SOP Title: Purification of Circulating free Nucleic Acids (cfDNA) from Plasma Protocol

1.0 Purpose and Scope

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

This procedure includes the following steps: lysing, lysate filtration with vacuum manifold, and purification of cfDNA using QIAamp Mini spin columns. Figure 1 briefly outlines this procedure.

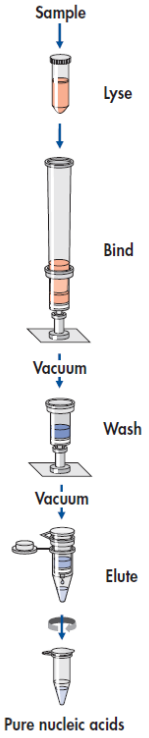


Figure 1: Illustration of the QIAamp Circulating Nucleic Acid Procedure (QIAamp Circulating Nucleic Acid Handbook, Qiagen).

Related documents:

- QIAamp circulating Nucleic Acid Handbook, **link to document.**
- QIAvac 24 Plus Handbook, **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
- SOP_TapeStation_4200_V1.0.doc
- Plasma_and_Buffy_Coat_Separation_From_Whole_Blood_Protocol_v1.0.2

Refer to section 5.0 Appendix for information regarding modifications to the manufacturers supplied protocols that were incorporated in this SOP.

2.0 Materials

Workspace		
Biosafety cabinet for all steps with plasma until after column wash		
Sample Prep room for all steps on column and after		
Equipment and associated consumables		
Medstore	50mL Polypropylene Conical Centrifuge Tubes, 25 tubes/bag (20 bags/case)	TB50-500
	Eppendorf Conical Tubes, 15 mL, Sterile, pyrogen-, DNase-, RNase-, human and bacterial DNA-free, colorless, 500 tubes (10 bags × 50 tubes) (Do not substitute, high g force tubes)	0030122151
	Serological Pipettes, 10mL, sterile, individually wrapped, 200/cs	357551
	Sharpie Permanent Marker	37001
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, (use rotor FA-45-48-11, Aerosol tight)	
	Eppendorf Centrifuge 5424, non-refrigerated, (use rotor FA-45-24-11, Aerosol tight)	
VWR	Tube Micro Clear 1500ul PK250	22234-044
Fisher	Fisherbrand* Premium Microcentrifuge Tubes 2mL, pk/500 (rnase/dnase safe/free)	5408138
Qiagen	QIAvac 24 Plus	19413
	VacConnectors	19407
	VacValves	19408
	QIAvac Connecting System	19419
Agilent	Agilent Tapestation 4200	
	TapeStation Analysis Software A.02.01 SR1	
	Plate Foil Seal	5067-5154
	Genomic DNA ScreenTape (105 samples, 7 tapes); 4°C	5067-5365
	Genomic DNA Reagents (Genomic DNA Sample Buffer 4°C; Genomic DNA Ladder, -20°C)	5067-5366
ThermoFisher	Qubit 3.0 Fluormeter	Q33216
	Qubit dsDNA HS Assay Kit (HS Reagent, HS Buffer, RT; HS standards 1 & 2; 4°C)	Q32854
	Qubit Assay Tubes	Q32856
	Water bath	
Reagent kits		
Qiagen	QIAamp Circulating Nucleic Acid Kit (50) (Note if processing 10 mls of plasma per patient, kit is sufficient for only 25 samples)	55114
	Qiagen Proteinase K (4°C)	

	QIAamp Mini columns (4°C)	
Grand and Toy	Sharpie Permanent Marker	37001
Commonly used reagents		
MedStore/Greenfield Specialty Alcohols	Ethanol, anhydrous 100% (brown bottle), case of 12X 500ml	P006EAAN
	Ethanol, anhydrous 100%, 4X4L white jug (cleaning only)	P016EAAN
	Nuclease-free water	W4502-1L
	2-Propanol (for molecular biology, >=99%), 500mL(Sigma-Aldrich)	I9516-500ML
	LAVO PRO 6 BLEACH 6% 5L (3 bottles per case), (Sodium Hypochlorite 6%), each	1952B

3.0 General Guidance:

- De-identified study codes must be used on all documentation.
- Whole blood and derivatives including plasma may contain infectious agents. Wear safety glasses and a lab coat in case of splattering. Immediately stop work if you experience a poke injury or ocular exposure; seek help! If ocular exposure occurs, proceed immediately to eye wash station and rinse eye for a minimum of 5 minutes. Report exposure to Debbie Kolozsvari, OICR Health and Safety (X7933), and/or Manager. Change gloves frequently when soiled.
- Upon kit receipt, store QIAamp Mini columns and proteinase K at 4°C. All other buffers may be stored at room temperature in respective boxes.
- Never store blood collection vials (K3EDTA, STRECK) in freezers as they will crack!
- Use Eppendorf brand centrifuge tubes (PN0030122151) rated for 19,000g. Other 15ml tube brands may fracture at high speed!
- Record all volumes of plasma processed per patient using the worksheet in section 7.1 of this protocol. Plasma volumes should be recorded with accuracy of 1.0 mls +/-0.1 mL, or 1 gradation mark on a 10ml pipette. These values may be used to track circulating nucleic acid concentration per ml of plasma.
- 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 pipets and bench surfaces with Peroxide wipes, then wipe with 70% ethanol (made from bulk ethanol, 4L).
- Dispose of waste material (Buffers ACL, ACB and ACW1) containing guanidine hydrochloride in liquid waste storage bucket. Guanidine hydrochloride reacts with acids, bases and bleach!
- 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.
- 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.
    - **Do not add carrier RNA to any buffers!**
- Up to 5 mls of plasma may be processed over 1 column. If routinely processing 10 mls of plasma over 1 purification column, you will require double the amount of buffers. For processing 50X10ml plasma samples, purchase 2 QIAamp Circulating Nucleic Acid Kits (50).

Table 1: cfDNA yield for Specified Cancer Types

Cancer Type	Average cfDNA Yield (ng/mL of plasma)
Normal (Healthy Donor)	7.3
Ovarian	18.6
Prostate	39.2
Pancreatic	52.3
Multiple Myeloma	57.6
Melanoma	210.7
Lung	356.8
Liver	49.9
Gyne	5.7
Gastrointestinal	28.7
Endometrial	67.5
Colorectal	54.5
Breast	80.0
All	53.6

Reference: https://www.nature.com/articles/ncomms15086 [1]

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) Turn on a water bath and set to 60°C
    a. Ensure the bath has enough water for 50mL tubes.
3) Remove the plasma samples from the -80°C freezer and allow to thaw at room temperature.
    a. If plasma is stored in 1.5 or 2mL tubes expect 30-40 minutes for thawing; if in 15mL tubes expect 60 minutes.
    b. Before thawing plasma, wipe the outside of the tube with 70% EtOH and inspect for cracks. If damaged, place the tube inside a fresh 50mL tube and let the plasma drain into the 50mL tube as it thaws.
    c. Once thawed, keep the plasma on ice until ready to proceed.
4) Record the plasma volume(s) (+/-0.1mL) in the Lysis and Binding Work Sheet (section 5.3) to calculate respective amounts of reagents to be used in steps 7-9.
    a. If the plasma volume is greater than 10mL for a given sample, split it in half and process as two separate samples throughout the protocol.
5) Transfer the plasma into a new 15mL tube (Eppendorf PN0030122151, rated for 19,000g).
    a. Quick spin the original tubes (1.5mL, 2mL or 15mL) and transfer any remaining plasma.
    b. Put all tips and tubes in contact with plasma in a 2% bleach solution overnight.
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. 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 for each sample

8) Transfer the supernatant of your sample to their corresponding 50mL tube containing respective Proteinase K. Leave behind ~0.5mL to prevent disturbing the cell pellet. Put the tube and tip into a 2% bleach solution overnight.
9) Add Buffer ACL to the sample in the ratio of **800µL of ACL per 1mL of plasma.**

Amount of ACL may be different for 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 exterior of the tube with 70% EtOH.

13) Add Buffer ACB to the samples in the ratio of **1.8mL of ACB per 1mL of plasma.**

Amount of ACB may be different for each sample

14) Close the lids and 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.
15) Incubate samples for 10 minutes on ice.

3. Vacuum Manifold Setup

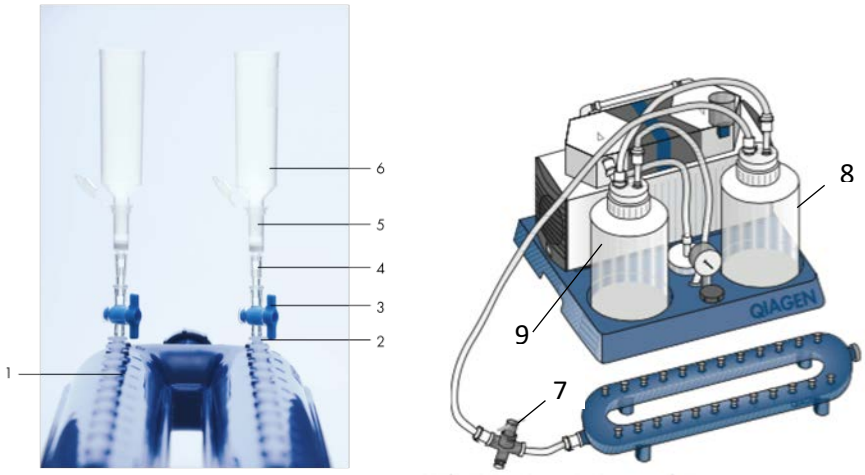


Figure 2: The QiaVac Vacuum Manifold.

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
7. Main Vacuum Valve
8. Waste Jug
9. Over-Flow Waste Jug

16) Connect the vacuum manifold to the vacuum line.
17) Close the manifold hole, located on the right side, with the black screw cap or the white screw cap valve. Do not over-tighten.
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. Distribute utilization of the manifold ports to ensure equal vacuum is achieved across multiple column purification columns.
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) The Plasma Sample Manifold Location Tracking Sheet (section 5.2) may be used to track sample position on the manifold. Add no more than 20 mls of plasma sample mixture at one time into the tube extender of the QIAamp Mini Column and turn the vacuum pump on. Add additional volumes of plasma sample mixture to the tube extender as sufficient liquid is pulled down the tube extender through the Mini Column. This process may take 20-40 minutes, depending on sample volume. Maintain a pressure of between -300 mbar and -800 mbar. If vacuum is weak, close the VacValve of a few columns to maintain pressure, or adjust the source vacuum line to increase vacuum pressure.
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 the total volume of all of the plasma sample mixture has passed through a column, close the VacValve (Figure 2, 3) to prevent excessive drying. When all columns are empty and VacValves closed, remove and discard tube extenders.
25) Use the main vacuum valve (Figure 2, 7) to close the vacuum, release pressure on the manifold and then open each of the VacValves.

5. Wash

26) With the main valve still closed, add 600µL of Buffer ACW1 to the QIAamp Mini Column, then open the main valve to allow the buffer to pass through the column.
27) Close the main valve, release pressure in the manifold and then add 750µL of Buffer ACW2 to the QIAamp Mini Column. Open the main valve to allow the buffer to pass through the column.
28) Close the main valve, release pressure in the manifold and then add 750µL of 96-100% EtOH to the QIAamp Mini Column. Open the main valve to allow it to pass through the column.
29) Close the main valve and release pressure in the manifold. Close the lid of the QIAamp Mini columns, remove from the vacuum manifold and place into a clean 2mL collection tube. Put the VacValves into a container for washing and discard the VacConnectors.
30) Centrifuge the column and collection tube at full speed for 3 minutes to dry the membrane (Eppendorf 5424, or 5427).
31) Place the QIAamp Mini column in a clean 1.5mL DNA low bind elution tube.
32) Open the column lids slightly and place the column/collection tubes into the thermomixer set at 56°C for 6 minutes.

6. Elute Nucleic Acids

33) Remove the columns from the heat and add **80µL** of Buffer AVE onto the center of the membrane, being careful to not pipette the liquid onto the side of the tube.
34) Incubate the tube for 10 minutes at room temperature.
35) Spin the tubes for 1 minute at full speed in Eppendorf 5424, or 5427.
36) Reload the eluate from step 33 directly onto the membrane. Incubate the sample for 10 minutes and then spin at full speed for 1 minute.
37) Quantify the yield of eluent using Qubit and store at -80°C (SOP_Qubit_Assay_for_Nucleic_Acid_Quantification.doc).
38) Run all purified cfDNA extractions on an Agilent Genomic DNA ScreenTape to assess the presence of genomic contamination, or all fragments greater than 1000bp. Record % genomic contamination in sample tracking sheets. Follow SOP_TapeStation_4200_V1.0.doc.

8. Clean-up

Waste liquids contain guanidine hydrochloride and cannot be bleached or mixed with acids or bases!!

39) Empty the vacuum manifold into an appropriate biohazard waste container and then wash with soap and water, followed by 70% ethanol. Leave to dry.
40) Soak the luer plugs in 70% ethanol for 30 minutes then remove and let dry.

41) Wash the VacValves in soap and water, and then soak in 70% ethanol for 30 minutes. Remove and let dry.
42) Empty the waste jug (Figure 2, 8) and waste over-flow jug (Figure 2, 9) into an appropriate biohazard waste container. Wash with soap and water followed by 70% ethanol. Let dry.

5.0 Quality Control

5.1 cfDNA Genomic Contamination Evaluation and cfDNA Size Distribution Assessment

Verify each purified cfDNA sample contains a cfDNA peak centred around 150-190bp. The following screenshots depict the presence and absence of genomic contamination (% of fragments >1000bp) in cfDNA, identified using a Agilent Genomic TapeStation trace. If genomic contamination exceeds 20%, notify manager before proceeding with further assay. Record % genomic contamination in project running sheet.

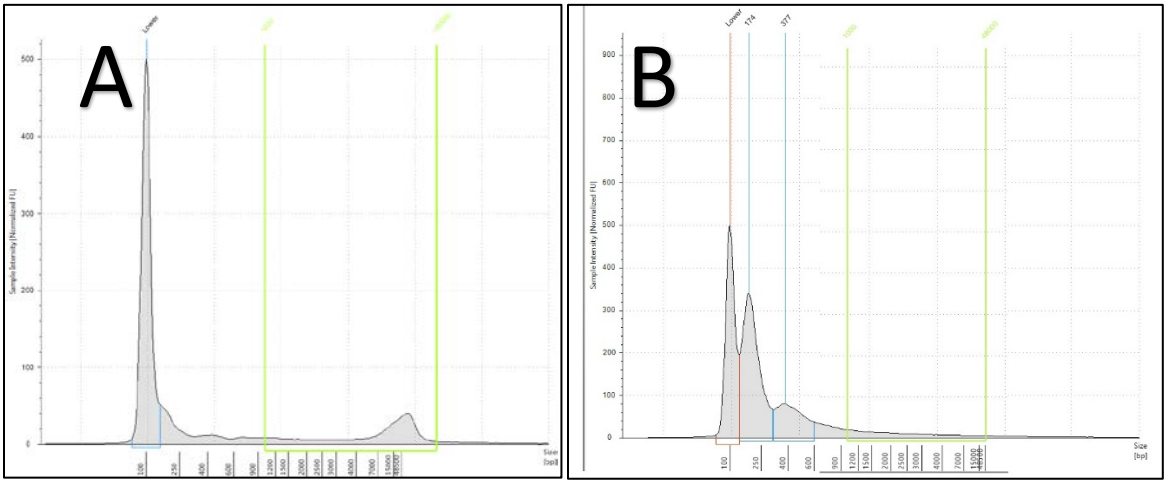
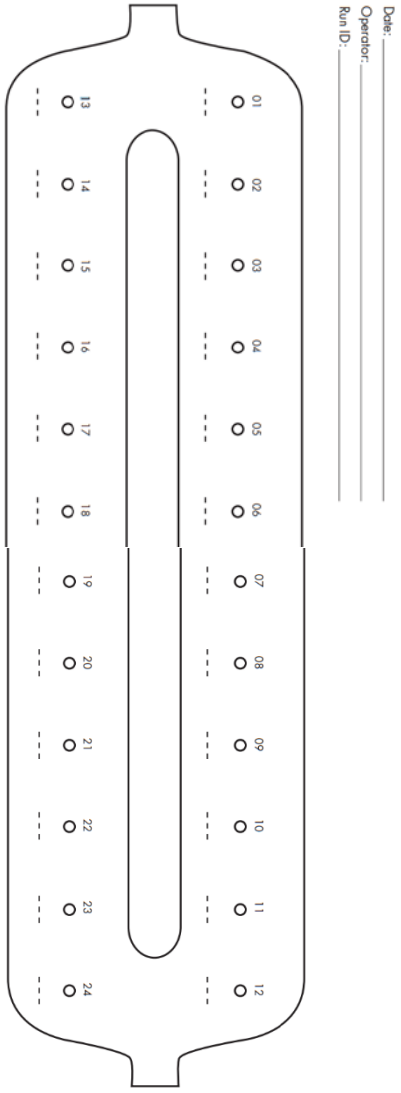


Figure 3: Agilent Genomic TapeStation trace of representative cfDNA samples. (A) cfDNA sample with genomic contamination. High molecular weight DNA (genomic) is observed around 48000bp, approximately 54% of fragments are over 1000bp in size. (B) Ideal cfDNA sample with low genomic contamination. There is a high amount of expected shorter DNA fragments <1000bp and centred around ~174bp, with concatemers around ~377bp; approximately 10% of fragments are over 1000bp in size.

5.2 Plasma Sample Manifold Location Tracking Sheet. (QIAamp Circulating Nucleic Acid Handbook, Qiagen).



5.3 Lysis and Binding Work Sheet (Step 2)

Addition of reagents to plasma, Step 2, sub steps 7-9. Calculations per mL of plasma.

			Prot K (ul)	ACL (mL)	ACB (mL)
	Plasma Sample	Volume (mls)	x100ul	x0.8mL	x1.8mL
Ex.	INS-C-01-cfDNA-01	4.6 mLs	460 ul	3.68 mLs	8.28 mL
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					
17					
18					
19					
20					
21					
22					
23					
24					

6.0 Revision History

Version Number	Date *(yyyy-mm-dd)*	History of change and editing author
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
1.0.3	2018-04-05	Edits/update by Kayla
1.0.4	2018-09-04	Edits/update by Kayla
1.0.5	2018-09-12	Added genomic tape station QC stage, DT
1.0.6	2018-09-27	Minor updates and edits by Kayla, DT. Altered elution volume and repeat nucleic acid elution.
1.0.7	2018-11-05	Added genomic TapeStation trace examples (6.0)
1.0.8	2019-06-13	Minor revisions
1.0.9	2019-07-10	DT-Added additional component part #s, reagent temperature storage conditions. Updated protocol name, subsection titles. Edited TapeStation text, guidance. Updated manifold tracking diagram, updated lysis and binding worksheet with reference example. Updated references.

7.0 References

1. Kis, O., et al., *Circulating tumour DNA sequence analysis as an alternative to multiple myeloma bone marrow aspirates.* Nat Commun, 2017. **8**: p. 15086.