SOP Title:

Purification of Circulating Nucleic Acids from Plasma Protocol

1.0 Purpose and Scope

This standard operating procedure (SOP) is for the purification of circulating nucleic acids from blood plasma samples at PM-OICR TGL.

This protocol consists of cell lysing, sample filtration using a vacuum, and purification of nucleic acids using the QIAamp Mini spin columns on a vacuum. Figure 1 briefly outlines this procedure.

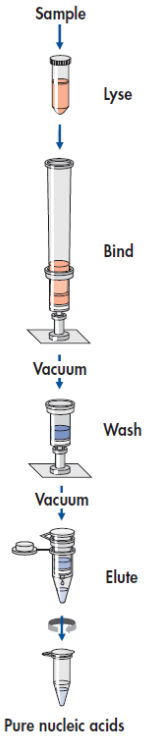


Figure 1: Illustration of the QIAamp Circulating Nucleic Acid Procedure (QIAamp Circulating Nucleic Acid Handbook, Qiagen).

Related documents:

- Manufacturer-supplied protocol for purification of circulating nucleic acids, **link to document.**

Related TGL documents:

- YYYY_MM_DD_SAMPLE SUBMISSION FORM_PI_Lastname_Firstname_TGL.xls
- SOP_Qubit_Assay_for_Nucleic_Acid_Quantification.doc

Refer to section 5.0 Appendix for information regarding the modifications and adjustments with respect to the manufacturer-supplied protocols that were incorporated in this SOP. Refer to the end of the document for a sample tracking print-out.

2.0 Materials

Workspace		
Sample prep room for all steps		
Equipment and associated consumables		
Medstore	50mL Polypropylene Conical Centrifuge Tubes, 25 tubes/bag (20 bags/case)	TB50-500
	Eppendorf Conical Tubes 15 mL (Do not substitute!)	0030122151
Eppendorf	ThermoMixer C, with 24 x 1.5 mL SmartBlock, 120 V	
	Rotor FA-45-6-30, 6x50ml with A-T lid (5804/10) (5820715006, Use with Eppendorf R810R)	
	Adapters 15ML CON F/50ML CON BOREHOLES PK/2 (5820717009, Use with Eppendorf R810R)	
	Eppendorf Centrifuge 5810 R, refrigerated, (use rotor FA-45-6-30)	
	Eppendorf Centrifuge 5427 R, refrigerated,	
	Eppendorf Centrifuge 5424, non-refrigerated, without rotor, rotary knobs, 230 V/50 – 60 Hz	
VWR	Tube Micro Clear 1500ul PK250	22234-044
Fisher	Fisherbrand* Premium Microcentrifuge Tubes 2mL, pk/500 (rnase/dnase safe/free)	5408138
Reagent kits		
Qiagen	QIAamp Circulating Nucleic Acid Kit (50)	55114
	QIAvac 24 Plus	19413
	VacConnectors	19407
	VacValves	19408
	QIAvac Connecting System	19419
Grand and Toy	Sharpie Permanent Marker	37001
ThermoFisher	Qubit dsDNA HS Assay Kit	Q32854
	Qubit Assay Tubes	Q32856
Commonly used reagents		
MedStore/Greenfield Specialty Alcohols	Ethanol anhydrous 100% (brown bottle), case of 12X 500ml	P006EAAN
	Ethanol anhydrous 100%, 4X4L white jugs (cleaning only)	P016EAAN

	Nuclease-free water	W4502-1L
	2-Propanol (for molecular biology, >=99%), 500mL(Sigma-Aldrich)	I9516-500ML

3.0 General Guidance:

- De-identified study codes must be used on all documentation.
- Wear safety glasses in case of splattering, plasma may contain infectious agents. Report poke injuries and ocular exposure to Debbie Kolozsvari, OICR Health and Safety (X7933), and/or Manager.
- Never store blood collection vials (EDTA, STRECK) in freezers, they will crack!
- Use Eppendorf brand centrifuge tubes (PN0030122151) rated for 19,000g. Other 15ml tube brands may fracture at high speed!
- When opening a new kit, record all lot numbers in TGL master lot tracking sheet (R:\Lot_tracking_forms\ 18_xx_xx_QIAamp_Circulating_Nucleic_Acid__Kit_lot_tracking.docx, associate all samples to the master lot tracking sheet using a letter key ("A", "B") in sample tracking sheet
- Do not mix and match reagents from multiple kits.
- Before beginning work every day, wipe down all pipetors and bench surfaces with Peroxide wipes, then wipe with 70% ethanol (made from bulk ethanol, 4L).
- Dispose of waste material containing guanidine hydrochloride in waste storage bucket. Guanidine hydrochloride reacts with acids and bases!
- Use molecular grade H_20 and anhydrous ethanol (brown bottle only!). Always use private stock ethanol and H_20 aliquots to minimize risk of contamination between technicians.
- Buffers bottles should be mixed/swirled prior to each use.
- Date all solutions in box with date received and date of resuspension, 1st use, and ethanol/isopropanol addition where appropriate
- Change gloves frequently if they become soiled with salts/solutions.
- Record all QC steps (quants, dilutions) in original sample submission sheet, including MISO LIMS IDs, (LIB, LDI) master lot tracking references, protocol version and include your name so that user/tech can be traced in sample sheet.
- Record and highlight all unusual observations, errors, or other issues in sample sheet.
- When opening a new kit, prepare the following buffers:
    - Add 200mL of 100% isopropanol to the 300mL of buffer ACB concentrate to obtain 500mL. Mix well.
    - Add 25mL of 96-100% EtOH to the 19mL of buffer ACW1 concentrate to obtain 44mL. Mix well.

- Add 30mL of 96-100% EtOH to the 13mL of buffer ACW2 concentrate to obtain 43mL. Mix well.

4.0 Procedure

1. Sample Preparation

1) Remove the standard rotor (A-4-81) in the Eppendorf 5810R centrifuge and replace with rotor FA-45-6-30. Insert 15ml adapters PN5820717009 into rotor. Pre-cool the centrifuge to 4°C.
2) Record the volumes of plasma which are to be extracted for each sample. Use a worksheet to calculate respective amounts of reagents to be used.
    a. If the plasma volume is greater than 10mL for a given sample, split it in half and treat as two separate samples throughout the protocol.
3) Turn on a water bath and set to 60°C
    a. Ensure the bath has enough water for 50mL tubes.
    b. Ensure there are enough slots for a whole sample batch of 50mL tubes to fit.
4) Remove the plasma samples from the -80°C freezer and allow to thaw at room temperature.
    a. If in 2mL tubes expect 30-40 minutes, if in 15mL tube expect 60 minutes.
    b. Before thaw, wipe the outside of the tube with 70% EtOH and inspect for cracks. If damaged, place the tube in a separate 50mL tube and let the plasma drain into the 50mL as it thaws, then transfer to a new 15mL tube (Eppendorf PN0030122151 only; rated for 19,000g!) .
    c. Once thawed, keep the plasma on ice until ready for high speed spin.
5) If the plasma is in 1.5 or 2mL tubes, quick spin and transfer plasma to 15mL tubes.
    a. After transferring, re-spin the 1.5 or 2mL tubes, collect any remaining plasma and transfer it to the corresponding 15mL tubes.
6) Spin the 15mL Eppendorf tubes containing the samples at 4°C for 5 minutes at max 16000 x g (10,921RPM with rotor FA-45-6-30).

2. Cell Lysis and Binding

7) Label one 50mL centrifuge tube per sample. Add Qiagen Proteinase K into each tube in the amount of **100µL of ProtK per 1mL of plasma**.

Amount of ProtK may be different each sample

8) Add your plasma sample to their corresponding 50mL tubes.
9) Add Buffer ACL to the sample in the amount of **800µL of ACL per 1mL of plasma.**

Amount of ACL may be different each sample

10) Mix by pulse-vortexing for 30 seconds.

a. Ensure that a visible vortex forms in the tube for adequate mixing.
b. If the volume of lysate in the sample is high and does not vortex properly, quickly invert the tube in a flicking motion to move around the lysate, and re-vortex.

11) Incubate the samples in the 60°C water bath for 1 hour.
12) Before putting the tubes back on the bench, wipe down the outside with 70% EtOH.

13) Add Buffer ACB to the samples in the amount of **1.8mL of ACB per 1mL of plasma.**
Amount of ACB may be different each sample
14) Close the lids and mix by pulse-vortexing for 30 seconds.
15) Incubate samples for 10 minutes on ice.

3. Vacuum Manifold Setup

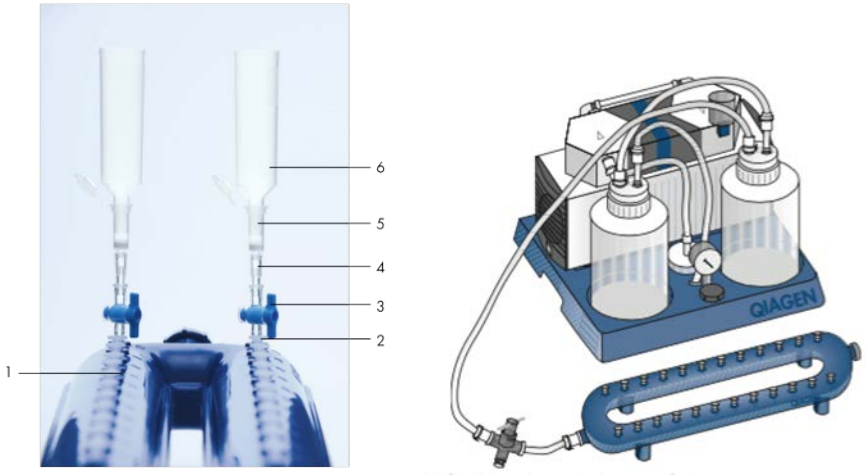


Figure 2: The vacuum manifold.

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. Tube Extender

16) Connect the vacuum manifold to the vacuum line.
17) Close the manifold hole, located on the right side, with the black screw cap.
18) Insert Luer plugs (Figure 2, 2) into Leur ports that will not be used for filtration. These plugs are reusable, do not throw out.

19) Insert VacValves (Figure 2, 3) into the vacuum manifold slots which will be used for filtration. These valves are reusable, do not throw out.
20) Insert the rest of the components into the VacValves in the following order:
    i. VacConnectors (Figure 2, 4)
    ii. QIAamp Mini Column (Figure 2, 5)
    iii. 20mL tube extender (Figure 2, 6)

4. Filtration

21) Add your sample into the tube extender of the QIAamp Mini Column and turn the vacuum pump on. Maintain pressure between -300 mbar and -800 mbar.
22) When all plasma has been added, quick spin the 50 mL centrifuge tubes and transfer over any residual sample into the tube extender.
23) Turn on the thermomixer C and set to 56°C.
24) When all sample has passed through the columns, close the VacValves (Figure 3, 3). Remove and discard the tube extenders.

5. Wash

25) With the VacValves still closed, add 600µL of Buffer ACW1 to the QIAamp Mini Column, then open the VacValves to allow the buffer to pass through the column.
26) Close the VacValves and add 750µL of Buffer ACW2 to the QIAamp Mini Column, then open the VacValves to allow the buffer to pass through the column.
27) Close the VacValves and add 750µL of 96-100% EtOH to the QIAamp Mini Column, then open the VacValves to allow it to pass through the column.
28) Close the lid of the column and remove from the vacuum manifold. Discard the VacConnector. Place the column in a clean 2mL collection tube.
29) Centrifuge the column and collection tube at full speed for 5 minutes to dry the membrane (Eppendorf 5424, or 5427).

6. Elute Nucleic Acids

30) Place the QIAamp Mini column in a clean 1.5mL DNA low bind elution tube.
31) Add 80µL of Buffer AVE onto the center of the membrane, being careful to not pipette the liquid onto the side of the tube.
    a. Decrease the amount of buffer added to 50µL if yields are expected to be low.
32) Incubate the tube for 10 minutes at room temperature.

33) Spin the tubes for 1 minute at full speed.
34) Repeat the elution step a second time into the same tube. Add the same volume of Buffer AVE directly onto the membrane, incubate the sample for 10 minutes and then spin at full speed for 1 minute.
35) Quantify the yield of eluent using Qubit and store at -80°C.

7. Optional Additional DNA Recovery

36) Move the column to a new elution tube. Add 30µL of Buffer AVE onto the center of the membrane and incubate at 4°C overnight.
37) The next morning, let the tubes come to room temperature. Spin the columns for 1 minute at full speed.
38) Quantify the second eluent using Qubit and then store at -80°C.
    a. For high yielding samples, the second eluent can contain as much as 30% cfDNA as the first eluent.
    b. If primary elution yield insufficient, second elution yield may be utilized; keep elutions separate.

5.0 Revision History

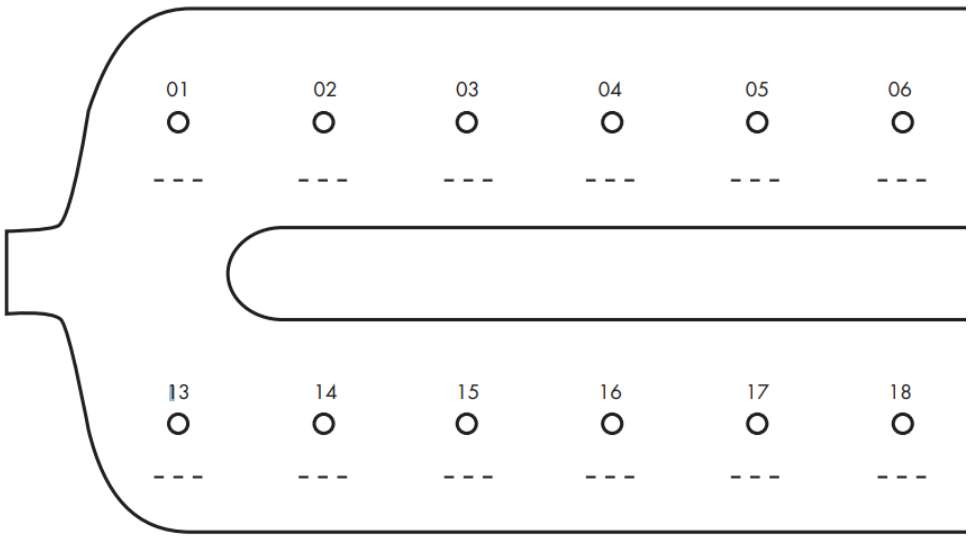
Version Number	Date *(yyyy-mm-dd)*	History of change
2016.02.003	2017-06-27	Working SOP for Pugh lab (created by EH, edits by Iulia Cirlan)
1.0.1	2018-02-15	Edits and formatting for TGL by Kayla, edits DT
1.0.2	2018-03-23	DT approved

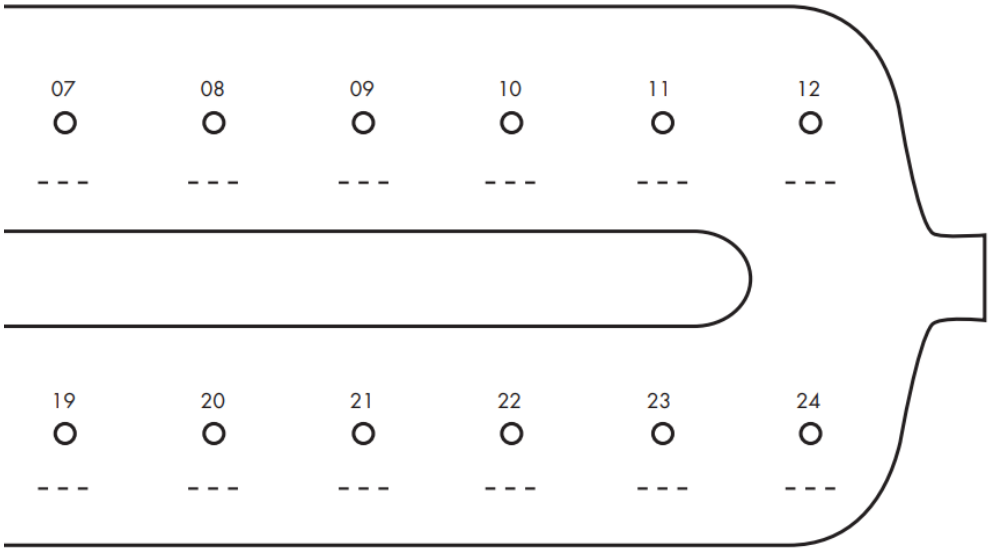
6.0 Tracking Sheet

Date: _____

Operator: _____

Run ID: _____

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