

Supplementary Protocol for User Self-Validation

QIAamp[®] DSP Circulating NA Kit Protocol for Purification of Circulating miRNA From 1 to 3 ml of Human Serum, Plasma, or Urine

This protocol describes how to isolate circulating miRNA (including total circulating RNA) from 1 to 3 ml of human serum, plasma, or urine using the QIAamp DSP Circulating NA Kit. Please note that this protocol requires Buffer ATL (cat. no. 939016), which needs to be purchased separately.

Note: It is the user's responsibility to validate performance using this combination for any procedures used in their laboratory according to local requirements, laws, and regulations.

Equipment and reagents to be supplied by user

- Buffer ATL as additional lysis buffer, cat. no. 939016 (up to 1 ml per sample)
- Pipettes (adjustable)
- Sterile pipette tips (pipette tips with aerosol barriers are recommended to help prevent cross-contamination)
- 1.5 ml or 2 ml nuclease-free microtube
- Water bath or heating block capable of holding 50 ml centrifuge tubes at 60°C*
- Heating block or similar at 56°C capable of holding 2 ml wash tubes*
- Ice
- 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
- Vortexer
- Ethanol (96–100%)[†]
- Isopropanol (100%)[†]
- 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 manufacturer's recommendations.

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

Warnings and precautions

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. Take off contaminated clothing and wash before reuse. Dispose of contents/container to an approved waste disposal plant.

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. Dispose of contents/container to an approved waste disposal plant.

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.

Note: Use of carrier RNA is not recommended with this miRNA protocol.

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 hours 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, serum, and urine storage and handling

It is recommended to perform the sample preparation and nucleic acid isolation immediately after sample collection, especially for RNA. For short-term storage the samples should be stored at 2–8°C. It is the user's responsibility to evaluate the storage conditions for the procedures used in their laboratory.

Storing eluted nucleic acids

Eluted nucleic acids are collected in 1.5 ml elution tubes (provided). Storage at –30°C to –15°C is recommended for DNA and –90°C to –60°C for RNA downstream applications. It is the user's responsibility to evaluate the storage conditions for the procedures used in their laboratory.

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 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 1).

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 mbar or 760 mm Hg) and can be measured using the QIAvac Connecting System (see Figure 1). The protocols require a vacuum pump capable of producing a vacuum of -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.

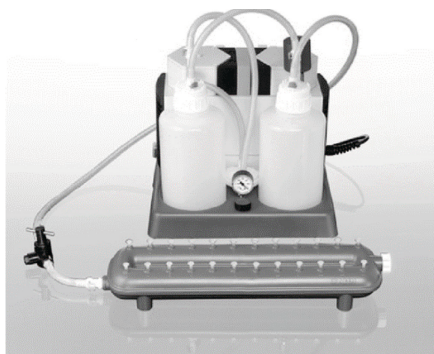


Figure 1. QIAvac 24 Plus, QIAvac Connecting System, and Vacuum Pump

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 2). 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 2).
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 2).
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 2).
Note: Make sure that the column extender is firmly inserted into the QIAamp Mini column to avoid leakage of sample.
6. For nucleic acid purification, follow the instructions on page 10. 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 2 for Warnings and precautions.

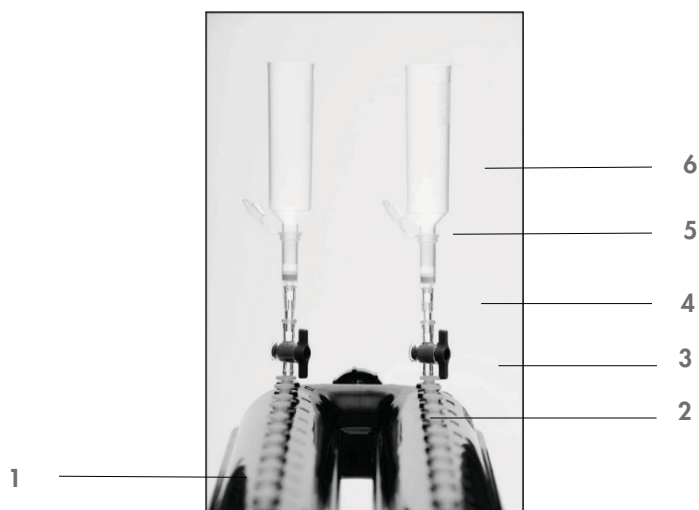
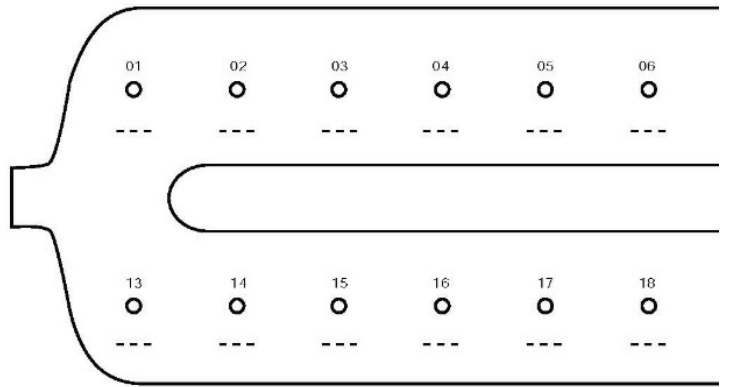


Figure 2. Setting up the QIAvac 24 Plus with QIAamp Mini columns using VacValves, VacConnectors, and Column Extenders.

- | | | | |
|----------|---|----------|--------------------|
| 1 | QIAvac 24 Plus vacuum manifold | 4 | VacConnector |
| 2 | Luer slot of the QIAvac 24 Plus (closed with luer plug) | 5 | QIAamp Mini column |
| 3 | VacValve* | 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 3 to avoid the mix-up of samples. This figure can be photocopied and labeled with the names of the samples.

* Must be purchased separately.



Date: _____

Operator: _____

Run ID: _____

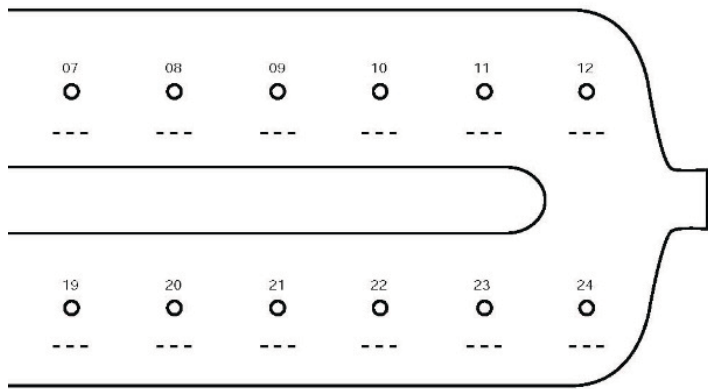


Figure 3. Labeling scheme for tubes and QIAamp Mini columns for use on the QIAvac 24 Plus vacuum system

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.

Important notes

- To obtain cell-free nucleic acids from urine, it is recommended to centrifuge the sample at 1900 x *g* for 10 (±1) minutes and only use the supernatant for nucleic acid extraction. This will remove cellular materials from the sample.
- 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 to 3 ml).
- Set up the QIAvac 24 Plus as described on page 5.
- 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.
- Equilibrate Buffer AVE to room temperature for elution in step 15.
- Ensure that Buffer ACB, Buffer ACW1, and Buffer ACW2 have been prepared (addition of isopropanol or ethanol) according to the instructions on page 9.

* Contains chaotropic salt. See page 1 **Error! Bookmark not defined.** for **Warnings and precautions**.

† Contains sodium azide as a preservative.

Procedure

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

| Setup | A | B | C |
|------------------------------|------|------|-----|
| ProtK (µl) | 130 | 270 | 400 |
| Urine, plasma, or Serum (ml) | 1 | 2 | 3 |
| ACL (ml) * | 1.1 | 2.1 | 3.2 |
| ATL (ml) | 0.33 | 0.67 | 1.0 |

* Without carrier RNA.

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

Make sure that a visible vortex forms in the tube. To ensure efficient lysis, it is essential that Proteinase K, the sample, Buffer ACL, and Buffer ATL 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) min.
4. Place the tube back on the lab bench and unscrew the cap.
5. Add Buffer ACB and isopropanol to the lysate in the tube. Choose the volume according to setup from step 1.

| Setup | A | B | C |
|------------------|-----|-----|-----|
| ACB (ml) | 3.0 | 6.0 | 9.0 |
| Isopropanol (ml) | 2.3 | 4.7 | 7.0 |

6. Close the cap and mix thoroughly by pulse-vortexing for 15 to 30 s.

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

7. Incubate the mixture in the tube for 5 (±1) min 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” on page 5). 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 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 (approx. 15 ml when starting with a 3 ml sample) may need up to 20 min 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, see Equipment and reagents to be supplied by user, page 1).

Note: To avoid cross-contamination, be careful not to cross-neighbor QIAamp Mini columns while column extenders are removed.

10. Apply 600 μ l 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 μ l 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 μ l 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 \times *g*; 14,000 rpm) for 3 (\pm 0.5) min.
14. Place the QIAamp Mini column into a new 2 ml wash tube. Open the lid and incubate the assembly at 56°C (\pm 1°C) for 10 (\pm 1) min to dry the membrane completely. If using the same heating block is used in step 3, reduce the temperature by 4°C.
15. Place the QIAamp Mini column in a clean 1.5 ml elution tube (provided) and discard the wash tube from step 14. Carefully apply 20–150 μ l of Buffer AVE to the center of the QIAamp Mini column membrane. Close the lid and incubate at room temperature for 3 (\pm 0.5) min.

Important: Ensure that the elution Buffer AVE is equilibrated to room temperature (15–25°C). If elution is done in small volumes (<50 μ l), 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 μ l 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 \times *g*; 14,000 rpm) for 1 min to elute the nucleic acids.

Note: Orient the elution tube 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).

Document Revision History

| Date | Changes |
|---------|--|
| 10/2019 | Initial release |
| 12/2019 | Added Buffer ATL to the "Equipment and reagents to be supplied by user". |
| 06/2022 | Precised the initial note that Protocol/Kit Combination is not covered by performance studies. |

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