Standard Operating Procedure (SOP) for FFPE RNA/DNA Co-Isolation Using the Allprep FFPE Kit and the Highpure miRNA Kits

I. SCOPE AND PURPOSE

Tissues that have been formalin-fixed and paraffin embedded (FFPE) present a challenge to molecular biology. Formalin fixation causes a wide variety of chemical modifications and damage to nucleic acid, including crosslinking and strand breaks. Paraffin embedding renders tissues insoluble in common molecular biology buffers and aggregates nucleic acid in a manner that lowers quality and further complicates efficient extraction.

This protocol uses two technologies: the AllPrep FFPE kit (Qiagen) which is well-suited for purification of DNA, and the Highpure miRNA kit, which is well-suited for purification of RNA (including miRNA). The initial preparation section of this procedure is responsible for deparaffinization and decrosslinking specific to FFPE-derived tissues. The RNA Prep and DNA Prep sections are similar to standard column-based extraction protocols used on fresh or frozen tissues.

The purpose of this SOP is to establish a procedure for co-isolating DNA and RNA from FFPE specimens and applies to all trained Biospecimen Core Resource laboratory personnel.

II. PROCEDURE

A. Safety Precautions

1. Wear personal protective equipment such as lab coats, goggles and gloves.
2. Bloodborne pathogens can be present in the unfixed frozen tissue (refer to SOP S009, "Bloodborne Pathogen and Exposure Control Plan" found in the BCR Safety Manual). Use all universal precautions.
3. Buffers AL and AW1 contain guanidine hydrochloride, which can form reactive compounds when combined with bleach. If liquid containing these buffers is spilled, clean with a laboratory detergent and water; if the liquid contains potentially infectious agents, follow with 1% sodium hypochlorite to disinfect.

B. Quality Control

1. The incoming tissue samples have a printed label with a 2D barcode and human readable format. The 2D barcode contains the internal LabVantage ID; the human readable has the internal LabVantage ID, CCG BCR barcode, and TSS identifier.
2. Working labels (containing the internal LabVantage ID, CCG BCR barcode, and scannable 2D barcode) are printed from LabVantage and used throughout the extraction process. Labels are printed that match the original LabVantage ID (to follow the subportion) and the corresponding newly created (DNA or RNA) LabVantage IDs. Four label colors (Blue, Green, Red, and Yellow) are rotated with each sample. Example: Sample A has blue working labels; Sample B has green working labels, etc.
3. Final storage labels (internal LabVantage ID (DNA or RNA), CCG BCR barcode, TSS identifier, and 2D barcode) are printed for storage in Matrix 2D barcode tubes.

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NOTE: For BLGSP FFPE analytes, DNA is denoted with an "E" and RNA is denoted with an "S". All other projects use "D" for DNA and "R" for RNA.

4. Barcode readers are used throughout the process whenever transferring samples from one tube to another.
5. Samples are tracked in LabVantage. Every subportion or analyte is tracked with its position in a freezer or refrigerator. When a technologist processes a sample, LabVantage displays the user name as having custody of that sample until the sample is checked in to a storage location.
6. DNA and RNA analyte stocks are stored as a primary single tube aliquot (when possible) with a smaller secondary aliquot for sample quality control. RNA quality is measured by RIN/DV200 using Agilent Bioanalyzer (see SOP M002, "RNA Nano Assay") and quantified by Spectrophotometer (see SOP BCR-MGL-EQP-1 "BIO-MATE UV-Visible Spectrophotometer"). DNA quality is evaluated for integrity by agarose gel electrophoresis (see SOP M003, "Gel Electrophoresis with the E-gel System"), quantified by PicoGreen Assay (see SOP M017, "Picogreen DNA Quantification Manual") and genotypic identity using SNP loci (see SOP M010 "Tissue Matching by SNP Analysis"). Primary stock aliquots should not be subject to numerous freeze thaw cycles.
7. No aliquot of original specimen, DNA or any other reagent should ever be returned to the original container after sampling.
8. Any deviations from the protocol as written should be documented on extraction worksheets and at the sample level in LabVantage. Protocol deviations that have the potential to compromise analyte quality (pre-analytical variables) should also be documented with an incident report. Pre-analytical variables include, but are not limited to, abnormal sample condition upon arrival (cracked tubes or clotted blood), temperature excursions (in storage freezer or during extraction), abnormal analyte appearance (cloudy or colored analyte elution), and identity failures.
9. The isolation kit is tested against predetermined specifications to ensure consistent product quality.
10. All new lots of reagents are tested in parallel with the one in current use before being put into use. All kit components must be quality control tested and used together thereafter. All reagents supplied in a kit must be used only with other reagents in the same kit lot number; reagents with identical lot numbers cannot be used interchangeable between kit lot numbers. Results are recorded on positive control extraction worksheets. All QC results are recorded in the Quality Control notebook.
11. At each step in the DNA isolation, the supernatant or pellet that should not contain the DNA is retained until after isolation and quantitation is completed.
12. RNA is extremely susceptible to degradation by ribonucleases that are ubiquitous in the environment. To ensure preservation of target RNA or RNA probes, special precautions are needed.
    a. Bench space is wiped down at the beginning of each extraction session with RNase Zap. Pipettes are wiped down with RNase Zap once a week or as needed.

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b. Gloves should always be worn throughout the process and should be changed frequently to prevent cross-contamination and transfer of ubiquitous RNases.
c. Only sterile, disposable plasticware and pipettes that are dedicated strictly to RNA work and certified RNase-free should be used to prevent cross-contamination with RNases from shared equipment.
d. Containers and tubes with samples must be kept covered when possible during the entire procedure to ensure they remain dust- and RNase-free.
e. All reagents must be made with RNase-free materials and chemicals, and containers and tubes with samples must be kept covered when possible during the entire procedure to ensure they remain dust- and RNase-free.

C. Specimen Information –

1. Type: FFPE tissue (blocks or unstained slides)
2. Handling Conditions: Follow standard precautions when handling all tissue samples. Samples should be stored refrigerated until isolation is commenced.
3. Sample Preparations: FFPE samples are prepared for isolation by Histology; blocks are cut into 10 µM scrolls and placed into a 2-mL safe-lock tube. Optimally, scrolls should be isolated within 24 hours fromtime cut. FFPE unstained slides are scraped by the Histology department and can be stored in the refrigerator until ready for isolation.
4. Indications for Study: This procedure is used when DNA and RNA are needed from the same piece of FFPE tissue. DNA is isolated from the AllPrep FFPE kit and RNA is isolated using the HighPure miRNA kit.

D. Required Equipment, Supplies and Reagents

1. Equipment

a. PPE (Gloves, Lab coat)
b. UV visible spectrophotometer
c. Capsule centrifuge
d. Digital dry bath
e. Microcentrifuge
f. Multi-channel and single channel pipettes
g. Vortexer
h. DNA/RNA Concentrator

2. Supplies

a. Roche Highpure miRNA kit (Roche #05080576001; no substitution)
b. AllPrep FFPE kit (Qiagen #80234; no substitution)
c. 1.5 mL microcentrifuge tubes (Fisher, 05-408-137)
d. 2.0 mL Safe-lock tubes (Fisher, 05-402-7)
e. 0.5 mL microcentrifuge tubes (Fisher, 05-408-128)
f. Matrix 0.5 mL ScrewTop Tube (ThermoScientific #3745)
g. Matrix ScrewCap (Direct Resource #4477 [RED, BLUE, GRE, PUR])
h. Pipettes, 10 µL – 1000 µL, single or multichannel

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i. Filtered tips for pipettes
j. Sodium dodecyl sulfate (SDS) (Fisher #BP166-100)

k. Wet and dry ice
l. Insulating trays for dry ice

3. Reagents

a. Heptane (VWR #BDH1127-4LP; no substitution)
b. Methanol (VWR #BDH1135-4LP; nosubstitution)
c. 100% molecular grade ethanol (Sigma # E7023-6X500mL)
d. RNase-Free DNase (Qiagen #79254)
e. PureLink RNase A (Invitrogen #12091-021)

4. Water, Molecular Biology Reagent (Sigma #W4502)Tris-EDTA Buffer, 100X (Sigma #T9285)
   a. Sodium Hydroxide, 5.0 M (Sigma #S8263)
5. Colored labels: THT, B, WHT 1x0/5”W0.375”DIA: Blue (Y1439894), Red (Y1439892), Green (Y1439893), Yellow (Y1439895) (Brady)
6. Thermal Transfer Printer Labels (Freezerbondz, # THT-152-492-3)
   a.

Notes:
It is possible to substitute disposable materials and certain equipment from other vendors, unless otherwise noted, as long as they are the equivalent to the item described above.

In the event that a reagent or disposable item either becomes contaminated or is suspected of being contaminated, it must be discarded.

E. Reagent Preparation

Note: All reagents are labeled with reagent name, concentration, date opened/prepared, expiration date, storage conditions, and appropriate hazard labeling.

1. 10% SDS: Dissolve 5 g of SDS in 40 mL of Sigma water, and then add Sigma water to 50 mL. This stock solution is stable for 6 months at room temperature.
2. DNase stock solution is prepared by dissolving lyophilized DNase 1 enzyme in 550 µL water that is provided by the kit. Mix gently by inverting the vial. Do not vortex reconstituted DNase 1. DNase 1 is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the vial. For long-term storage of DNase 1, remove the stock solution from the vial, divide it into single-use aliquots, and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.

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3. The Proteinase K provided from the Roche Highpure miRNA kit must be dissolved prior to use. Dissolve the Proteinase K in 4 mL Elution buffer, aliquot and store at -15 to -25°C for up to one year after preparation.
4. Wash Buffer is provided in the Roche HighPure miRNA kit. Before using for the first time, add 40 mL 100% ethanol to the Wash Buffer bottle. Store at room temperature for up to 6 months after adding ethanol.
5. Buffers AW1 and AW2 are supplied as a concentrate in the AllPrep FFPE kit. Before use for the first time, add the appropriate volume of ethanol (96-100%) as indicated on the bottle; for example, add 25 mL of ethanol to the unopened bottle of AW1 to obtain a total of 44 mL, or add 30 mL of ethanol to the unopened bottle of AW2 to obtain a total volume of 43 mL. Write the open and expiration dates; both buffers are stable at room temperature for up to one year after adding ethanol.
6. 0.1X TE is made by diluting a stock solution of 100X TE. Add 1 mL of 100XTE to 999 mL of deionized water. This reagent may be stored at room temperature for up to one year.
7. To prepare 50 mM NaOH: dilute 10 mL of stock 5M NaOH with 990 mL deionized water. This reagent may be stored at room temperature for up to one year.
8. All solutions in the Roche HighPure miRNA kit should be clear. If any solution contains a precipitate, warm the solution prior to use at 37°C to dissolve the precipitate.
9. Before starting the procedure, check whether precipitate has formed in Buffer AL and ATL in the AllPrep FFPE Kit. If necessary, dissolve by heating to 70°C with gentle agitation.
10. The QIAamp MinElute spin columns from the AllPrep FFPE Kit should be immediately stored at 2–8°C upon receipt in the lab. The buffers can be stored at room temperature (15–25°C). Under these conditions, the kit components can be kept for at least 9 months without any reduction in performance.
11. The Proteinase K supplied in the AllPrep FFPE Kit is in a specially formulated storage buffer and is stable for at least 1 year after delivery when stored at room temperature (15–25°C).
12. Buffer AW1 contains guanidine thiocyanate. PPE must be used when handling this reagent.

F. Deparaffinization and Cell Lysis

5. Add 8 x 10 µM scrolls with ~150 mm^2 tissue surface area each to a 2 mL safe lock tube. The number of scrolls can be adjusted to obtain a total tissue surface area of ~1,200 mm^2 (e.g., 16 scrolls with ~75 mm^2 surface area or 12 scrolls with ~100 mm^2 surface area).
6. In the fume hood add 1.4 mL **heptane**, vortex for 10 seconds, and incubate at room temperature for 10 minutes.
7. In the fume hood add 70 µL **methanol**, vortex for 10 seconds, and centrifuge for 2 minutes at 9,000 x g.

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8. Remove the supernatant via pipette without disturbing the pellet. This waste contains heptane and methanol. The supernatant is kept in a 50 mL conical tube at the bench and poured into the glass waste bottle (located in the hood) after the isolation is complete.
9. Add 1 mL 100% **ethanol** to each pellet and mix by brief vortexing.
10. Centrifuge for 2 minutes at 14,000 x g.
11. Remove the supernatant without disturbing the pellet. Retain the supernatant until after the isolation is complete.
12. Leave the cap open and incubate the tissue pellet for 12 minutes at 37°C in the CentriVap.
13. Add 300 µL of **Paraffin Tissue Lysis Buffer** (Highpure miRNA kit), 48 µL **10% SDS** and 120 µL **Proteinase K** working solution (Highpure miRNA kit) to each pellet. Pulse vortex 3 x 5 seconds to mix.
14. Incubate the tubes on a dry block at 55°C for 45 minutes.
15. Centrifuge the samples for 15 minutes at 16,000-20,000 x g. Remove from the centrifuge carefully, insuring the pellet is not disturbed.
16. Transfer the supernatant to a new 2 mL safe-lock tube and continue with the **RNA Extraction** below with the supernatant.
17. Close the tube with the pellet. Continue with the **DNA Extraction** below with the pellet. Note that the RNA and DNA isolation can be conducted simultaneously as there are two long incubation steps in the DNA protocol.

G. **DNA Extraction (Qiagen AllPrep FFPE reagents and columns) –** *Note that the first two steps should be performed before starting the RNA Extraction if performing simultaneously. The RNA Extraction can be completed during step 2 and 3.*

1. Using the pellet from Deparaffinization and Cell Lysis section, re-suspend in 180 µL of **Buffer ATL** (AllPrep FFPE Kit) and 40 µL of **proteinase K** (AllPrep FFPE Kit). Mix by vortexing.
2. Incubate at 56°C in a water bath for 1 hour.
3. Incubate at 90°C in a heat block for 2 hours.
4. Briefly centrifuge at 2,000 x g to remove drops from the inside of lid.
5. Allow sample to cool for 2 minutes at room temperature.
6. Add 4 µL **RNase A** (20 mg/mL). Invert 5 times. Incubate for 2 minutes at room temperature. Briefly spin down.
7. Add 200 µL of **Buffer AL** and vortex for 5 seconds. Briefly spin down.
8. Add 200 µL of 100% **ethanol** and vortex for 5 seconds. Briefly spin down.
9. Transfer entire sample to a QIAamp MinElute spin column (AllPrep FFPE kit) place in a 2 mL collection tube. Centrifuge for 1 minute at 8,000 x g.
10. Inspect columns. If liquid has not passed through the column, then centrifuge at 16,000 x g for 2 minutes and re-inspect. Repeat this step until all liquid has passed through the column and record how many times this step was conducted on the isolation worksheet.
11. Place column in a new collection tube.

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12. Add 700 µL of **Buffer AW1** to the column. Centrifuge for 30 seconds at 16,000 x g. If the entire sample does not go through, then repeat at 16,000 x g for 2 minutes. Repeat again if necessary at 16,000 x g for 1 minute. Record on the isolation worksheet. Place column in a new collection tube.
13. Add 700 µL of **Buffer AW2** to the column. Centrifuge for 30 seconds at 16,000 x g. If the entire sample does not go through, then repeat at 16,000 x g for 2 minutes. Repeat again if necessary at 16,000 x g for 1 minute. Record on the isolation worksheet. Place column in a new collection tube.
14. Add 700 µL of 100% **ethanol** to the column. Centrifuge for 30 seconds at 16,000 x g. If the entire sample does not go through, then repeat at 16,000 x g for 2 minutes. Repeat again if necessary at 16,000 x g for 1 minute. Record on the isolation worksheet.
15. Place the column in a new 2 mL collection tube. Centrifuge for 5 minutes at 16,000 x g to dry the column.
16. Place the column in a clean labeled 1.5 mL microcentrifuge tube. Add 100 µL of 0.1X TE to the column membrane and incubate at room temperature for 1 minute.
17. Centrifuge for 1 minute at 16,000 x g. Discard the spin column and store the sample at in a -80°C freezer or proceed with analysis immediately.

H. RNA Extraction (HighPure miRNA reagents/columns)

1. Take the supernatant from the Deparaffinization and Cell Lysis section.
2. In the fume hood, add 800 µL of **Binding Buffer** (Highpure miRNA kit) to each sample tube and vortex 15 seconds. Briefly spin down the sample.
3. In the fume hood, add 544 µL **Binding Enhancer** (Highpure miRNA kit) to each tube. Vortex 15 seconds. Briefly spin down the sample.
4. Add 700 µL of sample to the High Pure Filter and centrifuge for 30 seconds at 13,000 x g. Discard the flow through in biohazardous waste. Repeat three times with the remaining sample.
5. Centrifuge filter at 14,000 x g for 30 seconds to dry the filter and discard the flow through in biohazardous waste.
6. Add 500 µL of **Wash Buffer** working solution (HighPure miRNA kit) and centrifuge for 30 seconds at 13,000 x g. Discard the flow through in biohazardous waste.
7. Add 300 µL of **Wash Buffer** working solution and centrifuge for 30 seconds at 13,000 x g. Discard the flow through in biohazardous waste.
8. Centrifuge the filter for 1 minute at 14,000 x g to dry the filter.
9. Place the filter into a fresh 1.5 mL Eppendorf tube. Add 100 µL of **Elution Buffer** (HighPure miRNA kit) and incubate for 1 minute at room temperature. Centrifuge for 1 minute at 13,000 x g. Place sample on ice until ready to add the working DNase solution.
10. Make the working DNase solution. Note: DNase I is supplied lyophilized and should be reconstituted as described in "Reagent Preparation Section" before making the working DNase solution. The working DNase solution should be made fresh just prior to use.

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a. 1st Dilution: 1 µL DNase 1 (1500 Kunitz units) + 10 µL Buffer RDD; Mix by gently flicking the tube, and centrifuge briefly to collect residual liquid from the sides of the tube.
b. Working dilution: 1 µL of 1st dilution DNase 1 + 19 µL water (provided by DNase Kit); mix by gently flicking the tube, and centrifuge briefly to collect residual liquid from the sides of the tube.
c. Place the working dilution on ice until ready for use.

11. Add 1 µL of working DNase solution to the eluant. Gently mix by inverting the tube and incubate 30 min in a 37°C water bath.
12. After incubation, briefly spin down the sample.
13. In the fume hood, add 325 µL of **Binding Buffer,** briefly vortex, and briefly spin down the sample.
14. In the fume hood, add 210 µL **Binding Enhancer**, vortex 15 seconds, and briefly spin down the sample.
15. Add the entire sample to a new filter tube and centrifuge for 30 seconds at 13,000 x g. Discard the flow through in biohazardous waste.
16. Centrifuge the filter tube at 14,000 x g for 30 seconds to dry the filter. Discard the flow through in biohazardous waste.
17. Add 500 µL of **Wash Buffer** working solution and centrifuge for 30 seconds at 13,000 x g. Discard the flow through.
18. Add 300 µL of **Wash Buffer** working solution and centrifuge for 30 seconds at 13,000 x g. Discard the flow through in biohazardous waste.
19. Centrifuge the filter tube for 1 minute at 14,000 x g to dry the filter.
20. Place the filter into a labeled 1.5 mL microcentrifuge tube. Add 100 µL of **Elution Buffer** incubate for 1 minute at room temperature.
21. Centrifuge for 1 minute at 13,000 x g. Discard the column and store sample in a liquid nitrogen freezer or proceed with QC immediately.

I. Quantification and Normalization of RNA Samples

1. Prepare a set of 0.5 mL tubes with the unique sample identifier and portion number followed by R (for RNA). Add 98 µL of 50 mM sodium hydroxide to each tube.
2. Add 2 µL of the concentrated stock sample to the sodium hydroxide. Vortex for at least 5 seconds to ensure that the diluted sample is well mixed and briefly spin down.
3. Read the absorbance at 260 nm, 280 nm, and 320 nm in the spectrophotometer using a quartz cuvette.
4. Desired sample concentration may vary among projects.
   a. If the concentration is above the desired concentration use the known volumes and concentrations to calculate the amount of DEPC-treated water to add to the sample to yield a final desired concentration as shown below.
   b. If the concentration of the RNA sample is less-than the desired concentration, concentrate to the desired volume (see example below) using the speedvac with no heat.

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5. After diluting or concentrating samples, repeat steps 1-3 to confirm that the sample is within the target concentration range.
6. When the samples are at the target concentration, transfer to a labeled matrix tube (this is the primary sample aliquot). The matrix tube has a max capacity of 500 µL. If the sample has a volume larger than 500 µL, the sample must be split into multiple matrix tubes and aliquots must be created in LabVantage per BCR-SOP-A005, "LabVantage User Manual". Create a second aliquot for RIN/DV200 determination per BCR-SOP-M002, "RNA NanoAssay".

J. Quantification and Normalization of DNA Samples – Refer to BCR-SOP-M017, "PicoGreen DNA Quantification (Manual).

K. Sample Storage – PRNA samples are stored in the liquid nitrogen freezer; DNA samples are stored in a -80°C freezer.

L. Sample Calculations

1. Example: Samples that need to be diluted (concentrations >0.165 µg/µL)

[(current concentration/desired concentration) x current volume] – current volume
= [(0.21 µg/µL / 0.165 µg/µL) x 18 µL] - 18 µL
= 4.9 µL (volume of diluent to add)

Final sample volume: 22.9 µL (18 µL + 4.9 µL)

2. Example: Samples that need to be concentrated (concentrations <0.165 µg/µL)

[(current concentration/desired concentration) x current volume] – current volume
= [(0.08 µg/µL / 0.165 µg/µL) x 18 µL] - 18 µL
= 9.3 µL (volume to remove during concentration)

Final sample volume: 8.7 µL (18 µL – 9.3 µL)

III. REFERENCES

A. BCR-REF-001, "BCR Acronym List"
B. BCR-REF-002, "BCR Glossary"
C. BCR-SOP-A005, "LabVantage User Manual"
D. BCR-SOP-M002, "RNA NanoAssay"
E. BCR-SOP-M017, "PicoGreen DNA Quantification (Manual)
F. HighPure miRNA Isolation Kit, Roche, March 2013
G. AllPrep DNA/RNA FFPE Handbook, Qiagen, June 2012

IV. COMPREHENSIVE REVISION HISTORY

A. Changes made the Version **2**, Effective Date 8/16/2016
1. Minor word, spelling, and grammatical changes made throughout
2. Updated section II.E., including:

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a. Added specific storage temperatures
b. Changed the stability of buffers from 6 months to up to one year
c. Added instructions to wear PPE when handling guanidine thiocyanate

3. Updated section II.F., including
   a. Added Cell Lysis
   b. Clarified contents and disposal of supernatant
4. Reorganized flow of procedure so that DNA Extraction is now before RNA Extraction.
5. In the RNA Extraction section, clarified the procedure for making the working DNase solution.
6. Updated the procedure in section II.I. for Quantification and Normalization of RNA Samples
7. Removed the details of section II.J. for Quantification and Normalization of DNA samples and left reference to BCR-SOP-M017, "PicoGreen DNA Quantification (Manual)
8. Removed the Criteria for Repeating Extraction and Sample Pooling in section II.K.

B. Version 1, Effective Date 08/04/14 - New

Signatures

Approved By: **Signature on file** **Date:** **Date on file**

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