SOP Title:

Qiagen AllPrep DNA/RNA FFPE Kit Extraction Protocol

1.0 Purpose and Scope

This standard operating procedure (SOP) is for the simultaneous purification of both genomic DNA and total RNA, including microRNA, from FFPE samples at PM-OICR TGL.

This protocol consists of de-paraffinization using Citrisolv followed by purification of nucleic acids using either the RNeasy MinElute or QIAamp MinElute spin columns for RNA and DNA, respectively. Figure 1 briefly outlines this procedure.

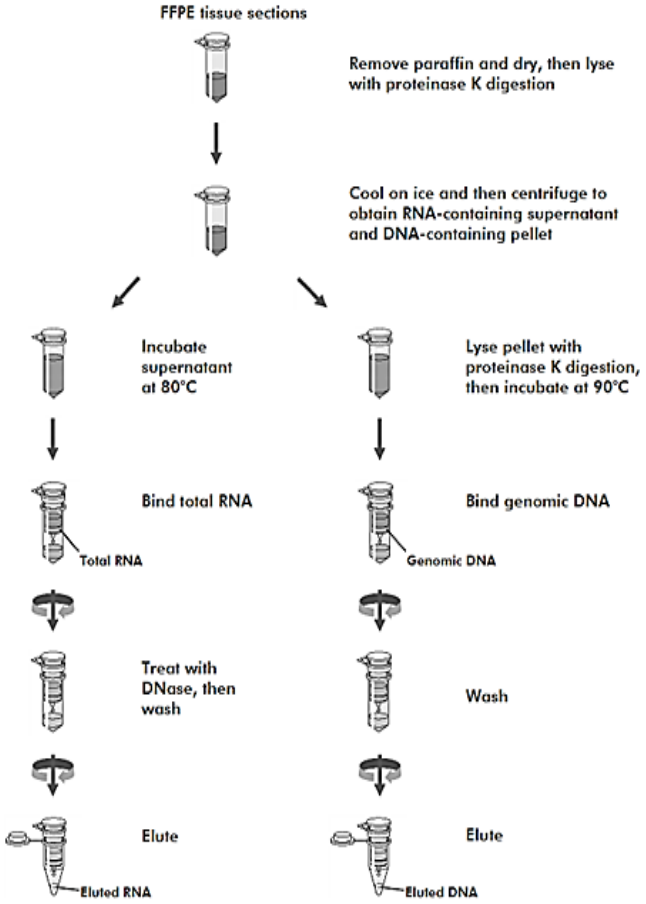

Figure 1: Illustration of the AllPrep DNA/RNA FFPE Co-Isolation and Purification Procedure (AllPrep DNA/RNA FFPE Handbook, Qiagen).

Related documents:

- Manufacturer-supplied protocol for purification of DNA and RNA, **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

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.

2.0 Materials

Workspace	
Sample prep room for all steps	

Equipment and associated consumables		
Agilent	Agilent TapeStation 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 5424, non-refrigerated, without rotor, rotary knobs, 230 V/50 – 60 Hz	
VWR	TUBE MICRO CLR 1500UL PK250	22234-044
	Disposable Safety Scalpels, Sterile, Integra™ Miltex	21909-672
Fisher	Fisherbrand* Premium Microcentrifuge Tubes 2mL, pk/500 (rnase/dnase safe/free)	5408138
Reagent kits		
Qiagen	Qiagen AllPrep DNA/RNA FFPE kit, n=50	80234
	Qiagen Collection Tubes (2ml) (Additional tubes in excess of kit) (additional tubes, not included)	19201
	Qiagen RNase A (2.5mls, 100mg/ml; 7000 units/mL) (not included)	19101
	RNase-Free DNase Set (50) (additional DNase, beyond what is included in kit)	79254
Fisher	CitriSolv™ Hybrid Solvent and Clearing Agent, Fisherbrand™	04-355-121
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:

PM-OICR TGL recommends the following for pathology tissue lab requests: 10 micron sections, minimum n=12, 1 H&E at the top of the sectioning stack to establish tumor cellularity, 10 unstained slides cut onto uncharged slides (if study includes immunohistochemistry assays, charged slides are required), and 1 H&E at the end of the sectioning stack to verify tumor cellularity. If there are discrete areas of tumor, please request the pathologist to circle regions on the two requested H&E slides and score the % tumor cells, % necrosis for the circled region. If the entire tissue section is tumor, the entire tissue may be circled and scored. Ideally at total surface area of >150 mm^2 (15mm^2 tumor surface area X 10 slides) will yield sufficient DNA and RNA for exome and RNA sequencing libraries; macro dissection of multiple slides may be required. A maximum of 600mm^2 of tumor tissue (100mm^2 tumor surface area X 6 slides) may be extracted over 1 purification column set. Tumors with a total surface area of <150mm^2 should be prioritized for DNA or RNA extraction only; separate protocols apply. This information may be detailed in PM-OICR TGL submission sheets. De-identified study codes must be used on all documentation.

- When opening a new kit, record all lot numbers in TGL master lot tracking sheet (R:\Lot_tracking_forms\ 17_07_10_Qiagen_AllPrep_DNA_RNA_FFPE_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).
- Record quants, dilutions, and other QC information in individual sample sheet.
- Use molecular grade H_20 and anhydrous ethanol (brown bottle only!). Always use private ethanol and H_20 aliquots to minimize risk of contamination between technicians.
- 10 mM Tris pH=8.0 can be made by diluting 100 µl of 1M stock in 9900 µl of molecular grade H_20.
- Buffers bottles should be mixed/swirled prior to each use.

- Store FFPE tissue slides at room temperature (long term storage). Process tissue curls as available. Curls may be stored at 4C prior to extraction.
- Date all solutions in box with date received and date of resuspension, 1st use, and ethanol/isopropanol addition where appropriate
- When opening a new kit add isopropanol to buffer FRN, and anhydrous ethanol (brown bottle) to RPE, AW1, AW2 in volumes indicated on bottles and reconstitute DNase I with 550 ul molecular grade H20. When reconstituting DNaseI do not open the vial. Inject lyophilized DNaseI bottle through septum using a rnase/dnase free syringe. Dispose of needle in sharps container. Mix by inversion, then aliquot and store in -20*°C. Never vortex DNaseI solution. Solution may be stored for 9 months, do not refreeze aliquots. Working aliquots should be stored at 2-8°C for a maximum of 6 weeks.* Order part 79254 if running low on DNaseI.
- Purification columns (RNeasy MinElute and QIAamp MinElute), Proteinase K, and lyophilized DNaseI should be stored at 2-8°C upon receipt of kit, after 9 months components may exhibit decreased efficiency
- If precipitate is noticed in buffers RLT, ATL or AL, gently agitate and warm until clear.
- If columns become "messy" with residual liquid on rims of tubes or caps, blot on a stack of folded Kimwipes to remove excess liquid prior to centrifuging. Clean the centrifuge with peroxide wipes/ethanol when contaminated with trace amounts of buffers/eluate.
- Change gloves frequently if they become soiled with salts/solutions.
- **All solutions containing guanidine hydrochloride should be discarded in appropriate waste container labeled with "guanidine hydrochloride/guanidine thiocyanate". DO NOT MIX WITH BLEACH. TOXIC VAPOURS WILL FORM!** *If you are unsure, all solution bottles indicate if they contain guanidine compounds, or you can refer to manufacturer-supplied protocol or MSDS.*
- Record all QC steps in original sample submission sheet, not running sample 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.

4.0 Procedure

1. Macro dissection

1) Using a measuring slide (figure 2) estimate the approximate tumor tissue surface area on H&E slide. Ideally at total surface area of >150 mm^2 (15mm^2 tumor surface area X 10 slides) will yield sufficient DNA and RNA for exome and RNA sequencing libraries; macro dissection of multiple slides may be required. A maximum of 600mm^2 of tumor tissue (100mm^2 tumor surface area X 6 slides) may be extracted over 1 purification column set. Tumors with a total surface area of <150mm^2 should be prioritized for DNA or RNA extraction only; separate protocols apply.

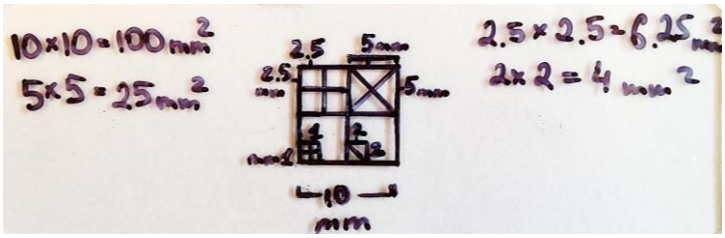

Figure 2: Picture of the microscope slide used for area determination.

2) Using a fine point marker (Sharpie Permanent Marker, Ultra Fine Point, Black) and the H&E slide marked by the pathologist as a guide, outline the area of interest indicated by the pathologist on the **backside** of all slides designated for macro dissection (do not touch marker to paraffin side, use on glass side only).
3) Using a disposable scalpel, scrape the area of interest in precise, long strokes and move scrapings to a labelled 1.5ml tube. Continue until all slides from the sample have been processed. You may use the same blade for all slides from the same sample block.
4) Ensure to clean working area between samples using 70% EtOH, as well as changing out blades and gloves.

Scraping can be performed the day before extraction. Tubes containing scrapings/curls may be stored at 4°C until extraction.

2. Deparaffinization

1) Add 1 ml CitriSolv to each sample, vortex vigorously. Centrifuge at full speed for 2 min.
2) Remove the supernatant. Leave a trace of supernatant behind if necessary to avoid disturbing the pellet.
3) Add 1 ml EtOH (96–100%) to the pellet, vortex for 10 s. Centrifuge at full speed for 2 min.

4) Carefully remove the supernatant, leaving the pellet intact. If necessary, use a fine pipette tip to remove any remaining EtOH.
5) Set the tubes with open lids to incubate at room temperature (or at up to 37°C) for 10 minutes or longer for residual ethanol to evaporate.
6) Add 150 µl Buffer PKD to re-suspend the pellet. Add 10 µl Proteinase K and mix by vortexing.
7) Incubate the tube at 56°C for exactly 15 min. Do not exceed this time, or genomic DNA will be released and co-purify with RNA!
8) Incubate on ice for 3 min (required for efficient precipitation).
9) Centrifuge for 15 min at 20,000 x g.
10) Without disturbing the DNA containing pellet (may be difficult to see), transfer **140 µL** of RNA containing supernatant (leaving ~20 µL behind) to a new 2.0 ml Safe-Lock centrifuge tube for RNA purification. Keep the DNA pellet for DNA extraction.

11) If performing DNA extraction on same day, begin proteinase K digestion as outlined in step 12, otherwise freeze pellet in -80*°C. If DNA extraction is the same day and you do not immediately proceed to step 12, place DNA pellet on ice, or keep in fridge until ready to begin*

12) If isolating DNA same-day, perform the following DNA Purification Steps on. If samples were at 4C, or stored at *-80°C* allow DNA pellet to warm to room temperature before proceeding.
    a. *Confirm ATL has no precipitate prior to use. If it does, heat at 70°C to dissolve! Re-suspend DNA pellet in 180 µl Buffer ATL and add 40 µl Proteinase K. Vortex to mix.
    b. Incubate samples at 56°C for 1 hour with periodic agitation to improve lysis. Heat second block to 90°C in preparation for step 12c, or if using same block, remove sample from block after 1 hour 56°C incubation and keep at room temperature until the block reaches 90°C, then place sample back in 90°C block.
    c. Incubate samples at 90°C for 2 hours **without agitation**. (Proceed to section 4.0, Purification of Genomic DNA). **Use weighted block on top of tubes to ensure tubes do not pop open during incubation.

3. Purification of total RNA

13) Incubate the supernatant containing RNA from Step 10 at 80°C for 15 min.
    Incubate for exactly 15 mins for maximum RNA yields.
14) Briefly spin down the tube containing RNA. Add 320 µl Buffer RLT. Mix by vortexing or pipetting.
15) Add 1120 µl EtOH (96‑100%). Mix well by vortexing or pipetting. Some precipitates may be visible upon addition of ethanol.

16) Transfer a maximum of 700 µl (of 1580 ul) of the sample to an RNeasy MinElute spin column placed in a 2 ml collection tube. Close the lid and centrifuge for 30 s at ≥8000 x g (≥10,000 rpm).
17) **Reload** the flow-through onto the spin column and centrifuge again for 30 s at ≥8000 x g (≥10,000 rpm). **Discard** the flow-through (keep collection tube).
18) Loading the remaining residual sample on the same column, reloading the flow through one time, before discarding flow through. Repeat these steps until total volume 1580 is passed over column.
19) Add 350 µl Buffer FRN to the RNeasy MinElute spin column. Centrifuge with closed lid for 15 s at ≥8000 x g (≥10,000 rpm). **Discard** the flow-through (keep collection tube).
20) Prepare the following master mix: combine 10 µl DNase I stock solution with 70 µl Buffer RDD per sample. Mix by gently by inverting the tube (avoid strong agitation to prevent physical denaturation of enzyme). *Do not refreeze DNase aliquots, store at 4°C for a max of 6 weeks after thawing.
21) Add 80 µl of the DNase I mix from Step 19 directly to the RNeasy MinElute spin column membrane. Ensure all DNase I mix is on column membrane and does not wick to side of plastic o-ring housing. Incubate for 15 min at room temperature.
22) Add 500 µl Buffer FRN to the RNeasy MinElute spin column. Centrifuge with closed lid for 15 s at ≥8000 x g (≥10,000 rpm). **Save the flow-through** (contains RNA including small RNAs).
23) Place the RNeasy MinElute spin column in a new 2 ml collection tube. Apply the flow-through from step 21 to the spin column.
24) Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). **Discard** the flow-through (**keep** collection tube).
25) Add 500 µl Buffer RPE to the RNeasy MinElute spin column. Centrifuge with closed lid for 15 s at ≥8000 x g (≥10,000 rpm). **Discard** the flow-through (**keep** collection tube).
26) Repeat step 25, this time discarding both collection tube and flow-through.
27) Place the RNeasy MinElute spin column in a new 2 ml collection tube. Open the lid, and centrifuge at full speed for 5 min. **Discard** collection tube with flow-through.
28) Place column in new collection tube or 1.5ml tube and spin for 1 minute to ensure all ethanol is removed.
29) Place the RNeasy MinElute spin column in a new 1.5 ml collection tube. Add 50 µl of RNase-free water directly to the spin column membrane. Incubate for 10 min at room temperature. Centrifuge at full speed for 1 min to elute the RNA. Repeat with another 50 µl of RNase-free water. Store purified RNA at -80°C.

The dead volume of the RNeasy MinElute spin column is approximately 2 µl: elution with 50 µl RNase-free water results in a 48 µl eluate.

4. Purification of DNA

30) Ensure **steps 12 a, b, c** are complete before continuing.
31) Briefly spin down the tube. Allow sample to cool to room temperature and then add 4 µl RNase A (100 mg/ml) (or equal available dilution). Incubate for 2 min at RT.
32) Ensure Buffer AL has no precipitate prior to use. If it does, heat at 70°C until dissolved. Prepare the following master mix: combine 200 µl of precipitate free Buffer AL and 200 µl EtOH (96–100%) for each sample, and mix thoroughly by vortexing. *
33) Add 400 µl of mix from Step 30 to each sample, vortexing immediately upon addition. A precipitate may form, but will not impact purification.
34) Transfer the entire sample to a **QIAamp MinElute** spin column placed in a 2 ml collection tube. Close the lid and centrifuge for 1 min at ≥8000 x g (≥10,000 rpm).
35) **Reload** the flow-through onto the spin column and centrifuge again for 1 min at ≥8000 x g (≥10,000 rpm). **Discard** the flow-through.
36) Place the **QIAamp MinElute** spin column in a new 2 ml collection tube. Add 700 µl Buffer AW1 and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. **Discard** the flow-through (**keep** collection tube).
37) Add 700 µl Buffer AW2 to the **QIAamp MinElute** spin column. Centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). **Discard** the flow-through (**keep** collection tube).
38) Add 700 µl ethanol (96–100%) to the **QIAamp MinElute** spin column. Centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). **Discard** the collection tube with the flow-through.
39) Place the **QIAamp MinElute** spin column in a new 2 ml collection tube. Centrifuge the column with an open lid at full speed for 5 min. **Discard** the collection tube with the flow-through.
40) Place column in new collection tube or 1.5ml tube and spin for 1 minute to ensure all ethanol is removed.
41) Place the **QIAamp MinElute** spin column in a new 1.5 ml tube labelled for storage. Add 50 µl of 10 mM Tris pH=8.0 directly to the spin column membrane. Close the lid gently, and incubate for 10 min at room temperature. Centrifuge at full speed for 1 minute.
42) Load another 50 µl of 10 mM Tris pH=8.0 directly onto the spin column membrane. Incubate for 10 min at room temperature. Centrifuge at full speed for 1 min to elute the DNA. Store purified DNA at -20°C.

*The dead volume of the **QIAamp MinElute** spin column is 5 µl: elution with 50 µl 10 mM Tris results in a 45 µl eluate.*

5. Assess Quality and Quantity of the DNA and RNA

1) Use Qubit HS dsDNA assay using TGL SOP to quantify the DNA (SOP_Qubit_Assay_for_Nucleic_Acid_Quantification.docx.).
2) Use Qubit HS RNA assay using TGL SOP to quantify the RNA (SOP_Qubit_Assay_for_Nucleic_Acid_Quantification.docx.).
3) 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_Library_Prep_V.1.3)

5.0 Revision History

Version Number	Date *(yyyy-mm-dd)*	History of change
1.0	2016-11-29	Working SOP for Pugh lab
1.1	2017-07-13	Edits and formatting for TGL by Kayla
1.1.1	2017-07-17	Edits by Dax Torti
1.1.2	2017-08-10	Posted to web
1.1.2.1	2017-08-18	Updated tumor surface area recommendations (DT)
1.1.2.2	2017-09-21	Minor edits of text by Kayla, increasing clarity