September 2024

Supplementary Protocol

Protocol: Purification of cell-free DNA from 10 mL Urine from the PAXgene® Urine Liquid Biopsy Tube using the QIAamp® Circulating Nucleic Acid Kit

This protocol is for purification of cell-free DNA from 10 mL of human urine stabilized in the PAXgene Urine Liquid Biopsy Tube. It is a supplementary protocol related to the handbook of the QIAamp Circulating Nucleic Acid Kit (cat. no. 55114).

Important: Urine sample must be collected and stabilized using the PAXgene Urine Liquid Biopsy Set. For specimen collection and stabilization, read the *PAXgene Urine Liquid Biopsy Set (20) Handbook*.

For purification of cfDNA from the PAXgene Urine Liquid Biopsy Tube, read the *QIAamp Circulating Nucleic Acid Kit Handbook*, paying careful attention to the "Safety Information" and "Important Notes" sections before following this supplementary protocol.

For Research Use Only. This protocol is not intended for the diagnosis, prevention, or treatment of a disease.

Further information

- *QIAamp Circulating Nucleic Acid Kit Handbook*: **www.qiagen.com/HB-0202**
- *PAXgene Urine Liquid Biopsy Set Handbook:* **www.qiagen.com/HB-3553**
- Safety Data Sheets: **www.qiagen.com/safety**
- BD Regulatory Documents: **regdocs.bd.com/regdocs/sdsSearch**
- Technical assistance: **support.qiagen.com**

Equipment and reagents to be supplied by the user

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

- Pipettes (adjustable)
- Sterile pipette tips (pipette tips with aerosol barriers are recommended to help prevent cross-contamination)
- Water bath capable of holding 50 mL centrifuge tubes at 60°C. Heating block or similar at 56°C (capable of holding 2 mL collection tubes).
- Microcentrifuge
- 50 mL centrifuge tubes
- QIAvac 24 Plus vacuum manifold (cat. no. 19413)
- QIAvac Connecting System (cat. no. 19419) or equivalent
- Vacuum Pump (cat. nos. 84010 [USA and Canada], 84000 [Japan], or 84020 [rest of the world]) or equivalent pump capable of producing a vacuum of –800 mbar to –900 mbar
- Ethanol (96–100%)*
- Isopropanol (100%)
- Crushed ice

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

Important points before starting

- The content of the kit is sufficient for 25 preparations when using this Supplementary Protocol rather than the 50 preparations specified in the *QIAamp Circulating Nucleic Acid Kit Handbook*. For 50 preparations starting from 10 mL stabilized urine, 220 mL of Buffer ACL (1x, cat. no. 939017), 300 mL of Buffer ACB (1x, cat. no. 1069275), and 30 mL of QIAGEN Proteinase K (3x, containing 10 mL each, cat. no. 19133) must be purchased separately.
- Ensure that the kit boxes are intact and undamaged and that the buffers have not leaked. Do not use a kit that has been damaged.
- When using a pipette, ensure that it is set to the correct volume and that the liquid is carefully and completely aspirated and dispensed.
- To obtain cell-free DNA (cfDNA) from urine, it is recommended to centrifuge urine samples to separate cells from the urine supernatant intended for cfDNA isolation. Please follow the instructions for urine centrifugation given in the protocol below.
- All centrifugation steps in the protocol are carried out at room temperature (15–25°C).
- Switch off vacuum between steps to ensure that a consistent, even vacuum is applied during protocol steps.

Things to do before starting

- Equilibrate samples to room temperature (15–25°C).
- Set up the QIAvac 24 Plus as described in the *QIAamp Circulating Nucleic Acid Handbook* (cat. no. 55114).
- Heat a water bath to 60°C for use with 50 mL centrifuge tubes in step 8.
- Heat a heating block to 56°C for use with 2 mL collection tubes in step 20.
- Equilibrate Buffer AVE to room temperature (15–25°C) for elution in step 21.
- Ensure that Buffer ACB, Buffer ACW1 and Buffer ACW2 have been prepared according to the instructions in "Important Notes" (cat. no. 55114; *QIAamp Circulating Nucleic Acid Handbook*)
- Ensure that the lyophilized carrier RNA is solved in Buffer AVE according to the instructions in "Important Notes" (cat. no. 55114; *QIAamp Circulating Nucleic Acid Handbook*).
- Add carrier RNA reconstituted in Buffer AVE to Buffer ACL according to instructions in Table 1 (differs from QIAamp Circulating Nucleic Acid handbook Handbook).

Table 1. Volumes of Buffer ACL and carrier RNA (dissolved in Buffer AVE) required for processing 10 mL urine samples

Number of samples	Buffer ACL (mL)	Carrier RNA in Buffer AVE (µL)
1	8.8	5.6
2	17.6	11.3
3	26.4	16.9
4	35.2	22.5
5	44.0	28.1
6	52.8	33.8
7	61.6	39.4
8	70.4	45.0
9	79.2	50.6
10	88.0	56.3
11	96.8	61.9
12	105.6	67.5
13	114.4	73.1
14	123.2	78.8
15	132.0	84.4
16	140.8	90.0
17	149.6	95.6
18	158.4	101.3
19	167.2	106.9
20	176.0	112.5
21	184.8	118.1
22	193.8	123.8
23	202.4	129.4
24	211.2	135.0

Procedure

1. Centrifuge the PAXgene Urine Liquid Biopsy Tube at room temperature (15–25°C) for 15 min at 1900 × *g* using a balanced swing-out bucket centrifuge. If braking is preferred, it is recommended to use medium level braking, but should be validated for your specific workflow.
2. Decant the supernatant into a 15 mL conical bottom centrifugation tube, making sure to not disturb the pellet (cellular fraction).
3. Centrifuge the 15 mL conical bottom centrifugation tube at room temperature (15–25°C) for 10 min at 1900 × *g* using a balanced swing-out bucket centrifuge. Transfer the urine supernatant into a new tube . For storage of urine supernatant please refer to the handbook of the PAXgene Urine Liquid Biopsy Set (RUO). For manual cfDNA isolation please follow this protocol.
4. Pipette 1.0 mL QIAGEN Proteinase K into a 50 mL tube (not provided).
5. Add 10 mL of urine supernatant from step 3 into the 50 mL tube.
6. Add 8.0 mL of Buffer ACL (with carrier RNA as needed; compare Table 1). Close the cap and mix by pulse-vortexing for 30 s.

   Note: Make sure a visible vortex forms in the tube. To ensure efficient lysis, it is essential that the samples and buffer ACL are mixed thoroughly to yield a homogeneous solution.
7. A precipitate may form upon mixing of the components. This will redissolve during the lysis incubation and does not affect the yield of nucleic acids.

   Note: Do not interrupt the procedure at this time. Proceed immediately to step 8 to start the lysis incubation.
8. Incubate at 60°C for 30 min.
9. Place the tube back on the lab bench and unscrew the cap.
10. Add 18 mL of Buffer ACB to the lysate, close the cap, and mix thoroughly by pulse-vortexing for 15–30 s.

    Note: Make sure a visible vortex forms in the tube.
11. Incubate the lysate–Buffer ACB mixture for 10 min on ice.
12. Insert the QIAamp Mini column into the VacConnector on the QIAvac 24 Plus.
13. Insert a 20 mL tube extender into the open QIAamp Mini column.
14. Make sure that the tube extender is firmly inserted into the QIAamp Mini column to avoid leakage of sample.

    Note: Keep the collection tube for the dry spin in step 19.
15. Carefully apply the lysate from step 11 into the tube extender of the QIAamp Mini column. Switch the vacuum pump on. When all lysates have been drawn through the columns completely, switch the vacuum pump off and release the pressure to 0 mbar. Carefully remove and discard the tube extender.

    Note: The lysate must be loaded into the tube extender in 2 aliquots. The column should not run dry before the second aliquot is applied. 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-contaminations, be careful not to cross neighboring QIAamp columns while tube extenders are removed.
16. Apply 600 µL of Buffer ACW1 to the QIAamp Mini column. Leave the lid of the column open and switch on the vacuum pump. After all of Buffer ACW1 has been drawn through the QIAamp Mini column, switch off the vacuum pump and release the pressure to 0 mbar.

17. Apply 750 µL of Buffer ACW2 to the QIAamp Mini column. Leave the lid of the column open and switch on the vacuum pump. After all of ACW2 has been drawn through the QIAamp Mini column, switch off the vacuum pump and release the pressure to 0 mbar.
18. 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 of the ethanol has been drawn through the QIAamp Mini column, switch off the vacuum pump and release the pressure to 0 mbar.
19. 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 collection tube (saved from step 14) and centrifuge at full speed (20,000 × *g*, 14,000 rpm) for 3 min.
20. Place the QIAamp Mini column into a new 2 mL collection tube, open the lid, and incubate the assembly at 56°C for 10 min to dry the membrane completely.
21. Place the QIAamp Mini column in a clean 1.5 mL elution tube and discard the collection tube from step 19. Carefully apply 75 µL of Buffer AVE to the center of the QIAamp Mini column membrane. Close the lid and incubate at room temperature (15–25°C) for 3 min.
22. Centrifuge at full speed (20,000 × *g*; 14,000 rpm) for 1 min to elute the cfDNA.

For storage of cfDNA eluates refer to *PAXgene Urine Liquid Biopsy Set Handbook*.

Document Revision History

Date	Changes
September 2024	Initial release

For up-to-date licensing information and product-specific disclaimers, see the respective PreAnalytiX or QIAGEN kit handbook or user manual. PreAnalytiX and QIAGEN kit handbooks and user manuals are available at **www.preanalytix.com**, **eifu.bd.com**, and **www.qiagen.com**, or can be requested from QIAGEN Technical Service or your local distributor.