June 2022

Supplementary Protocol for User Self-Validation

QIAamp® DSP Circulating NA Kit Protocol for Purification of Circulating Nucleic Acids From 1 to 5 ml of Human Urine

This protocol describes how to isolate circulating DNA and RNA from 1 to 5 ml of human 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 56°C*
- 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)

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.

* 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.

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.

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 13. This solution should be prepared fresh. 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

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 11. 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 1 for Warnings and precautions.

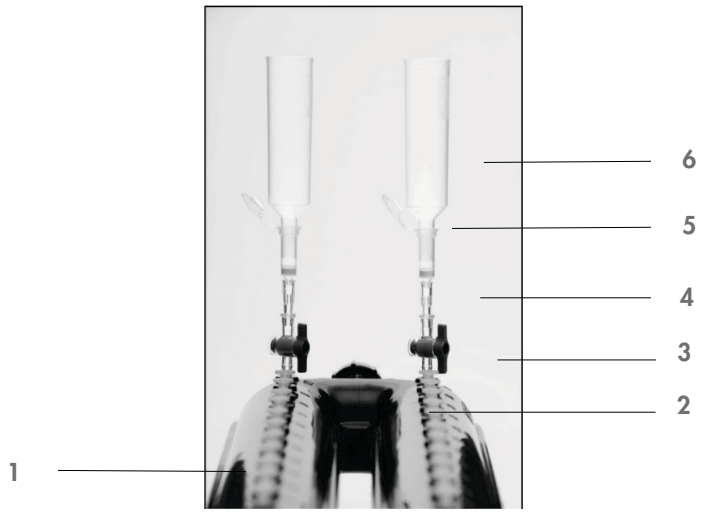
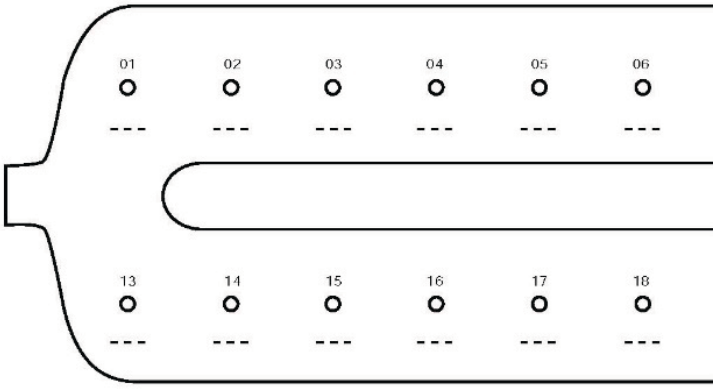


Figure 2. Setting up the QIAvac 24 Plus with QIAamp Mini columns using VacValves, VacConnectors, and Column Extenders.

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.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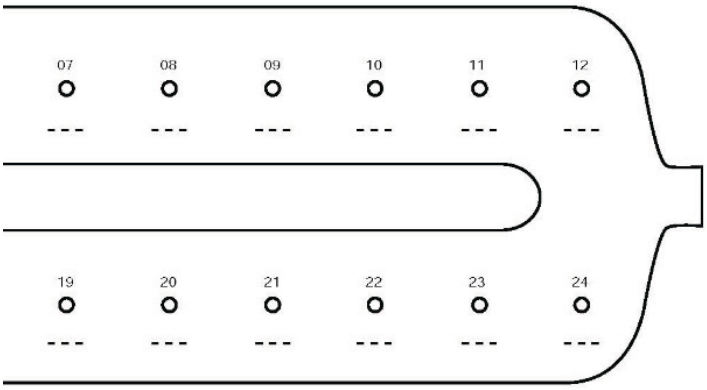

Figure 3. Labeling scheme for tubes and QIAamp Mini columns for use on the QIAvac 24 Plus vacuum system

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.

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 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°C to –15°C. Do not freeze–thaw the aliquots of carrier RNA repeatedly.

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

* Contains chaotropic salt. See page 1**Fehler! Textmarke nicht definiert.** for Warnings and precautions.
† Contains sodium azide as a preservative.

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 (Table 2).

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

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.)

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).
- Equilibrate samples to room temperature.
- Use phosphate-buffered saline to bring the volume of the sample to the nearest exact volume (1 to 5 ml).
- 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.
- Add carrier RNA reconstituted in Buffer AVE to Buffer ACL according to instructions in Table 2.
- Set up the QIAvac 24 Plus as described on page 3.
- 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.
- Heat a water bath or heating block to 56°C for use with 50 ml centrifuge tubes in step 3.

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

Setup for ml plasma	A	B	C	D	E	
	1 ml	2 ml	3 ml	4 ml	5 ml	
Number of samples	Buffer ACL					Carrier RNA in Buffer AVE (µl)
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

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	D	E
ProtK (µl)	100	200	300	400	500
Urine (ml)	1	2	3	4	5
ACL (ml)*	0.8	1.6	2.4	3.2	4
ATL (ml)	0.25	0.5	0.75	1	1.25

* Without carrier RNA.

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

Make sure that a visible vortex forms in the tube. 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) 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	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 s.

   Make sure that a visible vortex forms in the tube. 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) min 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” 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 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 (approx. 20 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, 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 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 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) min.
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 min to dry the membrane completely.

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 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) min.

    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 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	Specified the initial note that Protocol/Kit Combination is not covered by performance studies.

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.