Experimental Protocol

cfDNA preparation from plasma using the QIAamp® Circulating Nucleic Acid kit (Qiagen)

Total time for experiment: 2 hours

Reagents:

Sterile pipette tips with filters
50 ml centrifuge tubes
Ethanol (96–100%)*
Isopropanol (100%)
Crushed ice

Instruments and tools:

P20, p200 and p1000 pipette (Gilson)
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 collection tubes)
Microcentrifuge
QIAvac 24 Plus vacuum manifold (Qiagen ref: #19413)
QIAvac Connecting System (Qiagen ref: #19419) or equivalent
Vacuum Pump (Qiagen ref: #84010 [USA and Canada] or 84020 [rest of world]) or equivalent pump capable of producing a vacuum of –800 to –900 mbar

Prior considerations:

Some samples may require dilution with phosphate-buffered saline (PBS).
24 samples may be processed at a time with this system. However, ideally no more than 12 samples should be isolated at the same time when performed manually.
This protocol is for cfDNA isolation from 3ml of plasma, although the reagent volumes are also given for 1 and 2ml of plasma. Reagent volumes must be adjusted for other volumes, please see the QIAamp® Circulating Nucleic Acid kit (Qiagen) handbook.

Steps:

- Turn on the biological safety cabinet and clean it with an alcohol-based disinfectant.
- If samples have been stored frozen, retrieve plasma samples from the freezer and mark the lid of the cryogenic vials to track freeze-thaw cycles. Let plasma samples thaw at room temperature (15°C to 25°C).

- Set the water bath temperature to 60°C for use with 50 mL conical centrifuge tubes and verify the temperature of the water with an external thermometer.
- Set the heating block temperature to 56°C for use with 2 mL collection tubes and verify the temperature of the block with an external thermometer.
- Fill bucket with ice.
- Turn on the vacuum pump and check that it reaches -800 to -900 mbar vacuum pressure.
- Equilibrate Buffer AVE to room temperature (15 to 25°C) for elution steps.
- Prepare buffers (Buffer ACB, Buffer ACW1 and Buffer ACW2) and carrier RNA according to the instructions described below when using a new kit. If buffers and aliquots of carrier RNA dissolved in Buffer AVE have been previously prepared, proceed to the next step.

a. Buffer ACB – Before use, add 200 mL of isopropanol to 300 mL of Buffer ACB concentrate to obtain 500 mL of Buffer ACB. Mix well after adding isopropanol.
b. Buffer ACW1 – Before use, add 25 mL of ethanol to 19 mL of Buffer ACW1 concentrate to obtain 44 mL of Buffer ACW1. Mix well after adding ethanol.
c. Buffer ACW2 – Before use, add 30 mL of ethanol to 13 mL of Buffer ACW2 concentrate to obtain 43 mL of Buffer ACW2. Mix well after adding ethanol.
d. Carrier RNA – Add 1550 µL of Buffer AVE into the tube containing 310 µg of lyophilized carrier RNA. Mix thoroughly by pipetting up and down and split into 75 µL aliquots in 1.5 mL tubes. Label carrier RNA aliquots and store at -15 to -30°C. *N.B: Do not freeze-thaw the aliquots of carrier RNA more than 3 times.*

- Thaw aliquot(s) of carrier RNA dissolved in Buffer AVE at room temperature (15 to 25°C). Add carrier RNA dissolved in Buffer AVE to Buffer ACL, according to the number of samples in the table below and mix by gently inverting the tube 10 times.

■ 1 ml ▲ 2 ml ● 3 ml

Number of samples	Buffer ACL (ml) ■	▲	●	Carrier RNA in Buffer AVE (µl)
1	0.9	1.8	2.6	5.6
2	1.8	3.5	5.3	11.3
3	2.6	5.3	7.9	16.9
4	3.5	7.0	10.6	22.5
5	4.4	8.8	13.2	28.1
6	5.3	10.6	15.8	33.8
7	6.2	12.3	18.5	39.4
8	7.0	14.1	21.1	45.0
9	7.9	15.8	23.8	50.6
10	8.8	17.6	26.4	56.3
11	9.7	19.4	29.0	61.9
12	10.6	21.1	31.7	67.5
13	11.4	22.9	34.3	73.1
14	12.3	24.6	37.0	78.8
15	13.2	26.4	39.6	84.4
16	14.1	28.2	42.2	90.0
17	15.0	29.9	44.9	95.6
18	15.8	31.7	47.5	101.3
19	16.7	33.4	50.2	106.9
20	17.6	35.2	52.8	112.5
21	18.5	37.0	55.4	118.1
22	19.4	38.7	58.1	123.8
23	20.2	40.5	60.7	129.4
24	21.1	42.2	63.4	135.0

- Pipet 300 µL of Qiagen's Proteinase K into a 50 mL conical centrifuge tube for each plasma sample. (see table for other initial volumes of plasma).
- Add 3 mL of plasma to the 50 mL conical centrifuge tubes containing Proteinase K.
- Add 2.4 mL of Buffer ACL (containing carrier RNA) to each plasma sample. (see table for other initial volumes of plasma).
- Close the tube cap and mix by pulse vortexing (vortexing in short intervals) for 30 seconds. Then proceed immediately to the next step to start the lysis incubation. *N.B. samples can also be inverted for 30 seconds in order to avoid bubbles caused by vortexing.*

Reagent	1ml plasma	2ml plasma	3ml plasma
Proteinase K	100µl	200µl	300µl
ACL buffer	0.8ml	1.6ml	2.4ml
ACB Buffer	1.8ml	3.6ml	5.4ml

- For plasma samples from BD Vacutainer K2 EDTA tubes, incubate at 60°C (±1°C) in a water bath for 30 (±2) minutes. For plasma samples from Streck Cell-Free DNA BCT, incubate at 60°C (±1°C) in a water bath for 60 (±2) minutes.
- Take the tube out of the water bath, unscrew the cap and add 5.4 mL of Buffer ACB to the lysate in each tube. (see table for other initial volumes of plasma).
- Close the tube cap and mix thoroughly by pulse vortexing for 15-30 seconds.
- Incubate the lysate-Buffer ACB mixture for 5 (±1) minutes on ice.
- During the incubation period, complete set up of the QIAvac 24 Plus with QIAamp Mini columns. Insert the disposable VacConnectors, place the labeled QIAamp Mini columns into the VacConnectors on the QIAvac 24 Plus, and insert 20 mL tube extenders (extension adapters) into the open QIAamp Mini columns. Please see the QIAamp® Circulating Nucleic Acid kit (Qiagen) handbook for more details.
- Turn on the vacuum pump (set to -800 to -900 mbar) while the main vacuum valve remains closed.
- After incubation on ice, carefully transfer the lysate-Buffer ACB mixture into the tube extenders of the QIAamp Mini columns using a serological pipette and discard the tubes.

N.B: To avoid contamination do not perform work over the column extender of other samples when transferring the lysate.

- Open the main vacuum valve and let the lysates pass through the columns completely.

- Close main vacuum valve (while vacuum stays on) and release the vacuum pressure from the QIAvac 24 Plus to 0 mbar. Carefully remove and discard the tube extenders.

Note: To avoid contamination, be careful when removing the tube extenders so as to not perform work above other sample tubes. Apply 600 µL of Buffer ACW1 to the QIAamp Mini columns using a fresh pipette tip for each sample.

- Apply 600 µL of Buffer ACW1 to the QIAamp Mini columns using a fresh pipette tip for each sample.
- Leave the lid of the column open and open the vacuum valve to let Buffer ACW1 flow through the columns completely.
- Close the main vacuum valve (while vacuum stays on) and release the vacuum pressure from the QIAvac 24 Plus to 0 mbar.
- Apply 750 µL of Buffer ACW2 to the QIAamp Mini columns using a fresh pipette tip for each sample.
- Leave the lid of the columns open and open the vacuum valve to let Buffer ACW2 flow through the columns completely.
- Close the main vacuum valve (while vacuum stays on) and release the vacuum pressure from the QIAvac 24 Plus to 0 mbar.
- Apply 750 µL of ethanol to the QIAamp Mini columns using a fresh pipette tip for each sample.
- Leave the lid of the columns open and open the vacuum valve to let ethanol flow through the columns completely.
- Close the main vacuum valve and switch off the vacuum pump.
- Close the lid of the QIAamp Mini columns. Remove them from the vacuum manifold, and discard the VacConnector.

Note: Avoid touching the rim of the columns or lids. Change gloves if the rim of the columns or lids is touched accidentally to avoid cross-contamination.

- Place the QIAamp Mini columns into clean 2 mL collection tubes, and centrifuge at 20,000 x g for 3 (± 0.5) minutes. *N.B. This step can be repeated by applying the sample eluate again to the filter in order to increase the over cfDNA yield.*
- Place the QIAamp Mini columns into new 2 mL collection tubes. Discard the used 2 mL tubes.

Open the lids and incubate the assembly within heat block at 56°C (± 1°C) for 10 (± 1) minutes to dry the membrane completely. *N.B. Samples can be dried at room temperature for 1 minute in order to avoid over-drying and difficulties in eluting the DNA, although* residual ethanol may interfere with downstream applications. Ensure recommended the drying of the sample is adequate.

- Place the QIAamp Mini columns into clean 1.5 mL elution tubes and discard the 2 mL collection tubes. Ensure that elution tubes are labeled with sample ID.
- Carefully apply 70 µL of Buffer AVE to the center of the QIAamp Mini membrane (without touching the membrane) using a fresh pipette tip for each sample.
- Close the lids and incubate at room temperature for 3 (± 0.5) minutes.
- Centrifuge at 20,000 x g for 1 minute to elute the nucleic acids.
- Carefully apply again 20-150µL of Buffer AVE to the center of the QIAamp Mini membrane. Close the lids and incubate at room temperature for 3 (± 0.5) minutes. Centrifuge at 20,000 x g for 1 minute to elute the nucleic acids. *N.B. The elution volume should be adjusted according to the downstream application. As a guide, an elution volume if 30µl is recommended for 1ml of the original volume of plasma, 50 µl for 2ml and 70µl for 3ml. 5µl of the original elution volume may be lost therefore it is important to apply the elution buffer directly to the center of the filter.*
- Discard the QIAamp Mini columns.
- Close 1.5 mL elution tubes tightly and store at 2°C to 8°C (when continuing within 24 hours) or at -15°C to -30°C (up to a maximum of 8 days).
- Discard the liquid waste and clean and decontaminate the QIAvac 24 Plus following instructions in the QIAvac 24 Plus Handbook (Qiagen)

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