overview of the QIAamp DSP Circulating NA Kit procedure

Lysing samples

Free-circulating nucleic acids in biological fluids are usually bound to proteins or enveloped in vesicles, requiring an efficient lysis step in order to release nucleic acids for selective binding to the QIAamp Mini column. Hence, samples are lysed under highly denaturing conditions at elevated temperatures in the presence of proteinase K and Buffer ACL, which ensures inactivation of DNases and RNases and release of nucleic acids from bound proteins, lipids, and vesicles.

Adsorption to the QIAamp Mini column membrane

To allow optimal binding of the circulating nucleic acids to the membrane, binding conditions are adjusted by addition of Buffer ACB to the lysate. Lysates are then transferred onto a QIAamp Mini column, and circulating nucleic acids are adsorbed from a large volume onto the silica membrane as the lysate is drawn through by vacuum pressure. Salt and pH conditions ensure that the majority of proteins and other contaminants, which can inhibit PCR and other downstream enzymatic reactions, are not retained on the QIAamp Mini column membrane.

A vacuum manifold (e.g., the QIAvac 24 Plus with the QIAvac Connecting System) and a vacuum pump capable of producing a vacuum of ~800–900 mbar (e.g., QIAGEN® Vacuum Pump) are required for the protocol. A Vacuum Regulator should be used (part of the QIAvac Connecting System) for easy monitoring of vacuum pressure and convenient vacuum release.

Removal of residual contaminants

Nucleic acids remain bound to the membrane, while contaminants are efficiently washed away during 3 wash steps.

Elution of pure nucleic acids

Elution is performed using Buffer AVE. In a single step, highly pure circulating nucleic acids are eluted in Buffer AVE, equilibrated to room temperature. A flexible elution volume of 50–150 µl can be applied. If higher nucleic acid concentrations are required, the elution volume can be reduced to as low as 20 µl. Elution volumes lower than 50µl lead to higher concentrated nucleic acid eluates but may result in lower total yield.

The eluate volume recovered can be up to 5 µl less than the volume of elution buffer applied to the column.

Yield and size of nucleic acids

Yields of free-circulating nucleic acids isolated from biological samples are normally below 1 µg and are therefore difficult to determine with a spectrophotometer. The absolute yield of circulating DNA and RNA obtained from a sample using the QIAamp DSP Circulating NA Kit varies between samples from different individuals and also depends on other factors (e.g., certain disease states). In addition, carrier RNA present in the extracted nucleic acids is likely to dominate UV absorbance readings (see page 25). Quantitative amplification methods are recommended for determination of yields.

The size distribution of circulating nucleic acids purified using the QIAamp DSP circulating NA Kit can be checked by agarose gel electrophoresis or hybridization to a target-specific labeled probe[5] or a microfluidic electrophoresis solution (e.g. Agilent Bioanalyzer).

Description of protocols

Two different protocols are provided in this handbook.

The "Breeze Protocol: Purification of Circulating Nucleic Acids From 1–5 ml of Human Blood Plasma" (page 27) is for processing up to 5ml plasma in 1ml steps and has been optimized for low hands-on and turnaround time.

The "Classic Protocol: Purification of Circulating Nucleic Acids From 1–5 ml Human Blood Plasma" (page 32) is for processing up to 5 ml plasma in 1 ml steps and constitutes the unchanged protocol of QIAamp DSP circulating NA Kit Handbook Revision 3 (R3).

Materials Provided

Kit contents

QIAamp DSP Circulating NA Kit			(50)
Catalog no.			61504
Number of preps			50
QIAamp Mini	QIAamp Mini columns with Wash Tubes (WT) (2 ml)	COL	50
EXT	Column Extenders (20 ml)	COL EXT	2 x 25
WT	Wash Tubes (2 ml)	WASH TUBE	50
ET	Elution Tubes (1.5 ml)	ELU TUBE	50
VC	VacConnectors	VAC CON	50
ACL*	Lysis Buffer*	LYS BUF	220 ml
ACB*	Binding Buffer* (concentrate)	BIND BUF CONC	300 ml
ACW1*	Wash Buffer 1* (concentrate)	WASH BUF 1 CONC	19 ml
ACW2†	Wash Buffer 2† (concentrate)	WASH BUF 2 CONC	13 ml
AVE†	Elution Buffer† (purple caps)	ELU BUF	5 x 2 ml
PROTK	QIAGEN Proteinase K	PROTK	4 x 7 ml
Carrier	Carrier RNA (red caps)	CAR RNA	310 µg
	Handbook	HB	1

* Contains a chaotropic salt. See page 12 for Warnings and Precautions.

† Contains sodium azide as a preservative.

Materials Required but not Provided

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.

Make sure that instruments have been checked and calibrated according to the manufacturer´s recommendations.

For all protocols

- Pipettes (adjustable)
- Sterile pipette tips (pipette tips with aerosol barriers are recommended to help prevent cross-contamination)
- Water bath or heating block capable of holding 50 ml centrifuge tubes at 56°C or 60°C*
- Heating block or similar at 56°C capable of holding 2 ml wash tubes (only for the Classic Protocol)*
- Microcentrifuge (with rotor for 2 ml tubes)*
- 50 ml centrifuge tubes
- QIAvac 24 Plus vacuum manifold (cat. no. 19413)
- QIAvac Connecting System (cat. no. 19419) or equivalent
- Vacuum Pump (cat. no. 84010 [USA and Canada], 84000 [Japan], or 84020 [rest of world]) or equivalent pump capable of producing a vacuum of –800 to –900 mbar
- Ethanol (96–100%)†
- Isopropanol (100%)
- Crushed ice (only for the "Classic Protocol: Purification of Circulating Nucleic Acids From 1–5 ml Human Blood Plasma".)
- Some samples may require dilution with phosphate-buffered saline (PBS)
- Optional: VacValves (cat. no. 19408)

* Ensure that instruments have been checked and calibrated according to the manufacturer's recommendations.

† Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone.

Warnings and Precautions

For In Vitro Diagnostic Use

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.

WARNING	Risk of personal injury DO NOT add bleach or acidic solutions directly to the sample preparation waste.

Buffer ACL, Buffer ACB, and Buffer ACW1 contain guanidine salts, which can form highly reactive compounds when combined with bleach.

If liquid containing these buffers 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.

The following hazard and precautionary statements apply to components of the QIAamp DSP Circulating NA Kit.

Buffer ACB

Contains: guanidine thiocyanate. Danger! Harmful if swallowed. May be harmful in contact with skin or if inhaled. Causes severe skin burns and eye damage. Harmful to aquatic life with long-lasting effects. Contact with acids liberates very toxic gas. Wear protective gloves/ protective clothing/ eye protection/ face protection. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Immediately call a POISON CENTER or doctor/ physician.

Buffer ACL

Contains: guanidine thiocyanate. Danger! Harmful if swallowed. May be harmful in contact with skin or if inhaled. Causes severe skin burns and eye damage. Harmful to aquatic life with long-lasting effects. Contact with acids liberates very toxic gas. Wear protective gloves/ protective clothing/ eye protection/ face protection. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Immediately call a POISON CENTER or doctor/ physician.

Buffer ACW1

Contains: guanidine hydrochloride. Warning! Harmful if swallowed or if inhaled. Causes skin irritation. Causes serious eye irritation. Wear protective gloves/ protective clothing/ eye protection/ face protection.

Proteinase K

Contains: Proteinase K. Danger! Causes mild skin irritation. May cause allergy or asthma symptoms or breathing difficulties if inhaled. Avoid breathing dust/ fume/ gas/ mist/ vapors/ spray. Wear protective gloves/ protective clothing/ eye protection/ face protection. Wear respiratory protection. If exposed or concerned: Call a POISON CENTER or doctor/ physician. Remove person to fresh air and keep comfortable for breathing.

Reagent Storage and Handling

QIAamp Mini columns should be stored dry at 2–8°C. All buffers should be stored at room temperature (15–25°C). QIAamp Mini columns and buffers can be stored under these conditions until the expiration date on the kit box without showing any reduction in performance.

Lyophilized carrier RNA should be stored at room temperature (15–25°C) until the expiration date on the component label. Carrier RNA should be dissolved in Buffer AVE; dissolved carrier RNA should be immediately added to Buffer ACL as described on page 28 for the Breeze Protocol and on page 33 for the Classic Protocol. This solution should be prepared fresh and is stable at 2–8°C for up to 48 hours. Unused portions of carrier RNA dissolved in Buffer AVE should be frozen in aliquots at −30 to −15°C.

The QIAamp DSP Circulating NA Kit contains a ready-to-use proteinase K solution, which is dissolved in a specially formulated storage buffer. The proteinase K is stable until expiration date on the component label when stored at room temperature (15–25°C).

Specimen Storage and Handling

Blood storage and handling

To avoid degradation of cell-free nucleic acids and release of cellular nucleic acids, we recommend storage of whole blood for a maximum of 6 h at 2–8°C (e.g. EDTA samples). If using stabilized blood collection tubes, please consider the storage conditions provided by the manufacturer. We recommend validating these storage conditions in combination with your specific downstream application and target.

Plasma storage and handling

It is recommended to perform the plasma separation and nucleic acid isolation immediately after blood donation when using EDTA as anticoagulant, especially for RNA. For short-term storage the plasma can be stored up to 24 hours at 2-8°C.

For longer storage, plasma aliquots from stabilized as well as non-stabilized blood collection tubes can be stored at −20℃ (only for DNA as target) or −80℃ (DNA and RNA as target) for at least 4 weeks.

Storing eluted nucleic acids

Eluted nucleic acids are collected in 1.5 ml elution tubes (provided). The purified circulating nucleic acids can be stored for up to 24 hours at 2–8°C. For periods of storage longer than 24 hours, storage at –30 to –15°C is recommended for DNA and –90 to –60°C for RNA downstream applications.

Procedure

Important points before starting

The QIAvac 24 Plus

The QIAvac 24 Plus is designed for fast and efficient vacuum processing of up to 24 QIAGEN spin columns in parallel. Samples and wash solutions are drawn through the column membranes by vacuum instead of centrifugation, providing greater speed and reduced hands-on time in purification procedures.

In combination with the QIAvac Connecting System, the QIAvac 24 Plus can be used as a flow-through system. The sample flow-through is collected in a separate waste bottle.

For maintenance of the QIAvac 24 Plus, refer to the handling guidelines in the *QIAvac 24 Plus Handbook*.

Processing QIAamp Mini columns on the QIAvac 24 Plus

QIAamp Mini columns are processed on the QIAvac 24 Plus using disposable VacConnectors and reusable VacValves. VacValves (optional) are inserted directly into the luer slots of the QIAvac 24 Plus manifold and ensure a steady flow rate, facilitating parallel processing of different sample volumes. They should be used if sample flow rates differ significantly in order to ensure consistent vacuum. VacConnectors are disposable connectors that fit between QIAamp Mini columns and VacValves or between the QIAamp Mini columns and the luer slots of the QIAvac 24 Plus. They prevent direct contact between the spin column and VacValve during purification, thereby avoiding any cross-contamination between samples. VacConnectors are discarded after a single use. Due to the large solution volumes used, the QIAvac Connecting System (or a similar setup with waste bottles) is required (see Figure 2).

Handling guidelines for the QIAvac 24 Plus

- Always place the QIAvac 24 Plus on a secure bench top or work area. If dropped, the QIAvac 24 Plus manifold may crack.
- Always store the QIAvac 24 Plus clean and dry. For cleaning procedures, see the QIAvac 24 Plus Handbook.
- The components of the QIAvac 24 Plus are not resistant to certain solvents (Table 1). If these solvents are spilled on the unit, rinse it thoroughly with water.
- To ensure consistent performance, do not apply silicone or vacuum grease to any part of the QIAvac 24 Plus manifold.
- Always use caution and wear safety glasses when working near a vacuum manifold under pressure.
- Contact QIAGEN Technical Services or your local distributor for information concerning spare or replacement parts.
- The vacuum pressure is the differential pressure between the inside of the vacuum manifold and the atmosphere (standard atmospheric pressure 1013 millibar or 760 mm Hg) and can be measured using the QIAvac Connecting System (see Figure 2). The protocols require a vacuum pump capable of producing a vacuum or −800 to −900 mbar (e.g., QIAGEN Vacuum Pump). Higher vacuum pressures must be avoided. Use of vacuum pressures lower than recommended may reduce nucleic acid yield and purity and increase the risk of clogged membranes.

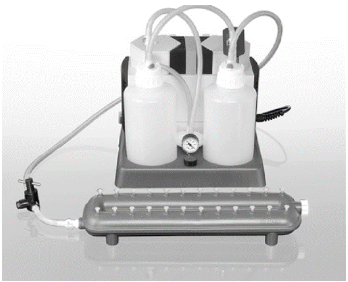


Figure 2. QIAvac 24 Plus, QIAvac Connecting System, and Vacuum Pump

Table 1. Chemical resistance properties of QIAvac 24 Plus

Resistant to		Not resistant to
Acetic acid	Chaotropic salts	Benzene
Chromic acid	Concentrated alcohols	Phenol
SDS	Sodium chloride	Chloroform
Tween® 20	Urea	Toluene
Chlorine bleach	Hydrochloric acid	Ethers
Sodium hydroxide		

Setup of the QIAvac 24 Plus vacuum manifold

1. Connect the QIAvac 24 Plus to a vacuum source. If using the QIAvac Connecting System, connect the system to the manifold and vacuum source as described in Appendix A of the *QIAvac 24 Plus Handbook*.
2. Insert a VacValve (optional) into each luer slot of the QIAvac 24 Plus that is to be used (see Figure 3). Close unused luer slots with luer plugs or close the inserted VacValve.

   VacValves should be used if flow rates of samples differ significantly to ensure consistent vacuum.
3. Insert a VacConnector into each VacValve (see Figure 3).

   Perform this step directly before starting the purification to avoid exposure of VacConnectors to potential contaminants in the air.
4. Place the QIAamp Mini columns into the VacConnectors on the manifold (see Figure 3).

   Note: Save the wash tube from the blister pack for use in the purification protocol.
5. Insert a column extender (20 ml) into each QIAamp Mini column (see Figure 3).

   Note: Make sure that the column extender is firmly inserted into the QIAamp Mini column in order to avoid leakage of sample.
6. For nucleic acid purification, follow the instructions in the protocols. Discard the VacConnectors appropriately after use.

Leave the lid of the QIAamp Mini column open while applying vacuum.

Switch off the vacuum between steps to ensure that a consistent, even vacuum is applied during processing. For faster vacuum release, a Vacuum Regulator should be used (part of the QIAvac Connecting System).

Note: Each VacValve can be closed individually when the sample is completely drawn through the spin column, allowing parallel processing of samples of different volumes or viscosities.

7. After processing samples, clean the QIAvac 24 Plus (see "Cleaning and Decontaminating the QIAvac 24 Plus" in the *QIAvac 24 Plus Handbook*).

   Note: Buffers ACL, ACB, and ACW1 are not compatible with disinfecting agents containing bleach. See page 12 for Warnings and Precautions.

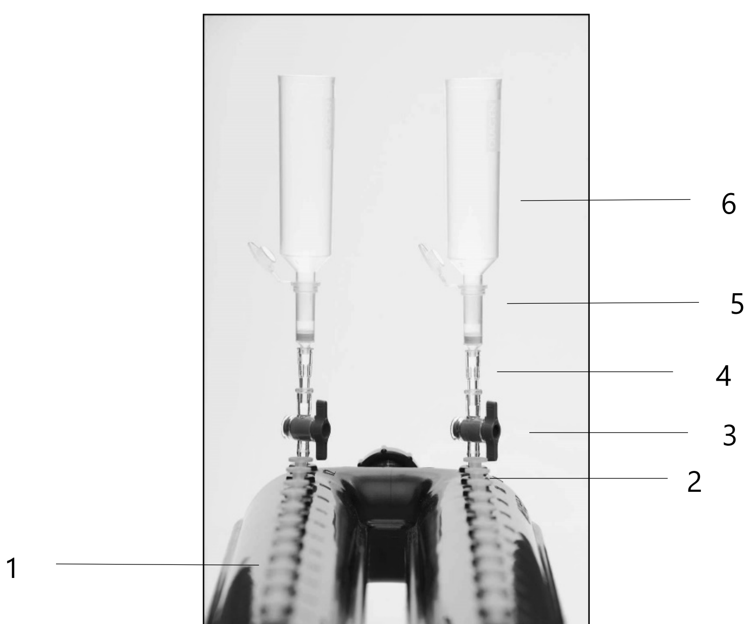


Figure 3. Setting up the QIAvac 24 Plus with QIAamp Mini columns using VacValves, VacConnectors, and Column Extenders.

1 QIAvac 24 Plus vacuum manifold

2 Luer slot of the QIAvac 24 Plus (closed with luer plug)

3 VacValve**

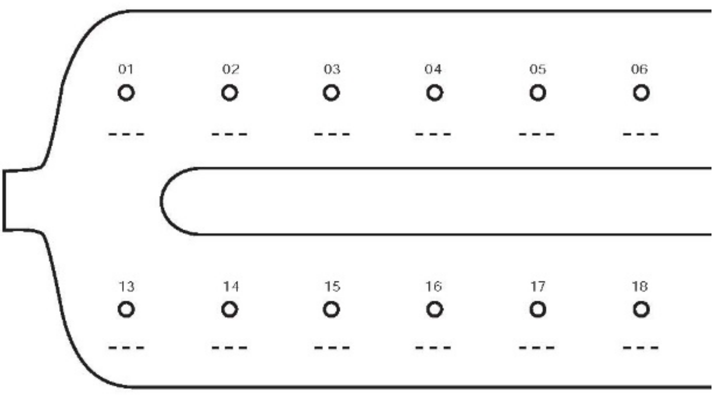
4 VacConnector

5 QIAamp Mini column

6 Column Extender

We recommend labeling the tubes and the QIAamp Mini columns for use on the QIAvac 24 Plus vacuum system according to the scheme in Figure 4 in order to avoid the mix-up of samples. This figure can be photocopied and labeled with the names of the samples.

* Must be purchased separately.



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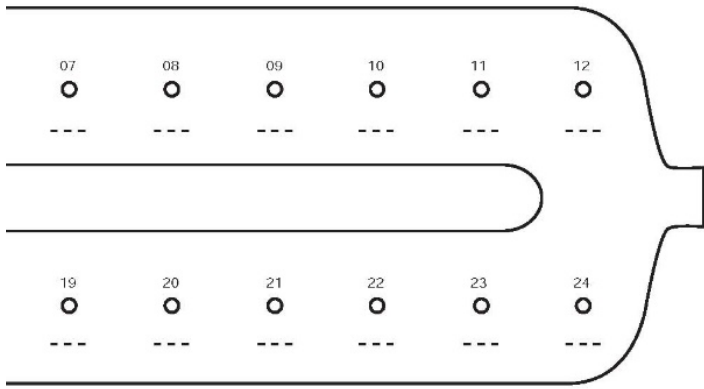


Figure 4. Labeling scheme for tubes and QIAamp Mini columns for use on the QIAvac 24 Plus vacuum system

Preparation of Buffers and Reagents

Buffer ACB

Before use, add 200 ml isopropanol (100%) to 300 ml Buffer ACB concentrate to obtain 500 ml Buffer ACB. Mix well after adding isopropanol.

Buffer ACW1 *

Before use, add 25 ml ethanol (96–100%) to 19 ml Buffer ACW1 concentrate to obtain 44 ml Buffer ACW1. Mix well after adding ethanol.

Buffer ACW2 †

Before use, add 30 ml ethanol (96–100%) to 13 ml Buffer ACW2 concentrate to obtain 43 ml Buffer ACW2. Mix well after adding ethanol.

Adding carrier RNA to Buffer ACL*

Carrier RNA serves 2 purposes: firstly, it enhances binding of nucleic acids to the QIAamp Mini membrane, especially if there are very few target molecules in the sample. Secondly, the addition of large amounts of carrier RNA reduces the chance of RNA degradation in the rare event that RNase molecules escape denaturation by the chaotropic salts and detergents in Buffer ACL.

The amount of lyophilized carrier RNA provided is sufficient for the volume of Buffer ACL supplied with the kit. The recommended concentration of carrier RNA has been adjusted so that the QIAamp DSP Circulating NA protocol can be used as a generic purification system

* Contains chaotropic salt. See page 12-13 for warnings and precautions.
† Contains sodium azide as a preservative.

compatible with many different amplification systems and is suitable for a wide range of RNA and DNA targets.

Different amplification systems vary in efficiency depending on the total amount of nucleic acids present in the reaction. Eluates from the kit contain both circulating nucleic acids and carrier RNA, and the amount of carrier RNA will greatly exceed the amount of circulating nucleic acids in most cases. Therefore, quantification of isolated circulating nucleic acids by UV-absorbance reading will not be adequate, as the results of such measurements are determined by the presence of carrier RNA.

To obtain the highest levels of sensitivity in amplification reactions, it may be necessary to reduce the amount of carrier RNA added to Buffer ACL.

For amplification systems involving oligo dT primers, no carrier RNA should be added during isolation of free-circulating nucleic acids.

Add 1550 µl Buffer AVE* to the tube containing 310 µg of lyophilized carrier RNA to obtain a solution of 0.2 µg/µl concentration. Dissolve the carrier RNA thoroughly, divide it into conveniently-sized aliquots, and store it at –30 to –15°C. Do not freeze–thaw the aliquots of carrier RNA more than 3 times.

Note that carrier RNA does not dissolve in Buffer ACL. It must first be dissolved in Buffer AVE and then added to Buffer ACL.

Calculate the volume of Buffer ACL–carrier RNA mix needed per batch of samples according to the tables in the protocols. Select the number of samples to be simultaneously processed.

Gently mix by inverting the tube or bottle 10 times. To avoid foaming, do not vortex.

* Contains sodium azide as a preservative.

Note: The sample-preparation procedure is optimized for a maximum of 1.0 µg of carrier RNA per sample. If lesser carrier RNA has been shown to be better for your amplification system, transfer only the required amount of dissolved carrier RNA to the tubes containing Buffer ACL. For each microgram of carrier RNA required per preparation, add 5 µl of dissolved carrier RNA to Buffer ACL. (Use of less than 1.0 µg carrier RNA per sample may be beneficial and must be validated for each particular sample type and downstream assay.)

Breeze Protocol: Purification of Circulating Nucleic Acids From 1–5 ml of Human Blood Plasma

This protocol is for purification of circulating DNA and RNA from 1–5 ml of human blood plasma and has been optimized for low hands-on and turnaround time. For existing user-validated workflows using the QIAamp DSP circulating Kit version 1/R3, please refer to the "Classic Protocol: Purification of Circulating Nucleic Acids From 1–5 ml Human Blood Plasma" (page 32).

Important points before starting

- All centrifugation steps are carried out at room temperature (15–25°C).
- Switch off vacuum between steps to ensure that a consistent, even vacuum is applied during the protocol steps.
- **Note**: Vacuum Pump pressure should be between −800 to −900 mbar.
- Equilibrate samples to room temperature.
- Use phosphate-buffered saline to bring the volume of the sample to the nearest exact volume (1 ml to 5 ml).
- Set up the QIAvac 24 Plus as described on page 19.
- Heat a water bath or heating block to 56°C for use with 50 ml centrifuge tubes in step 3.
- Equilibrate the QIAamp Mini spin columns for at least 1 hour to room temperature before use.
- Ensure that Buffer ACB, Buffer ACW1, and Buffer ACW2 have been prepared (addition of isopropanol or ethanol) according to the instructions on page 24.
- Add carrier RNA reconstituted in Buffer AVE to Buffer ACL according to instructions in Table 2.

Table 2. Volume of Buffer ACL and carrier RNA (dissolved in Buffer AVE) required for processing 1–5 ml human blood plasma samples

Setup for ml plasma	A	B	C	D	E	Carrier RNA in Buffer AVE (µl)
	1 ml	2 ml	3 ml	4 ml	5 ml	
Number of samples	Buffer ACL (ml)					
1	0.9	1.8	2.6	3.5	4.4	5.6
2	1.8	3.5	5.3	7.0	8.8	11.3
3	2.6	5.3	7.9	10.6	13.2	16.9
4	3.5	7.0	10.6	14.1	17.6	22.5
5	4.4	8.8	13.2	17.6	22.0	28.1
6	5.3	10.6	15.8	21.1	26.4	33.8
7	6.2	12.3	18.5	24.6	30.8	39.4
8	7.0	14.1	21.1	28.2	35.2	45.0
9	7.9	15.8	23.8	31.7	39.6	50.6
10	8.8	17.6	26.4	35.2	44.0	56.3
11	9.7	19.4	29.0	38.7	48.4	61.9
12	10.6	21.1	31.7	42.2	52.8	67.5
13	11.4	22.9	34.3	45.8	57.2	73.1
14	12.3	24.6	37.0	49.3	61.6	78.8
15	13.2	26.4	39.6	52.8	66.0	84.4
16	14.1	28.2	42.2	56.3	70.4	90.0
17	15.0	29.9	44.9	59.8	74.8	95.6
18	15.8	31.7	47.5	63.4	79.2	101.3
19	16.7	33.4	50.2	66.9	83.6	106.9
20	17.6	35.2	52.8	70.4	88.0	112.5
21	18.5	37.0	55.4	73.9	92.4	118.1
22	19.4	38.7	58.1	77.4	96.8	123.8
23	20.2	40.5	60.7	81.0	101.2	129.4
24	21.1	42.2	63.4	84.5	105.6	135.0

Procedure: Breeze Protocol

1. Pipet QIAGEN Proteinase K, plasma, and Buffer ACL **in this order** into a 50 ml centrifuge tube (not provided).

Setup	**A**	**B**	**C**	**D**	**E**
ProtK (µl)	100	200	300	400	500
Plasma (ml)	1	2	3	4	5
ACL (ml)	0.8	1.6	2.4	3.2	4

2. Close the cap and mix by pulse-vortexing for 5 x 2 seconds.

   Make sure that a visible vortex forms in the tube. In order to ensure efficient lysis, it is essential that the sample and Buffer ACL are mixed thoroughly to yield a homogeneous solution.

   Note: Do not interrupt the procedure at this time. Proceed immediately to step 3 to start the lysis incubation.

3. Incubate at 56°C (±1°C) for 15 (±1) minutes.
4. Place the tube back on the lab bench and unscrew the cap.
5. Add Buffer ACB to the lysate in the tube. Choose the volume according to setup from step 1.

Setup	**A**	**B**	**C**	**D**	**E**
ACB (ml)	1.8	3.6	5.4	7.2	9

6. Close the cap and mix thoroughly by pulse-vortexing for 5 x 2 seconds.

   Make sure that a visible vortex forms in the tube. In order to ensure efficient lysis, it is essential that the lysate and Buffer ACB are mixed thoroughly to yield a homogeneous solution.

7. Incubate the lysate–Buffer ACB mixture in the tube for 5 (±1) minutes at room temperature.
8. Insert the QIAamp Mini column into the VacConnector on the QIAvac 24 Plus (see "Setup of the QIAvac 24 Plus vacuum manifold", page 19). Insert a 20 ml column extender into the open QIAamp Mini column.

   Make sure that the column extender is firmly inserted into the QIAamp Mini column in order to avoid sample leakage.

   Note: Keep the wash tube for the dry spin in step 13.
9. Carefully apply the lysate from step 7 into the column extender of the QIAamp Mini column. Switch on the vacuum pump. When all lysates have been drawn completely through the columns, switch off the vacuum pump and release the pressure to 0 mbar. Carefully remove and discard the column extender.

   Please note that large sample lysate volumes (about 18 ml when starting with a 5 ml sample) may need up to 20 minutes to pass through the QIAamp Mini membrane by vacuum force.

   For fast and convenient release of the vacuum pressure, the Vacuum Regulator should be used (part of the QIAvac Connecting System).

   Note: To avoid cross-contamination, be careful not to cross-neighbor QIAamp Mini columns while column extenders are removed.
10. Apply 600 µl Buffer ACW1 to the QIAamp Mini column. Leave the lid of the column open and switch on the vacuum pump. After all the Buffer ACW1 has been drawn through the QIAamp Mini column, switch off the vacuum pump and release the pressure to 0 mbar.
11. Apply 750 µl Buffer ACW2 to the QIAamp Mini column. Leave the lid of the column open, and switch on the vacuum pump. After all the Buffer ACW2 has been drawn through the QIAamp Mini column, switch off the vacuum pump and release the pressure to 0 mbar.

12. Apply 750 µl of ethanol (96–100%) to the QIAamp Mini column. Leave the lid of the column open and switch on the vacuum pump. After all the ethanol has been drawn through the spin column, switch off the vacuum pump and release the pressure to 0 mbar.
13. Close the lid of the QIAamp Mini column. Remove it from the vacuum manifold, and discard the VacConnector. Place the QIAamp Mini column in a clean 2 ml wash tube (from step 8), and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 (±0.5) minutes.
14. Place the QIAamp Mini column into a new 2 ml wash tube. Open the lid and incubate the assembly at room-temperature for 3 minutes to dry the membrane completely.
15. Place the QIAamp Mini column in a clean 1.5 ml elution tube (provided) and discard the 2 ml wash tube from step 14. Carefully apply 20–150 µl of Buffer AVE to the center of the QIAamp Mini column membrane. Close the lid and incubate at room temperature for 3 (±0.5) minutes.

    Important: Ensure that the elution Buffer AVE is equilibrated to room temperature (15–25°C). If elution is done in small volumes (<50 µl), the elution buffer has to be dispensed on the center of the membrane for complete elution of bound nucleic acids.

    Elution volume is flexible and can be adapted according to the requirements of downstream applications.

    Elution with smaller volumes of Buffer AVE leads to higher nucleic acid concentrations, but may result in lower total yield.

    The recovered eluate volume can be up to 5 µl less than the elution volume applied to membrane of the QIAamp Mini column.

    Note: For expected low NA yields, using a Low-bind tube is recommended for elution (not supplied).
16. Centrifuge in a microcentrifuge at full speed (20,000 x *g*; 14,0000 rpm) for 1 minute to elute the nucleic acids.

Classic Protocol: Purification of Circulating Nucleic Acids From 1–5 ml Human Blood Plasma

This protocol constitutes the unchanged protocol of the QIAamp DSP circulating NA Kit Handbook Revision 3 (R3) for use with e.g. existing user-validated workflows for 1-5 ml human plasma.

Important points before starting

- All centrifugation steps are carried out at room temperature (15–25°C).
- Switch off vacuum between steps to ensure that a consistent, even vacuum is applied during the protocol steps.

  Note: Vacuum Pump pressure should be between –800 to –900 mbar.
- Equilibrate samples to room temperature.
- Use phosphate-buffered saline to bring the volume of the sample to the nearest exact volume (1 ml to 5 ml).
- Set up the QIAvac 24 Plus as described on page 19.
- Heat a water bath or heating block to 60°C for use with 50 ml centrifuge tubes in step 3.
- Heat a heating block to 56°C for use with 2 ml wash tubes in step 14.
- Equilibrate the QIAamp Mini spin columns at least 1 hour to room temperature before use.
- Ensure that Buffer ACB, Buffer ACW1, and Buffer ACW2 have been prepared (addition of isopropanol or ethanol) according to the instructions on page 24.
- Add carrier RNA reconstituted in Buffer AVE to Buffer ACL according to instructions in Table 3.

Table 3. Volume of Buffer ACL and carrier RNA (dissolved in Buffer AVE) required for processing 1–5 ml human blood plasma samples

Setup for ml plasma	A	B	C	D	E	Carrier RNA in Buffer AVE (µl)
	1 ml	2 ml	3 ml	4 ml	5 ml	
Number of samples	Buffer ACL (ml)					
1	0.9	1.8	2.6	3.5	4.4	5.6
2	1.8	3.5	5.3	7.0	8.8	11.3
3	2.6	5.3	7.9	10.6	13.2	16.9
4	3.5	7.0	10.6	14.1	17.6	22.5
5	4.4	8.8	13.2	17.6	22.0	28.1
6	5.3	10.6	15.8	21.1	26.4	33.8
7	6.2	12.3	18.5	24.6	30.8	39.4
8	7.0	14.1	21.1	28.2	35.2	45.0
9	7.9	15.8	23.8	31.7	39.6	50.6
10	8.8	17.6	26.4	35.2	44.0	56.3
11	9.7	19.4	29.0	38.7	48.4	61.9
12	10.6	21.1	31.7	42.2	52.8	67.5
13	11.4	22.9	34.3	45.8	57.2	73.1
14	12.3	24.6	37.0	49.3	61.6	78.8
15	13.2	26.4	39.6	52.8	66.0	84.4
16	14.1	28.2	42.2	56.3	70.4	90.0
17	15.0	29.9	44.9	59.8	74.8	95.6
18	15.8	31.7	47.5	63.4	79.2	101.3
19	16.7	33.4	50.2	66.9	83.6	106.9
20	17.6	35.2	52.8	70.4	88.0	112.5
21	18.5	37.0	55.4	73.9	92.4	118.1
22	19.4	38.7	58.1	77.4	96.8	123.8
23	20.2	40.5	60.7	81.0	101.2	129.4
24	21.1	42.2	63.4	84.5	105.6	135.0

Procedure: Classic Protocol

1. Pipet QIAGEN Proteinase K, plasma, and Buffer ACL in this order into a 50 ml centrifuge tube (not provided).

Setup	A	B	C	D	E
ProtK (µl)	100	200	300	400	500
Plasma (ml)	1	2	3	4	5
ACL (ml)	0.8	1.6	2.4	3.2	4

2. Close the cap and mix by pulse-vortexing for 30 seconds.

   Make sure that a visible vortex forms in the tube. In order to ensure efficient lysis, it is essential that the sample and Buffer ACL are mixed thoroughly to yield a homogeneous solution.

   Note: Do not interrupt the procedure at this time. Proceed immediately to step 3 to start the lysis incubation.

3. Incubate at 60°C (±1°C) for 30 (±2) minutes.
4. Place the tube back on the lab bench and unscrew the cap.
5. Add Buffer ACB to the lysate in the tube. Choose the volume according to setup from step 1.

Setup	A	B	C	D	E
ACB (ml)	1.8	3.6	5.4	7.2	9

6. Close the cap and mix thoroughly by pulse-vortexing for 30 seconds.

   Make sure that a visible vortex forms in the tube. In order to ensure efficient lysis, it is essential that the lysate and Buffer ACB are mixed thoroughly to yield a homogeneous solution.

7. Incubate the lysate–Buffer ACB mixture in the tube for 5 (±1) minutes on ice.
8. Insert the QIAamp Mini column into the VacConnector on the QIAvac 24 Plus (see "Setup of the QIAvac 24 Plus vacuum manifold", page 19). Insert a 20 ml column extender into the open QIAamp Mini column.

   Make sure that the column extender is firmly inserted into the QIAamp Mini column in order to avoid sample leakage.

   Note: Keep the wash tube for the dry spin in step 13.
9. Carefully apply the lysate from step 7 into the column extender of the QIAamp Mini column. Switch on the vacuum pump applying a pressure of –800 to –900 mbar. When all lysates have been drawn completely through the columns, switch off the vacuum pump and release the pressure to 0 mbar. Carefully remove and discard the column extender.

   Please note that large sample lysate volumes (about 18 ml when starting with a 5 ml sample) may need up to 20 minutes to pass through the QIAamp Mini membrane by vacuum force.

   For fast and convenient release of the vacuum pressure, the Vacuum Regulator should be used (part of the QIAvac Connecting System).

   Note: To avoid cross-contamination, be careful not to cross-neighbor QIAamp Mini columns while column extenders are removed.
10. Apply 600 µl Buffer ACW1 to the QIAamp Mini column. Leave the lid of the column open, and switch on the vacuum pump. After all the Buffer ACW1 has been drawn through the QIAamp Mini column, switch off the vacuum pump and release the pressure to 0 mbar.
11. Apply 750 µl Buffer ACW2 to the QIAamp Mini column. Leave the lid of the column open, and switch on the vacuum pump. After all the Buffer ACW2 has been drawn through the QIAamp Mini column, switch off the vacuum pump and release the pressure to 0 mbar.

12. Apply 750 µl of ethanol (96–100%) to the QIAamp Mini column. Leave the lid of the column open, and switch on the vacuum pump. After all the ethanol has been drawn through the spin column, switch off the vacuum pump and release the pressure to 0 mbar.
13. Close the lid of the QIAamp Mini column. Remove it from the vacuum manifold and discard the VacConnector. Place the QIAamp Mini column in a clean 2 ml wash tube (from step 8), and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 (±0.5) minutes.
14. Place the QIAamp Mini column into a new 2 ml wash tube. Open the lid and incubate the assembly at 56°C (±1°C) for 10 (±1) minutes to dry the membrane completely.
15. Place the QIAamp Mini column in a clean 1.5 ml elution tube (provided) and discard the 2 ml wash tube from step 13. Carefully apply 20–150 µl of Buffer AVE to the center of the QIAamp Mini column membrane. Close the lid and incubate at room temperature for 3 (±0.5) minutes.

    Important: Ensure that the elution Buffer AVE is equilibrated to room temperature (15–25°C). If elution is done in small volumes (<50 µl), the elution buffer has to be dispensed on the center of the membrane for complete elution of bound nucleic acids.

    Elution volume is flexible and can be adapted according to the requirements of downstream applications.

    Elution with smaller volumes of Buffer AVE leads to higher nucleic acid concentrations, but may result in lower total yield.

    The recovered eluate volume can be up to 5 µl less than the elution volume applied to the QIAamp Mini column.

    Note: For expected low NA yields, using a Low-bind tube is recommended for elution (not supplied).
16. Centrifuge in a microcentrifuge at full speed (20,000 x *g*; 14,0000 rpm) for 1 minute to elute the nucleic acids.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of QIAamp DSP Circulating NA Kits is tested against predetermined specifications to ensure consistent product quality.

Limitations

The system performance for isolation of circulating, cell-free nucleic acids has been established using human plasma samples generated from the following Blood Collection Tubes:

- K2-EDTA (Beckton Dickinson, Cat. No. 367525)
- PAXgene Blood ccfDNA Tube (PreAnlaytiX, Cat. No. 768115)
- Cell-Free DNA BCT (Streck, Cat. No. 218962)

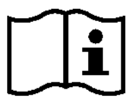
It is the user's responsibility to validate system performance for any procedures used in their laboratory, which are not covered by the QIAGEN performance studies.

To minimize the risk of a negative impact on the diagnostic results, adequate controls for downstream applications should be used. For further validation, the guidelines of the International Conference on Harmonization of Technical Requirements (ICH) in ICH Q2 (R1) Validation of Analytical Procedures: Test And Methodology are recommended.

Any diagnostic results that are generated must be interpreted in conjunction with other clinical or laboratory findings.

Symbols

Symbol	Symbol definition
Σ <N>	Contains reagents sufficient for <N> tests
	Use by
IVD	In vitro diagnostic medical device
	Upon arrival
	Open on delivery; store QIAamp Mini Spin columns at 2–8°C
REF	Catalog number
NUM	Number
LOT	Lot number
MAT	Material number
COMP	Components
VOL	Volume
ADD	Adding
	Temperature limitation

Symbol	Definition
	Manufacturer
	Consult instructions for use
	Write down current date after adding ethanol to the bottle
EtOH	Ethanol
	Write down current date after adding isopropanol to the bottle
IPA	Isopropanol
CONT	Contains
	Leads to
GITC	Guanidine thiocyanate
GuHCl	Guanidine hydrochloride
BRIJ 58	BRIJ 58
GTIN	Global Trade Item Number

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2. Hahn, S. and Zimmermann, B.G. (2010) Cell-free DNA in maternal plasma: has the size-distribution puzzle been solved? Clin Chem. **56**, 1210-1211.

3. Anfossi, S., Babayan, A., Pantel, K., and Calin, G. (2018) Clinical utility of circulating non-coding RNAs - an update. Nat Rev Clin Oncol **15**, 541-563.

4. Babayan, A. and Pantel, K. (2018) Advances in liquid biopsy approaches for early detection and monitoring of cancer. Genome Med **10**, 21.

5. Terrinoni, A.,et al (2019) The circulating miRNAs as diagnostic and prognostic markers. Clin Chem Lab Med. **57**, 932-953.

Contact Information

For technical assistance and more information, please see our Technical Support Center at **support.qiagen.com** or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit **www.qiagen.com**).

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For contact information, see back cover or visit **www.qiagen.com**.

		Comments and suggestions
Little or no nucleic acid in the eluate		
a)	Usage of non-stabilized plasma	Non-stabilized plasma samples may lead to accelerated DNA degradation. We recommend to follow CEN/TS 16835-3:2015. Repeat the purification procedure with new samples.
b)	Extended time between blood draw and plasma preparation	Nucleated blood cells may disintegrate and release genomic DNA into the plasma, diluting the target nucleic acid.
c)	Samples frozen and thawed more than once	Repeated freezing and thawing should be avoided as this may lead to DNA degradation. Always use fresh samples or samples thawed only once.
d)	Low concentration of target DNA in the samples	Plasma samples were left standing at room temperature for too long. Repeat the purification procedure with new samples **Note**: some individuals may have a low cell-free NA concentration in plasma; here, an increased sample volume and a low eluate volume should be chosen.
e)	Inefficient sample lysis in Buffer ACL	If QIAGEN Proteinase K was subjected to elevated temperature for a prolonged time, it can lose activity. Repeat the procedure using new samples and fresh QIAGEN Proteinase K.
f)	Buffer ACL–carrier RNA mixture not sufficiently mixed	Mix Buffer ACL with carrier RNA by gently inverting the tube of Buffer ACL–carrier RNA at least 10 times.
g)	Low-percentage ethanol used instead of 96–100%	Repeat the purification procedure with new samples and 96–100% ethanol. Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone.
h)	Buffer ACB prepared incorrectly	Check that Buffer ACB concentrate was reconstituted with the correct volume of isopropanol (not ethanol, see page 24).
i)	Buffer ACW1 or Buffer ACW2 prepared incorrectly	Check that Buffer ACW1 and Buffer ACW2 concentrates were diluted with the correct volume of ethanol (see page 24). Repeat the purification procedure with new samples.
j)	Buffer ACW1 or Buffer ACW2 prepared with 70% ethanol	Check that Buffer ACW1 and Buffer ACW2 concentrates were diluted with 96–100% ethanol (see page 24). Repeat the purification procedure with new samples.
DNA or RNA do not perform well in downstream enzymatic reactions		
a)	Little or no DNA in the eluate	See "Little or no nucleic acid in the eluate" above for possible reasons. Increase the amount of eluate added to the reaction if possible.

		Comments and suggestions
b)	Inappropriate elution volume used	Determine the maximum volume of eluate suitable for your downstream application. Reduce or increase the volume of eluate added to the downstream application accordingly. The elution volume can be adapted proportionally. **Note**: Elution with smaller volumes of Buffer AVE leads to higher nucleic acid concentrations but may result in a lower total yield.
c)	Buffers not mixed thoroughly	Salt and ethanol components of wash Buffer ACW2 may have separated out after being left standing for a long period between runs. Always mix buffers thoroughly before each run.
d)	Interference due to carrier RNA	If the presence of carrier RNA in the eluate interferes with the downstream enzymatic reaction, it may be necessary to reduce the amount of carrier RNA or to omit it altogether.
General handling		
a)	Clogged QIAamp Mini column	If the flow rate is reduced, vacuum time can be extended. Alternatively, close the VacValve, if used, and carefully remove the column extender–VacConnector–VacValve assembly from the QIAamp Mini column without losing any of the lysate in the column extender. Remove the QIAamp Mini column from the vacuum manifold, place it in a 2 ml wash tube and spin it at full speed until sample has completely passed through the membrane. Replace the column extender–VacConnector–VacValve assembly containing the remaining lysate. Switch on the vacuum pump, open the VacValve, and continue to load the remaining lysate. Repeat the above procedure if the QIAamp Mini Column continues to clog. Cryoprecipitates may have formed in plasma due to repeated freezing and thawing. These can block the QIAamp Mini column. Do not use plasma that has been frozen and thawed more than once. In case cryoprecipitates are visible, clear the sample by centrifugation for 5 minutes at 16,000 x *g*.
b)	Variable elution volumes	Different samples can affect the volume of the final eluate. The recovered eluate volume can be up to 5 µl less than the elution volume applied to the QIAamp Mini column.
c)	Vacuum pressure of –800 to –900 mbar not reached	The vacuum manifold is not tightly closed. Press down on the lid of the vacuum manifold after the vacuum is switched on. Check if vacuum pressure is reached. Gasket of QIAvac lid has worn out. Check the seal of the manifold visually and replace it if necessary. VacValves have worn out. Remove all VacValves and insert VacConnectors directly into the luer extensions. Insert QIAamp Mini columns into VacConnectors, close the lid of the columns, and switch on vacuum. Check if vacuum pressure is reached. Replace VacValves if necessary. Connection to vacuum pump is leaky. Close all luer extension with luer caps and switch on the vacuum pump. Check if vacuum pressure is stable after the pump is switched on (and the Vacuum Regulator valve is closed). Exchange the connections between pump and vacuum manifold if necessary. If the vacuum pressure is still not reached, replace the vacuum pump with a stronger one.

Appendix A: Recommendation for Blood Plasma Separation and Storage

For stabilization blood collection tubes (e.g. PAXgene ccfDNA tube or Streck Cell-Free DNA tube) please follow the manufacturer instructions for plasma separation and storage. We recommend validating these storage conditions in combination with your specific downstream application and target.

For non-stabilized BCT, we recommend to follow CEN/TS 16835-3:2015.

In order to isolate circulating, cell-free nucleic acids from blood samples, we recommend following this protocol, which includes a high g-force centrifugation step in order to remove cellular debris, thereby reducing the amount of cellular or genomic DNA and RNA in the sample.

1. Place whole EDTA Blood in BD Vacutainer® tubes (or other primary blood tubes containing EDTA as an anti-coagulant) in a centrifuge cooled down to 4°C with swing-out rotor and appropriate buckets.
2. Centrifuge blood samples for 10 minutes at 1900 x *g* (3000 rpm) at 4°C.
3. Carefully aspirate plasma supernatant without disturbing the plasma-cellular interface layer. About 4–5 ml plasma can be obtained from one 10 ml primary blood tube.

   Note: Plasma can be used for circulating nucleic acid extraction at this stage. However, the following high-speed centrifugation will remove additional cellular debris and contamination of the circulating nucleic acids by genomic DNA and RNA derived from damaged nucleated blood cells.
4. Aspirated plasma is transferred into a fresh centrifuge tube.
5. Centrifuge plasma samples for 10 minutes at 16,000 x *g* (in fixed-angle rotor) at 4°C.

   This will remove additional cellular nucleic acids attached to cell debris.

6. Carefully remove supernatant and transfer to a new tube without disturbing the pellet.
7. If plasma will be used for nucleic acid extraction on the same day, store at 2–8°C until further processing. For longer storage, plasma aliquots from stabilized as well as non-stabilized blood collection tubes can be stored at –20°C (DNA as target) or –80°C (RNA as target) for at least 4 weeks. Before using the plasma for circulating nucleic acid extraction, thaw plasma tubes at room temperature.
8. **Optional**: In order to remove cryoprecipitates, centrifuge plasma samples for 5 minutes at 16,000 x *g* (in fixed angle rotor).

   Optional: Transfer supernatant to a new tube and then begin with the circulating nucleic acid extraction 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 destroy RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Great care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the purification procedure. In order to create and maintain an RNase-free environment, the following precautions must be taken during pretreatment and use of disposable and non-disposable 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.

Disposable plasticware

The use of sterile, RNase-free, disposable polypropylene tubes is recommended throughout the procedure.

Ordering Information

Product	Contents	Cat. no.
QIAamp DSP Circulating NA Kit (50)	For 50 preps: QIAamp Mini columns, Column Extenders, VacConnectors, QIAGEN Proteinase K, Reagents, Buffers, and collection tubes	61504
Accessories		
QIAvac 24 Plus vacuum manifold*	Vacuum manifold for processing 1–24 spin columns: QIAvac 24 Plus Vacuum manifold, Luer Plugs, and Quick Couplings	19413
Vacuum Pump*	Universal vacuum pump	84010 [USA and Canada] 84000 [Japan] 84020 [rest of world]
QIAvac Connecting System*	System to connect vacuum manifold with vacuum pump: includes Tray, Waste Bottles, Tubing, Couplings, Valve, Gauge, and 24 VacValves	19419

* For use with vacuum protocols.

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.

Handbook Revision History

Date	Changes
R4 09/2019	Change of Intended Use to cell-free nucleic acids from human plasma only. Inclusion of "Breeze" protocol. No protocols for Urine and miRNA included. Update of safety information.

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