SOP Title:

Buffy Coat Qiagen AllPrep DNA/RNA/miRNA Universal Kit Extraction Protocol

1.0 Purpose and Scope

This standard operating procedure (SOP) is for the co-isolation of both DNA and RNA, including microRNA, from Buffy Coat samples at PM-OICR TGL.

This protocol consists of cell lysing and purification of nucleic acids using the Qiagen AllPrep Universal spin columns. Figure 1 briefly outlines this procedure.

Related documents:

- Manufacturer-supplied protocol for purification of nucleic acids using Qiagen AllPrep Universal kit, **link to document.**
- Manufacturer-supplied protocol for the Qiagen Gentra Puregene Blood Kit, link to document.

Related TGL documents:

- YYYY_MM_DD_SAMPLE SUBMISSION FORM_PI_Lastname_Firstname_TGL.xls
- SOP_Plasma_and_Buffy_Coat_Separation_From_Whole_Blood_Protocol_v1.0.2
- SOP_Qubit_Assay_for_Nucleic_Acid_Quantification.doc
- SOP_TapeStation_4200_V1.0

Refer to section 5.0 Appendix for information regarding the modifications and adjustments to this SOP.

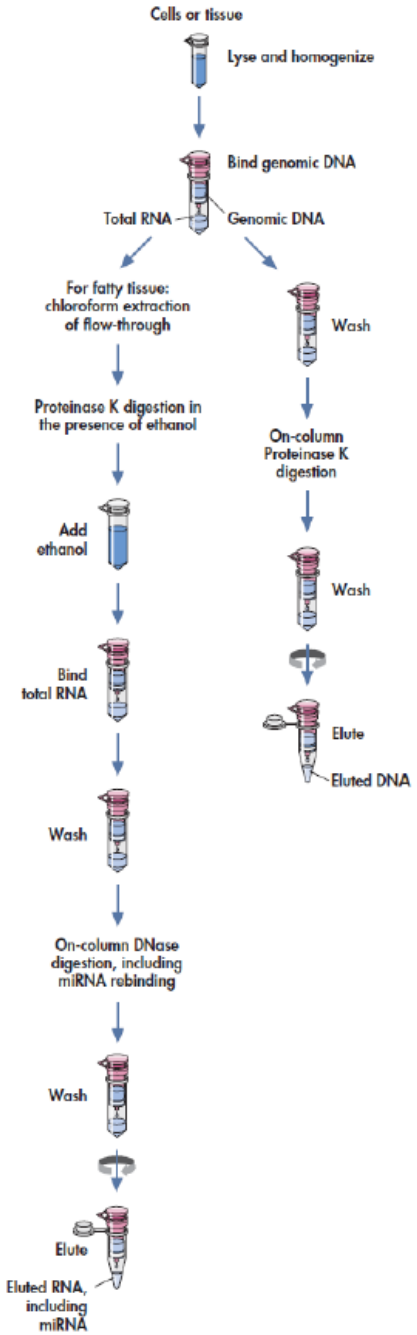

Figure 1: Illustration of the Qiagen AllPrep DNA/RNA/miRNA Universal Kit Extraction Protocol

Source: AllPrep DNA/RNA/miRNA Universal Handbook, Qiagen, 2014.

2.0 Materials

Workspace
Biosafety cabinet while working with blood samples
Sample prep room for all steps after cell lysis

Equipment and associated consumables		
Agilent	Agilent Tape Station 4200	
	High Sensitivity RNA ScreenTape	5067-5579
	High Sensitivity RNA ScreenTape Sample Buffer	5067-5580
	High Sensitivity RNA ScreenTape Ladder	5067-5581
Eppendorf	ThermoMixer C, with 24 x 1.5 mL SmartBlock, 120 V	
	Eppendorf Centrifuge 5810 R, refrigerated, Rotor A-4-81 with buckets	
	Eppendorf Adapters for 5810R, 15 ml tubes PN5810722004	
	Eppendorf Caps for 500mL Rectangular Buckets PN5810724007 (Aerosol tight)	
	Eppendorf Centrifuge 5424, non-refrigerated, Rotor FA-45-24-11 (Aerosol tight)	
VWR	TUBE MICRO CLR 1500UL PK250	22234-044
Fisher	EMD Millipore™ Calbiochem™ β-Mercaptoethanol, Molecular Biology Grade	444203250
MedStore	15mL Polypropylene Conical Centrifuge Tubes, 25 tubes/bag (20 bags/case)	TB15-500
ThermoFisher	Qubit 3.0 Fluorometer	Q33216
Reagent kits		
Qiagen	AllPrep DNA/RNA/miRNA Universal Kit (50)	80224
	Qiagen Collection Tubes (2ml) (Additional tubes in excess of kit) (additional tubes, not included)	19201
	RNase-Free DNase Set (50)	79254
	Qiagen Gentra Puregene Blood Kit (120 ml)	158467
ThermoFisher	1M Tris, pH=8.0, 100 mls	AM9855G
	Qubit RNA HS Assay Kit	Q32855
	Qubit dsDNA HS Assay Kit	Q32854
	Qubit Assay Tubes	Q32856
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 jugs (cleaning only)	P016EAAN
Medstore	Nuclease-free water	W4502-1L
	Needles 18G X 1-1/2" (disposable), 100/pk (BD # 305196)	2537-CABD305196
	Plastic Disposable Syringes, 1cc, 200/pk	2606-309659
	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.
- Whole blood, peripheral blood mononuclear cells (PBMCs) or buffy coat, may contain infectious agents. Wear safety glasses, gloves 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.
- 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). Wipe down work surface after the protocol is complete.
- Ensure proper protective equipment is worn during this protocol. Gloves, lab coat and goggles are required. Change gloves if they become soiled with blood derivatives or salts/solutions.
- When opening a new kit, record all lot numbers in TGL master lot tracking sheet (R:\Lot_tracking_forms\18_xx_xx_AllPrep_DNA_RNA_miRNA_Universal_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.
- **Dispose of waste material containing guanidine hydrochloride (Buffers RLT, FRN) in 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 stocks of ethanol and H_20 aliquots (i.e. do not take H_20 from 1L jug, make aliquots) to minimize risk of contaminating working solutions.
- Buffer bottles should be mixed/swirled prior to each use.
- Record all quality control steps (nucleic acid quantifications, dilutions) in original sample submission sheet, including MISO LIMS IDs, (LIB, LDI) master lot tracking references, protocol version. Include your name so that the technician/sample reagents used can be traced in the sample sheet.
- Record and highlight all unusual observations, errors or other issues in sample sheet.
- Date all solutions in box with date received, date of resuspension, 1st use, and ethanol/isopropanol addition where appropriate.
- Reconstitute DNase I with 500 µL nuclease-free water. Inject the water using a needle/syringe through the septum on the DNase I vial. Invert/flick to mix before aliquoting. Freeze aliquots at -20°C; aliquots are stable for 9 months. Once an aliquot is thawed, do not refreeze, store at 4°C for a maximum of 6 weeks. Vortexing will shear the DNaseI enzyme, diminishing enzymatic activity, only flick mix or gentle inversion prior to use.

- When opening a new kit, prepare the following buffers; indicate/mark the date and alcohol addition directly on the bottle:
  - Add 42 mL of 100% isopropanol to the 14 mL of buffer FRN concentrate to obtain 56mL. Mix well.
  - Add 44 mL of 96-100% (anhydrous) ethanol to the 11 mL of buffer RPE concentrate to obtain 56mL. Mix well.
  - Add 25mL of 96-100% (anhydrous) ethanol to the 19mL of buffer AW1 concentrate to obtain 44mL. Mix well.
  - Add 30mL of 96-100% (anhydrous) ethanol to the 13mL of buffer AW2 concentrate to obtain 43mL. Mix well.

4.0 Procedure

The Qiagen AllPrep DNA/RNA/miRNA Universal Kit will process a maximum 1.5 mL of whole blood, or $1x10^7$ leukocytes/PBMCs.

1. RBC lysis

NOTE: If buffy coat preparation does not contain any red blood cells, skip this section and begin at section 2, Cell Lysis.

1) Quickly thaw the frozen buffy coat samples in a 37°C water bath. Once thawed, keep on ice.
2) Estimate the volume of available buffy coat preparation. Calculate three times the amount of sample volume and add that amount of RBC Lysis Solution to a labelled 15 mL tube (from the Qiagen Gentra Puregene Blood Kit, PN158467).
3) Add the buffy coat sample to the 15 mL tube and invert to mix.
4) Incubate for 10 minutes at room temperature. Invert several times during incubation.
5) Centrifuge for 5 minutes at 2000 x g in the Eppendorf 5810R centrifuge.
6) Without disturbing the pellet, discard the supernatant.
7) Wash the pellet with RBC Lysis Solution using 2x the original sample volume (step 2) to remove any residual red blood cells.
8) Centrifuge for 5 minutes at 2000 x g.
9) Remove most of the supernatant, leaving 100-200 µL behind.
10) Vortex the sample to re-suspend the pellet in the residual supernatant/pellet.

2. Cell Lysis

11) In a fume hood, prepare Buffer RLT Plus. Make a master stock of 600ul of RLT Plus + 0.01% β-mercaptoethanol, sufficient for all samples. Add β-mercaptoethanol at a ratio of 10ul β-mercaptoethanol per 1ml of Buffer RLT Plus.
12) Add 600 µL of the cell lysis solution (RLT+ β-mercaptoethanol, step 11) to the re-suspended buffy coat pellet from step 10, or directly to the buffy coat of samples with no red blood cell contamination (step 1, RBC lysis).
13) Homogenize the sample on the Eppendorf thermomixer C for 10 minutes at 2000rpm at 20°C.
14) Centrifuge for 15 seconds at 8000 x g in the Eppendorf R5424 centrifuge.
15) Transfer the lysate to an AllPrep DNA Mini spin column placed in a 2 mL collection tube.
16) Centrifuge for 30 seconds at maximum speed.
17) Reload the flow through onto the column and repeat the centrifugation. **Save this flow through for RNA isolation.**

18) Place the AllPrep DNA Mini spin column into a new 2 mL collection tube and store at room temperature if planning to process immediately, or at 4°C for later DNA purification (step 37).

3. Total RNA Extraction

19) Add 80 µL of proteinase K to the flow through (from step 17) and mix by pipetting. Do not centrifuge.
20) Add 350 µL of 90-100% (anhydrous) ethanol and mix well by pipetting. Incubate for 10 minutes at room temperature.
21) Add 750 µL of 96-100% (anhydrous) ethanol and mix well by pipetting. Do not centrifuge.
22) Transfer 700 µL of the sample to the RNeasy mini spin column placed in a 2 mL collection tube. Centrifuge for 30 seconds at max speed. Discard the flow-through.
23) Repeat until the entire sample has been passed through the spin column, using the same collection tube.
24) Add 500 µL Buffer RPE to the spin column and spin for 30 seconds at max speed. Discard the flow through.
25) Prepare a DNase mix by combining 10 µL of DNase stock solution with 70 µL Buffer RDD per sample; make a sufficient master mix solution for all samples to be processed. Mix gently by inversion and quick spin.
26) Add 80 µL of the DNase mix directly to the column membrane and incubate for 15 minutes at room temperature.
27) Add 500 µL Buffer FRN and centrifuge for 15 seconds at max speed. **SAVE THE FLOWTHROUGH.**
28) Place the RNeasy mini spin column into a new 2 mL collection tube.
29) Add the flow through from step 27 to the column and centrifuge for 30 seconds at max speed. Discard the flow through.
30) Add 500 µL Buffer RPE and centrifuge for 30 seconds at max speed. Discard the flow through.
31) Add 500 µL of 96-100% (anhydrous) ethanol and centrifuge for 2 minutes at max speed.
32) Place the RNeasy mini spin column into a new 2 mL collection tube. Spin for 2 minutes at max speed to remove residual ethanol.
33) Place the RNeasy mini spin column into a labelled 1.5 mL tube.
34) Add 50 µL RNase-free water directly to the membrane and incubate for 5 minutes at room temperature. Centrifuge for 1 minute at max speed.
35) Add another 50 µL of RNase-free water directly to the membrane and incubate for 5 minutes at room temperature. Centrifuge for 1 minute at max speed.
36) Store Total RNA sample (100ul total) at -80°C.

3. DNA Purification

Pre-heat Buffer EB to 65°C in ThermoMixer C at least 30 minutes before use.

37) Add 350 µL Buffer AW1 to the AllPrep DNA Mini spin column (from step 18). Centrifuge for 30 seconds at maximum speed. Discard the flow through.
38) Make a mix of 20 µL of proteinase K and 60 µL of Buffer AW1 per sample; a master mix may be made for all samples being processed. Mix gently and quick spin.
39) Add 80 µL of the Prot K mix directly to the column membrane. Incubate for 5 minutes at room temperature.
40) Add 350 µL Buffer AW1. Centrifuge for 30 seconds at max speed. Discard the flow through.
41) Add 500 µL Buffer AW2. Centrifuge for 2 minutes at max speed.
42) Empty the collection tube and re-use it to perform a second spin (2 minutes at max speed) to remove residual ethanol.
43) Place the AllPrep DNA Mini spin column into a labelled 1.5 mL tube.
44) Add 50 µL of pre-warmed Buffer EB directly to the membrane. Incubate for 5 minutes at room temperature. Centrifuge for 1 minutes at max speed.
45) Add an additional 50 µL of pre-warmed Buffer EB directly to the membrane. Incubate for 5 minutes at room temperature. Centrifuge for 1 minute at max speed.
46) Store genomic DNA sample (100ul total) at -20°C.

4. Assess Quality and Quantity of the DNA and RNA

47) Use Qubit HS dsDNA assay using TGL SOP to quantify the DNA (SOP_Qubit_Assay_for_Nucleic_Acid_Quantification.docx).
48) Use Qubit HS RNA assay using TGL SOP to quantify the RNA (SOP_Qubit_Assay_for_Nucleic_Acid_Quantification.docx.).
49) Use high sensitivity RNA screen tape on RNA samples and record both RIN and DV200. These values and the shape of fragment distribution will be used to determine fragmentation time for RNA Library Prep (see 5.0 Appendix section 2 in SOP_Illumina_TruSeq_Stranded_Human_Total_RNA_Ribozero_Gold_Library_Prep_V.1.4.2)

5.0 Revision History

Version Number	Date *(yyyy-mm-dd)*	History of change
2017.02.007	2017-05-10	Working SOP for Pugh lab
1.0	2018-04-02	Edits and formatting for TGL by Kayla
1.0.1	2018-09-28	Edits by DT