

ETCTN
Biobanking and Molecular
Characterization Initiative (BMCI)
Laboratory Manual

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Prepared by

S. Percy Ivy, MD, National Cancer Institute

Tim Schulz, PhD, Technical Resources International

Kristie Magee, PhD, Technical Resources International

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1 OVERVIEW

The mission of the National Cancer Institute (NCI) Experimental Therapeutics Clinical Trials Network (ETCTN) is to develop new approaches to cancer treatment based on molecular characterization and biomarker assay development used for patient selection in early phase experimental therapeutic clinical trials. To that end, the NCI has established a central biorepository for the processing and retention of patient specimens across all ETCTN trials, and a central laboratory for molecular characterization at the Frederick National Laboratory for Cancer Research.

This document outlines the complete biospecimen flow, required materials, and Standard Operating Procedures (SOPs) for collection, processing, and shipment of tissue specimens (biopsy and whole blood) collected on NCI-sponsored ETCTN trials.

This document also outlines the procedures, required materials, and other requirements for whole exome sequencing (WES), messenger RNA sequencing (RNA-Seq), and whole genome sequencing (WGS) at the Molecular Characterization Laboratory (MoCha).

2 PURPOSE AND BIOSPECIMEN WORKFLOW

The purpose of this manual is to describe the biospecimen workflow for ETCTN trials contributing materials to the ETCTN Biorepository. This involves collection of specimens at the ETCTN sites and shipping it to the ETCTN biobank (Nationwide Children's Hospital, Columbus, OH) for storage and distribution to the MoCha laboratory. All central biomarker testing will be performed by the MoCha laboratory.

Biospecimen Workflow

Prior to specimen receipt by ETCTN Biorepository		
Site	Register patient and obtain informed consent. Order specimen shipment kit.	
	Collect specimens per protocol requirements (APPENDIX 2B, 2C, 2D, 2E)	
	Log samples into the Sample Tracking System (STS), RAVE (APPENDIX 2A)	
	Ship samples to ETCTN Biorepository	
ETCTN Biorepository		
Shipper/ Receiving Dock	Delivery of shipment to ETCTN Biorepository (Nationwide Children's). Specimens accepted Monday through Thursday only, not on holidays	
ETCTN Biorepository	Opening of shipping kit and examination of specimen containers	
	Receipt logged into STS and the ETCTN Biorepository Specimen Tracking and Reporting System (STARS)	
	Visually inspect specimens and labeling for any inconsistencies or issues.	
	TISSUE	BLOOD
	<p>Transfer of core biopsies in formalin into barcoded cassettes with one core each. Samples will be transported to the histology laboratory for loading in processor for overnight embedding (APPENDIX 4A). Alternatively, cell block procedure for scanty specimens (APPENDIX 4B).</p> <p>Frozen tissue specimens will be barcoded and banked in a liquid nitrogen vapor phase freezer until distribution.</p>	<p>Receive blood in EDTA and cfDNA Streck tubes at ambient temperature.</p> <p>Process whole blood in Streck tubes for PBMCs (APPENDIX 3A) and Plasma (APPENDIX 3A), and aliquot by 1-mL into cryovials.</p> <p>Process whole blood in EDTA for DNA using the Qiagent AllPrep DNA kit (APPENDIX 3F), followed by PicoGreen quantitation and agarose gel electrophoresis QC. Aliquot DNA into 2 x 250 µL aliquots and 1 stock vial.</p>

		Store plasma and DNA in a -80°C freezer until distribution.
		Aliquot and freeze plasma and serum.
	Removal of embedded tissue from processor(APPENDIX 4C).	
	FFPE blocks sectioned and the first slide stained for H&E (APPENDIX 3B). H&E slide transported to the pathologist for confirmation and demarcation of tumor(APPENDIX 3B). Additional unstained slides will be prepared for IHC. RNase-free unstained slides prepared for macrodissection and co-extraction (APPENDIX 3C).	
	Macrodissection of demarcated tumor (APPENDIX 3D)	DNA quantitation by PicoGreen (APPENDIX 3G), RNA quantitation by Nanoassay (APPENDIX 3H).
	Scan the marked H&E slide to a virtual image for future reference. Bank the physical H&E slide for long-term storage.	Store nucleic acid aliquots in a -80°C freezer until distribution.
	AllPrep DNA and RNA extraction from FFPE slide scrapings (APPENDIX 3E)	
	DNA quantitation by PicoGreen (APPENDIX 3G) and QC by agarose electrophoresis gel, and RNA quantitation by spectrophotometer and RNA Integrity Number (RIN) by Agilent (APPENDIX 3H). Aliquot into 2 x 250 ng and a stock vial. Store nucleic acid aliquots in a -80°C freezer until distribution.	

	Additional FFPE tissue remaining after creating unstained slides for IHC and nucleic acid extractions will be banked for long-term storage at ambient temperature.	
	As needed (see below)	
	In batches, ETCTN Biorepository will prepare and send at least 250 ng of DNA (from blood and tumor tissue) and 250 ng of RNA (from tumor tissue) by Overnight Shipping to MoCha lab (APPENDIX 3I)	
MoCha Sequencing Lab		
MoCha Sequencing Lab	Receipt of analysis package/biospecimens for sequencing	
	DNA library prepared for WES (APPENDIX 5B). RNA library prepared for RNA-Seq (APPENDIX 5C). DNA library prepared for WGS (APPENDIX 5D or 5E).	
	Library quantitation using ddPCR for WES, RNA-Seq, or WGS (APPENDIX 5F).	
	Denaturing and clustering of DNA and RNA libraries for sequencing (APPENDIX 5E).	
	Sequencing of WES, RNA-Seq, and WGS libraries (APPENDIX 5H).	
	Data analysis for WES (APPENDIX 5I) and RNASeq (APPENDIX 5J).	

3 COLLECTION OF SPECIMENS

Procedures described in this **Section 3** apply to the **ETCTN clinical sites** enrolling patients and collecting the biospecimens.

Figure 1 presents an overview of the collection and shipping procedures described in this manual.

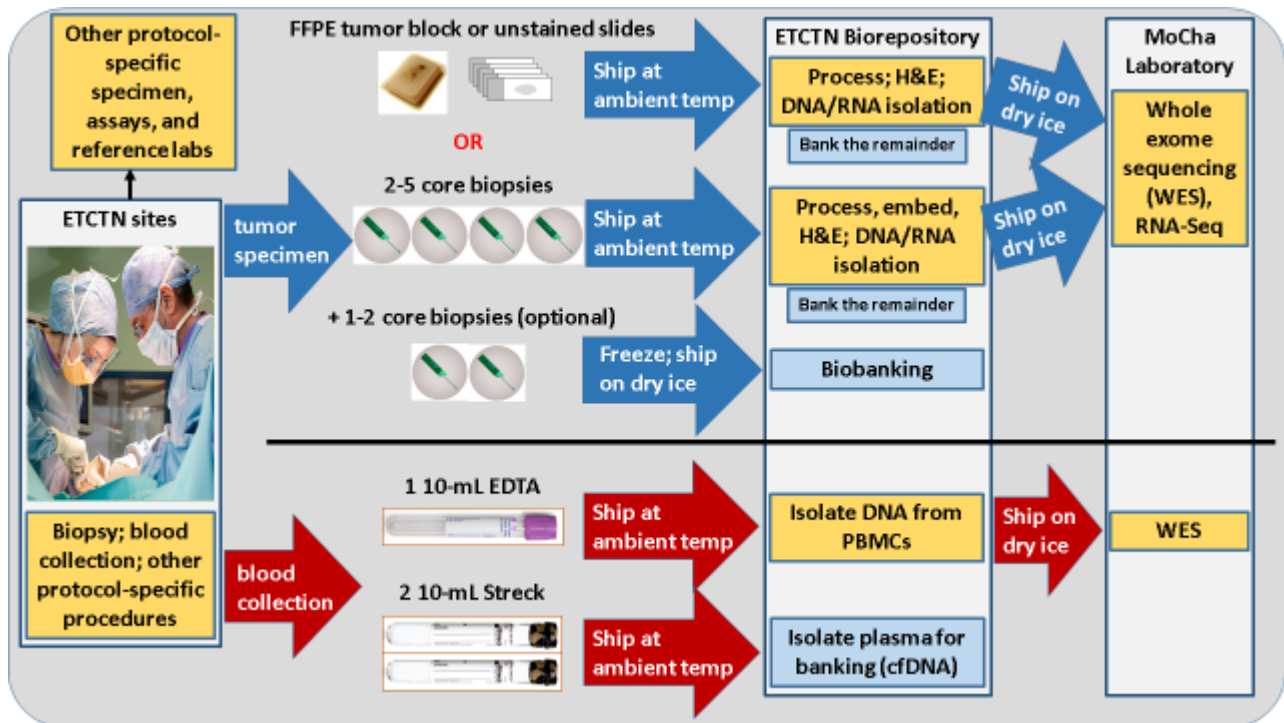


Figure 1. Workflow depicting overall disposition of biospecimens (tumor tissue and blood samples) obtained at ETCTN clinical sites for processing/biobanking at the ETCTN Biorepository and next-generation sequencing at the central laboratory (MoCha).

3.1 Specimen Procurement Kits, Scheduling, and Tracking Sample Collections

3.1.1 Specimen Shipping kit: Kits for the collection and shipment of specimens to the ETCTN Biorepository can be ordered online via the **Kit Management system** (<https://ricapps.nationwidechildrens.org/KitManagement>). Sites will need to set up an account in the Kit Management system and select a specific clinical trial protocol to request a kit. Each user may order two kit types per protocol per day (daily max = 6 kits). Kits are shipped ground, so please allow 5-7 days for receipt. A complete list of kit contents for each kit type is located on the Kit Management system website and in the instructions included with the kits.

3.1.2 Scheduling of Specimen Collections

Tumor tissue cores collected during biopsy procedures that are fixed in the formalin (provided by sites) and shipped on the same day as collection.

Tissue can be collected **Monday through Wednesday**, and shipped immediately overnight (FedEx Priority Overnight strongly preferred to avoid potential delays in specimen receipt and processing) for arrival on Tuesday through Thursday at the ETCTN Biorepository at Nationwide Children’s Hospital. Snap-frozen cores may be shipped Monday through Thursday, since the Biorepository does not need to perform additional time-sensitive pre-analytic processing on them.

Blood specimens should be collected at the same time as tumor tissue collections and shipped in the same package. If, for any reason, blood specimens are collected alone, fresh blood specimens may be collected and shipped Monday through Friday. Saturday delivery is only available for shipments of fresh blood.

Do not ship specimens the day before a US federal holiday. If you are unsure whether the ETCTN Biorepository will be able to receive specimens, then please contact the Biorepository using the contact information provided in Section 3.4.5.

3.1.3 Tracking of Specimens

It is required that all samples submitted on this trial be entered and tracked using the ETCTN Rave Sample Tracking System (STS) (See **Figure 2** for details).

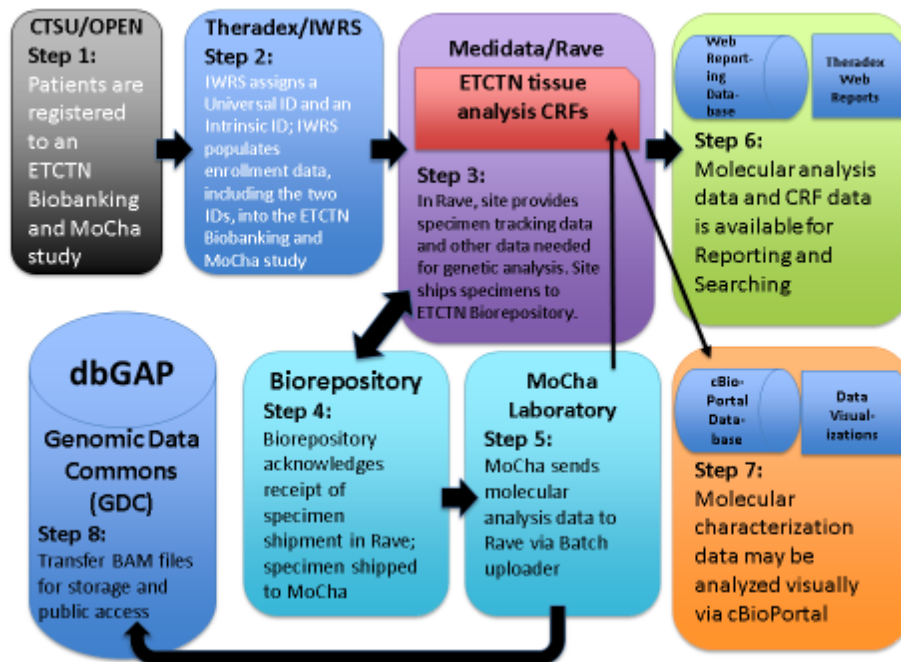


Figure 2. *Steps of specimen tracking via Medidata Rave.*

The STS is accessed through special Rave user roles: “CRA Specimen Tracking” for data entry at the treating institutions and “Biorepository” for users receiving the specimens for processing and storage at reference labs and the Biorepository. If a patient agrees to participate in the ETCTN Biobanking and Molecular Characterization Initiative, the specimen tracking data need to be entered in Rave under the treatment Protocol ####.

Important: Failure to complete required fields in STS may result in assay result reporting delays. Any case reimbursements associated with sample submissions will not be credited if samples are not logged into STS.

Additionally, please note that the STS software creates pop-up windows when reports are generated, so you will need to enable pop-ups within your web browser while using the software.

A user manual is available at
http://theradex.com/downloads/ETCTN_Specimen_Tracking_User_Guide.pdf.

A specimen transmittal form must be completed and a Shipping List report included with all sample submissions.

Please direct your questions or comments pertaining to the STS to link/e-mail.

The date and time of collection and shipping date must be entered into the STS for all submitted specimens. STS shipment manifest must be included in the package.

NOTE: The corresponding anatomical clinical pathology report (see Section 4.3.3) MUST be submitted with the specimen for central confirmation of histology, or the sample will not be processed. The pathology report must state the disease diagnosis made by the reviewing pathologist. If a new anatomical pathology report cannot be obtained, then the most recent available pathology report (identifying the malignancy) may be provided instead.

Detailed instructions for use of the STS can be found in **APPENDIX 2A**.

3.2 Biopsy Collection Procedure: Sample collection at clinical sites

Core needle biopsy (CNB) tumor tissues will be collected, for molecular profiling. Core biopsies at least 1 cm in length will be obtained through Interventional Radiology by a percutaneous approach using a 16-18-gauge needle. Only percutaneous biopsies will be performed on patients with solid tumors. However, excisional biopsy or endoscopic

biopsy is allowed if medically indicated and can be used for analysis. Biopsies will be sent for analyses as defined in the protocol. Two to five core biopsies will be used for FFPE, and (optionally, and contingent on sufficient material and patient consent) 1-2 additional cores will be immediately snap-frozen in vapor phase of Liquid Nitrogen (LN2) as snap-frozen tissue for biobanking.

3.2.1 Fine-Needle Aspirate (FNA)

If fine needle aspiration (FNA) is available, especially for sampling of bone lesions in order to assure specimen adequacy and to avoid specimen decalcification, use of FNA before core needle biopsy is preferred. Real-time cytopathologic immediate evaluation of the FNA specimen can confirm that the chosen target area of a lesion is satisfactory for obtaining the core needle biopsy specimens. This preferred procedure with on-site assessment increases the frequency of successful molecular testing for patient eligibility.

If real-time cytopathology assessment of FNA specimens is not available, collection of FNA specimens prior to core needle biopsy procurement is recommended.

Please refer to **APPENDIX 2B** for the FNA collection procedure and **APPENDIX 2C** for coverslipping instructions.

3.2.2 Collection of Biopsy Specimen using Kits

Specimen jars for collecting tissue in formalin should be labeled with the participant ID, biopsy date and time of collection, protocol number, and type of material. The date and time of collection and shipping date must be entered into the Sample Tracking System (STS) for all submitted specimens.

Specimen size requirement is as follows:

- Surface area of 25mm² is optimal. Minimum is 5mm².
- Volume: 1mm³ optimal. Minimum volume is 0.2mm³.

3.2.3 Excisional Cutaneous Biopsy Specimen Collection and Processing (ALTERNATE PROCEDURE)

These instructions also apply to additional specimens from medically necessary endoscopic, laparoscopic, or surgical procedures performed for clinical care.

1. Verify patient identification, enrollment in OPEN, and signed consent.
2. Complete Sample Submission Form and label blood collection tubes, cassettes and all specimen collection containers.
3. Obtain four 16-gauge or 18-gauge core needle biopsy specimens from the

region of the lesion in which the FNA (IF USED) yielded tumor, place two cores between sponges in each of the two cassettes (total of four cores), snap the cassette lids in place, place the two cassettes into the formalin-filled container as soon as possible after collection to prevent air drying, secure the container lid, and place the container into the shipping kit. If touch preparation slides of cores are made, allow to dry and then place them in the slide cartridge. Secure the slide container lid and place it in the shipping kit.

4. For pathologic examination of removed tissue, place a portion of the biopsy specimen in a labeled specimen container with fixative for transport to your Pathology Department. (This specimen container is not provided in the shipping kit.) The pathology report must be uploaded to Medidata Rave as soon as available.
5. Log the samples in the in the ETCTN Rave Specimen Tracking System, print the STS-generated shipping manifest and corresponding pathology report and place into the shipping kit.

3.2.4 Collection of Snap-Frozen Specimen

1. Pre-label the cryovial with the protocol, patient ID, patient initials, study visit, as well as the date of collection and snap-freeze the biopsy as per **APPENDIX 2D**.
2. Ship frozen on dry ice (Monday to Thursday) to the Biorepository (Nationwide Children's Hospital).

3.2.5 Formalin Fixed Paraffin-Embedded (FFPE) Tumor Sample for Research

Even when patients are able to provide a biopsy specimen, prior (archived) representative tumor tissue block may be requested from patients who consent "Yes" to "I agree to allow my tissue to be submitted for research." Tissue may be submitted with the initial biopsy material or separately.

If previously-collected FFPE will be submitted, then the following criteria must be met:

- Tissue must have been collected within 6 months prior to registration
- Formalin-fixed paraffin-embedded tumor tissue block(s) must be submitted. The optimal block is at least 70% tumor. Specimen size requirement is as follows:
 - Surface area: 25 mm² is optimal. Minimum is 5 mm².
 - Volume: 1 mm³ optimal. Minimum volume is 0.2 mm³, however the success of DNA extraction decreases at suboptimal tissue volume

If an existing block cannot be submitted, the following are requested, if available:

- One (1) H&E slide,
- Twenty (20) 4 μm unstained air-dried plus slides,

- One (1) or more core punches (minimum of 4 mm diameter) from tumor block.

3.3 Blood Samples

Whole blood (WB) samples will also be collected into 1 EDTA (purple-top) tube (10 mL) and 2 cell-free DNA (cfDNA) Streck tubes (2 × 10 mL). to obtain plasma / peripheral blood mononuclear cells (PBMCs). Please refer to **Figure 1**.

3.3.1 Collection in EDTA (purple top) Tube

Collect blood in EDTA tube, and gently invert 5-10 times to mix. Maintain specimens at ambient temperature (room temperature) during collection and transport. Blood (10mL) collected in an EDTA (purple top) tube will be processed for plasma and DNA (see **APPENDIX 3A**) at the biorepository.

3.3.2 Collection in Streck Tubes

Collect blood in two cfDNA Streck tubes (10 mL), Cat No. 218992, and invert to mix. **Note: blood must be thoroughly mixed to ensure preservation of specimen.** After collection, blood in cfDNA Streck tubes **should never be refrigerated**, as this will compromise the specimen. Blood in cfDNA Streck tubes is stable at room temperature. These tubes will be shipped to the ETCTN Biorepository for further processing. Upon receipt at the ETCTN Biorepository, blood in cfDNA Streck tubes will be processed for plasma and DNA (see **APPENDIX 3A**).

3.4 Shipping to the Biobank

Ship the core biopsies, EDTA tube, and Streck tubes as one shipment at ambient temperature **Monday through Wednesday** for overnight delivery to the Biorepository on Tuesday through Thursday. Note: If not collected the same day, blood can be shipped Monday through Friday for receipt Tuesday through Saturday. Do not delay shipment of tissue in formalin. Please use the same box to ship that was used to receive the kit contents.

3.4.1 Labeling of Specimens

Tissue samples are to be labeled with:

- the Rave generated specimen ID (which includes the treatment protocol number and universal patient ID)
- an Intrinsic ID,
- specimen type (P for primary or M for metastatic),
- collection date,
- surgical pathology ID (SPID) number, and
- block number from the corresponding pathology report.

Blood samples are to be labeled with:

- the Rave generated specimen ID (which includes the treatment protocol number and Universal Patient ID),
- an Intrinsic ID,
- specimen type (blood),
- and collection date.

3.4.2 Packing Instructions

The shipment of all human tissue samples must comply with appropriate regulations as specified by the carrier. Frozen samples should be sent on dry ice. At a minimum, all samples must be packaged within two containers with absorbent material between containers to control any spill or leakage. The outer container must be puncture resistant (e.g., cardboard mail tube, corrugated cardboard box). A biohazard sticker must be affixed to both the inner and outer containers.

1. Before packaging specimens verify that each specimen is labeled according to the instructions above and that the lids of all primary receptacles containing liquid are tightly sealed and wrapped in parafilm.
2. Place samples into a biohazard envelope with absorbent material and seal the envelope securely.
3. Place the biohazard envelope into a Tyvek envelope and seal securely.
4. Place the sample(s) and a copy of the shipping manifest and corresponding pathology report (when applicable) and other forms listed in section 3.4.3 into the insulated shipping container.
5. Place the lid on top of the container. Close the outer flaps and tape shut.
6. Attach a shipping label to the top of the shipping container.
7. Place an Exempt Human Specimen sticker to the side of the container.
8. Ship samples via overnight courier (FedEx preferred) to the address listed below.

Additional notes for specimen shipping:

Include a cold (not frozen) pack when shipping specimens during hot weather. In winter months, please include extra insulation, such as bubble wrap inside the shipping container, to prevent specimens from freezing.

When shipping slides, first place them in a plastic slide holder and stabilize by placing cotton or soft paper under the lid. Tape the lid so that it does not pop open during shipment (one piece of tape is sufficient).

3.4.3 Forms to be Included

Copies of the following forms and reports are required to be included with all submissions of pathology materials:

- The pathology report(s) on tumor from the most recent pre-diagnostic biopsy or surgery specimen(s).
IMPORTANT NOTE: Corresponding anatomical clinical pathology report **MUST** be submitted with the specimen for central confirmation of histology, or the sample will not be processed. This can be a prior pathology report on the same malignancy.
- Reports on immunologic, immunohistochemical, or molecular studies, if performed on the pre-trial materials
- A completed Sample Submission Form. This form is to be submitted with tissue collected for research only
- The Sample Tracking System (STS) generated shipping manifest
NOTE: The pathology report for the submitted biopsy material, if pathology review was performed locally, is required to be uploaded into Rave as soon as it is available.

3.4.4 Shipping Address

Ship to the address below the same day as the specimen collection using overnight courier (FedEx), early morning delivery option.

ETCTN Biorepository
Nationwide Children's Hospital
700 Children's Dr., WA1340
Columbus, OH 43205
Phone: (614) 722-2865
FAX: (614) 722-2897
Email: BPCBank@nationwidechildrens.org

FedEx Priority Overnight service is very strongly preferred. There is no central Courier account for this study. Sites are responsible for all costs for overnight shipment per sample shipment to the ETCTN Biorepository, utilizing the site screening and base intervention payments.

NOTE: The ETCTN Biorepository FedEx Account will not be provided to submitting institutions.

3.4.5 Contact Information for Assistance

For all queries, please use the contact information below:

ETCTN Biorepository
Toll-free Phone: (800) 347-2486
E-mail: BPCBank@nationwidechildrens.org

4 PROCESSING & STORAGE OF BIOSPECIMENS

Procedures described in this **Section 4** apply to the **ETCTN Biorepository**.

Core biopsies collected in kits (for FFPE) will be processed by the Biorepository using a Leica ASP6025 processor and paraffin embedded. Biopsies will be sectioned and H&E stained for Aperio scanning and storage of the image, as well as histopathologic analysis. Unstained sections will also be used to isolate DNA/RNA at the Biorepository. DNA/RNA will then be shipped to MoCha lab for sequencing applications. Any unused FFPE blocks will be stored at the Biorepository.

Snap-frozen biopsies received by the biobank will be shipped to the ETCTN Biorepository for biobanking.

Whole blood from both EDTA (purple top) and Streck tubes will be processed. Germline DNA will be isolated from blood; Streck tube samples will be used to isolate cell-free DNA (cfDNA) from plasma. DNA will be shipped to MoCha lab for analysis.

4.1 Processing and Paraffin Embedding of Formalin Fixed Tissue

See **Figure**.

- 4.1.1 The samples will be processed by the sites or ETCTN Biorepository per **APPENDIX 4A**. Cell block for scanty specimens or smears will be performed as per **APPENDIX 4B**.
- 4.1.2 Following processing, the tissue will be paraffin embedded (**APPENDIX 4C**) using a Thermo Histocenter.
- 4.1.3 Slides will be stained with H&E for image scanning and histopathological examination by a designated pathologist as per **APPENDIX 3B**. The H&E section will serve to confirm original diagnosis and also estimate percent tumor content. An adequate biopsy sample will contain at least 50% tumor cells with minimal necrosis and stromal tissue.
- 4.1.4 RNase-free slides: Slides free of RNase will be created as per **APPENDIX 3C**.
- 4.1.5 Macrodissection: For somatic DNA analysis, all specimens will undergo tumor enrichment using a standard macrodissection protocol (**APPENDIX 3D**) to ensure at least 50% tumor whenever possible. Alternate macro- or microdissection SOPs may be used as needed for protocol-specific analyses.

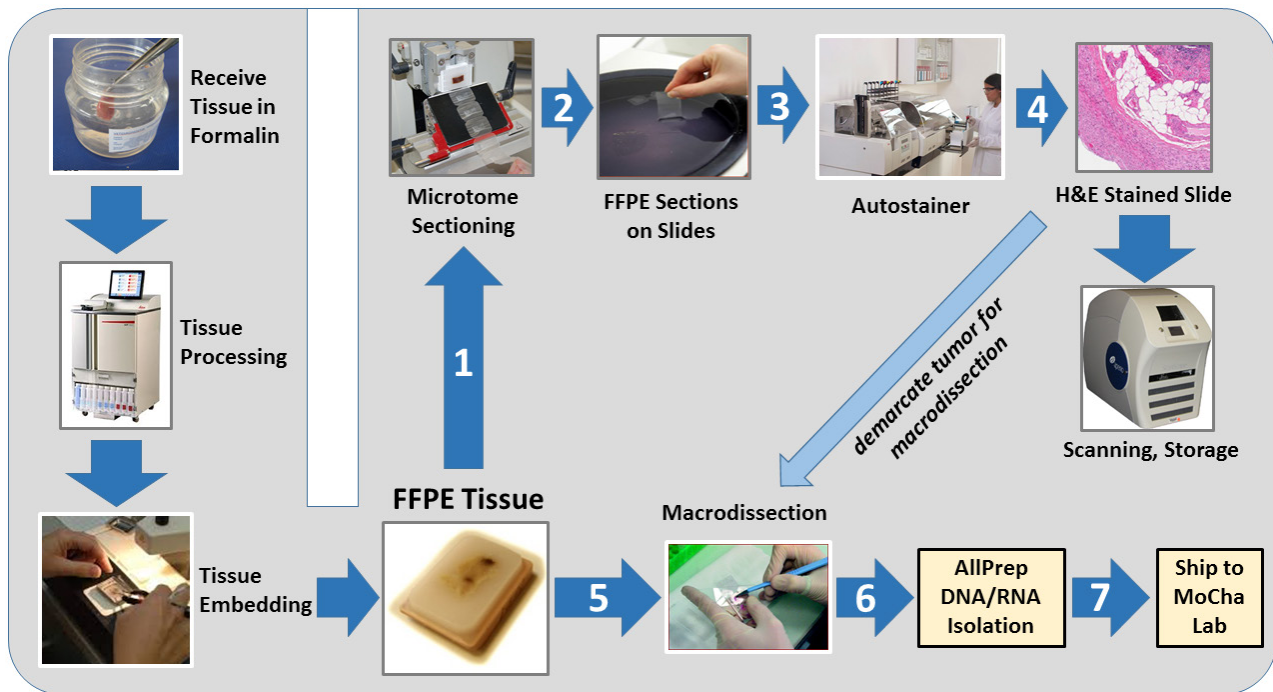


Figure 3. Biopsy workflow at the ETCTN Biorepository depicting tissue processing, embedding, sectioning, staining, macrodissection, and DNA/RNA isolation. First slide is intended for H&E staining; subsequent unstained RNase-free slides may be generated for biobanking.

4.2 Blood processing for Plasma/ PBMCs / Serum

4.2.1 PBMC processing

PBMCs will be isolated from both EDTA (purple top) and Streck tubes (**APPENDIX 2E**).

4.2.2 Plasma processing

Blood samples from Streck tubes will be processed to isolate cfDNA-containing plasma as per **APPENDIX 3A**.

4.2.3 Serum processing

Information on serum processing will need to be provided by the ETCTN Biorepository, and in some cases, another reference laboratory, as it is protocol- and assay-specific. Serum processing will be addressed on a per protocol basis.

4.3 Storage of Specimens at ETCTN Biorepository

Plasma samples will be stored in a -80°C freezer.

PBMCs will be stored in a vapor phase of liquid nitrogen (LN₂) freezer.

FFPE tissue blocks will be stored at ambient temperature.

Snap-frozen tissue biopsies will be stored in a LN₂ vapor phase freezer.

4.4 Isolation of DNA/RNA for Whole Exome Sequencing/Targeted Exome Sequencing (WES/TES) from FFPE specimens and Blood/PBMCs

Biopsy will be extracted for nucleic acids using the Qiagen FFPE All-Prep procedure. 10 uM sections will be obtained for extraction of DNA/RNA. Please refer to **APPENDIX 3E**. DNA will also be extracted from PBMCs as detailed in **APPENDIX 3F**.

4.4.1 DNA quantitation will be performed using PicoGreen as per **APPENDIX 3G**.

4.4.2 RNA quality evaluation will be performed using Bioanalyzer (Agilent) as per **APPENDIX 3H**.

4.5 Shipping of Specimen to Central Molecular Characterization (MoCha) Lab

All specimens that meet necessary quantity and quality will be sent for sequencing to the central MoCha laboratory (**APPENDIX 3I**).

5 SEQUENCING PROCEDURES

Procedures described in this **Section 5** apply to the **Molecular Characterization Laboratory (MoCha)**.

Next-generation sequencing assays (WES, RNA-Seq, WGS) will be performed at the Molecular Characterization Laboratory on the purified DNA and RNA aliquots provided by the Biorepository.

5.1 Whole-Exome Sequencing/Targeted-Exome Sequencing (WES/TES)

WES DNA libraries will be generated using the Agilent SureSelect XT Target Enrichment System as per **APPENDIX 5B**, and quantitated via digital droplet PCR (ddPCR) as detailed in **APPENDIX 5F**. Library samples are denatured, diluted and clustered on the cBot clonal amplification system (**APPENDIX 5G**) in preparation for sequencing on the Illumina HiSeq 2500 (**APPENDIX 5H**).

The pipeline and data analysis specifications for WES data from samples available as tumor-normal pairs is detailed in **APPENDIX 5I**.

5.2 RNA-Seq

RNA libraries will be generated using the Agilent SureSelect XT Target Enrichment System as specified in **APPENDIX 5C**, and quantitated via digital droplet PCR (ddPCR) as per **APPENDIX 5F**. Library samples are denatured, diluted and clustered on the cBot clonal amplification system (**APPENDIX 5G**) in preparation for sequencing on the Illumina HiSeq 2500 (**APPENDIX 5H**).

The pipeline and data analysis specifications for HiSeq RNA-Seq data is detailed in **APPENDIX 5J**.

5.3 Whole Genome Sequencing (WGS)

Whole genome sequencing (WGS) DNA libraries will be generated with PCR amplification (**APPENDIX 5D**) or without PCR amplification (**APPENDIX 5E**) using the KAPA HyperPlus Kit. The libraries will be quantitated via digital droplet PCR (ddPCR) as detailed in **APPENDIX 5F**. Library samples are denatured, diluted and clustered on the cBot clonal amplification system (**APPENDIX 5G**) in preparation for sequencing on the Illumina HiSeq 2500 (**APPENDIX 5H**).

APPENDIX 1: General Info

APPENDIX 1A: Contacts

1. ETCTN Biorepository

Nilsa Del Carmen Ramirez-Milan, M.D.
The Research Institute at Nationwide Children's Hospital
700 Children's Drive
Columbus, Ohio 43205
PH: (614) 722-5450
FAX: (614) 722-2899
Email: BPCBank@nationwidechildrens.org

2. Central MoCha Lab (for WES/RNAseq):

Dr. P. Mickey Williams/Dr. Biswajit Das/Dr. Anand Datta
MoCha Lab, Frederick National Laboratory for Cancer Research
Fort Detrick
Building 433 Room 302
Frederick, MD 21702

3. NCI:

Percy Ivy, M.D.
Cancer Therapy Evaluation Program (CTEP)
National Cancer Institute (NCI)
9609 Medical Center Drive
Bethesda, MD 20892-9728
PH: (240)-276-6565
Email: ivyp@ctep.nci.nih.gov

APPENDIX 2: SOPs for Sites

APPENDIX 2A: Using the Sample Tracking System

All registrations will be performed using the OPEN system, enrolling the patient in the treatment study. Patient IDs for each system will be generated after completion of a single step study, or after the first step of a multi-step study. Registered and Screen Failure registrations will both use this format.

Upon completion of registration, patients enrolling in a study participating in the ETCTN Biobanking and Molecular Characterization Initiative will be placed into the TAC-BIOSPECIMEN COLLECTION cohort, with all applicable registration data flowing from OPEN-IWRS into Rave.

Any data entry errors made during enrollment should be corrected in Rave.

Step 1: Upload Tumor Pathology, tumor profiling, genetic abnormalities, mutation and variant reports into Rave. Enter initial clinical specimen data into Rave:

- **Begin Specimen Tracking CRF:** To begin the tracking process, select protocol time point, specimen category and Labels Needed for each specimen. CRF generates unique Specimen ID.

Step 2: Print labels using report in EDC and collect specimen

- Label specimen and write collection date and time on each label
- Store labeled specimens

Step 3: Complete specimen data entry

- **Initialize Specimen CRF:** Click Save and data is derived from other CRFs.
- **Specimen Transmittal Form:** Enter Collection date and time, provide details on specimen format, type, source.

Step 4: When ready to ship, enter shipment information

- **Shipping Status CRF:** Enter tracking number, your contact information, recipient, ship date once for the 1st specimen in a shipment
- **Copy Shipping CRF:** Select additional specimens to add to an existing tracking number

Step 5: Print shipping list report and ship. Print two copies of the shipping manifest, one to provide in the Box, the other for your own records

Step 6: Click checkbox on **Shipping Status CRF** to email recipient

Standard specimen shipping routes:

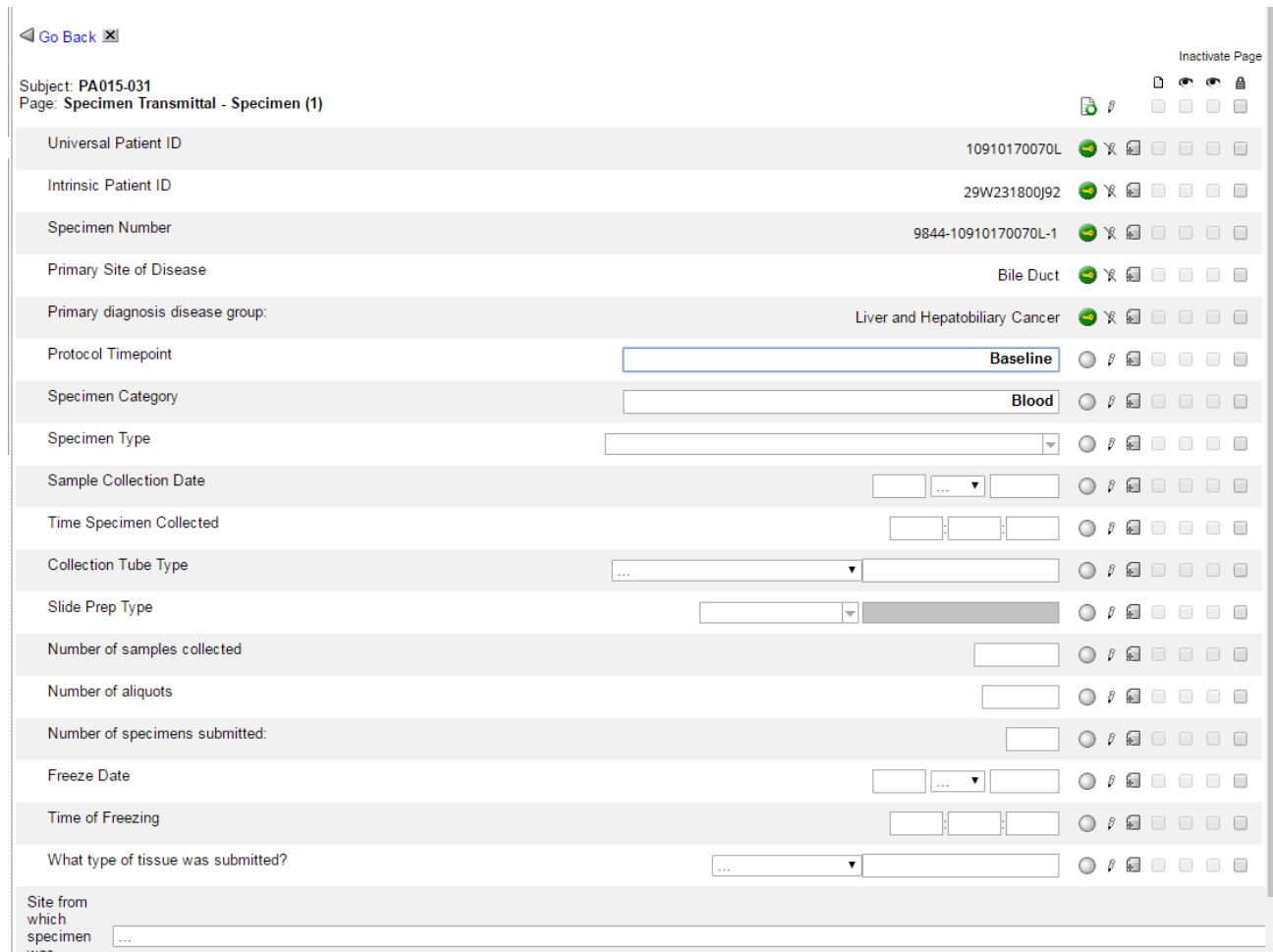
- Treating Site  Biorepository  MoCha Lab

BioRepository/Reference Lab Activities

Using the Biorepository role, the following actions can be undertaken:

- Acknowledge Receipt of Shipment
 - **Shipping Status CRF:** Update status for all specimens received
- Perform additional processing of a specimen
 - **Specimen Transmittal Form:** Reclassify specimen
- Ship specimens to other locations
 - **Shipping Status CRF:** Enter new shipping data and send email alert



















































































































Here is an example of a Specimen Transmittal Form:



◀ Go Back ✕

Inactivate Page

Subject: PA015-031
Page: Specimen Transmittal - Specimen (1)

Universal Patient ID	10910170070L						
Intrinsic Patient ID	29W231800J92						
Specimen Number	9844-10910170070L-1						
Primary Site of Disease	Bile Duct						
Primary diagnosis disease group:	Liver and Hepatobiliary Cancer						
Protocol Timepoint	<input type="text" value="Baseline"/>						
Specimen Category	<input type="text" value="Blood"/>						
Specimen Type	<input type="text"/>						
Sample Collection Date	<input type="text"/> ... <input type="text"/>						
Time Specimen Collected	<input type="text"/> : <input type="text"/> : <input type="text"/>						
Collection Tube Type	<input type="text"/> ... <input type="text"/>						
Slide Prep Type	<input type="text"/>						
Number of samples collected	<input type="text"/>						
Number of aliquots	<input type="text"/>						
Number of specimens submitted:	<input type="text"/>						
Freeze Date	<input type="text"/> ... <input type="text"/>						
Time of Freezing	<input type="text"/> : <input type="text"/> : <input type="text"/>						
What type of tissue was submitted?	<input type="text"/> ... <input type="text"/>						
Site from which specimen was	<input type="text"/>						

APPENDIX 2B: Collection of Fine Needle Aspirates

Title	
SOP	
Modified from	NCI-MATCH Protocol
Effective Date	

PURPOSE

The purpose of the procedure is to describe the collection method for palpable (superficial) and non-palpable (radiologic guided) fine needle aspirations (FNA).

SCOPE

This procedure applies to all technical personnel in cytology.

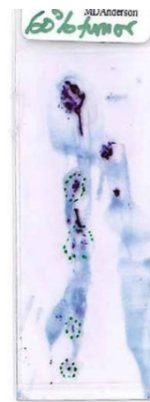
PROCEDURE

1. Verify patient identification, enrollment in OPEN, and signed consents for the associated treatment trial and for this study.
2. Complete Sample Submission Form and label frosted-tip slides, cassettes and all specimen collection containers.
3. Perform image-guided or visually-guided fine needle aspiration (FNA) of lesion.
4. If a cytopathologist and/or cytologic technologist is available on site, immediately fix one slide in 95% ethanol and air dry another slide for each FNA pass. The air dried slide will be stained by Diff-Quik method to assess tumor cell quantity and quality (see examples below).



Optimal specimen:

Satisfactory specimen:



If a cytopathologist and/or cytologic technologist is unavailable for immediate assessment, please skip Step 5 and proceed to Step 6 below.

5. If the first FNA specimen has low tumor cellularity (see illustrations above for examples of satisfactory FNA smears), repeat the FNA in a different location in larger sized lesions (>2 cm) or in a different lesion if one is accessible.
6. If on-site cytopathologic assessment is not available, collect tumor sample by FNA. Label slides and prepare smears for each FNA pass. Immediately fix one slide in 95% ethanol in a coplin jar and air dry another slide. Place the air dried slide in the slide cartridge provided. Secure the lids of the coplin jar and wrap with parafilm. Secure the lid of the slide container. Place both in the shipping kit.

APPENDIX 2C: Instructions for Coverslipping Fine Needle Aspirate Specimens

Title	
SOP	
Modified from	MD Anderson SOP, DP CYT 0011K
Effective Date	

PURPOSE

Mounting medium is a substance that acts as a permanent bond between the slide and the coverslip. It protects the cellular material from air-drying and shrinkage, and acts as an effective seal against oxygen to prevent stains from fading. It must be miscible with the clearing agent, and must have a refractive index that closely matches the specimen and glass slide to yield the best possible transparent image.

SCOPE

This procedure applies to all technical personnel in cytology.

SPECIMEN REQUIREMENT

Slide should be kept in xylene and not be allowed to air-dry prior to application of mounting media Supplies/equipment

SUPPLIES

1. Cytoseal XYL mounting medium
2. Dropper and bottle (optional)
3. Xylene
4. Paper towel
5. Gauze (4x4)
6. Coverslip
7. Slide warmer

SPECIAL SAFETY PRECAUTIONS

Disposable gloves
Disposable gowns

PROCEDURE

Method 1: 3-6 drops of Cytoseal XYL™ are placed on the face of the slide and the coverslip is gently rolled onto the slide until the mounting media spreads over the entire surface of the slide.

Method 2: Coverslip is placed on a paper towel and 3-6 drops of Cytoseal XYL™ are placed on the coverslip and the slide is then rolled onto the coverslip face down until the mounting media spreads over the entire surface of the slide

Using a paper towel or gauze excessive Cytoseal XYL is blotted and the slides are kept on slide warmer to be dried. If air bubbles form, remove by gently pressing on the slide. Wipe around the edges or clean with artist brush dipped in xylene as necessary.

APPENDIX 2D: Collection of Tissue for Snap-freezing and Viable Cryopreservation of Biopsies

1.0 PURPOSE

This Standard Operating Procedure (SOP) outlines standardized procedures for sites to snap freeze tumor tissue.

4.0 Materials and Equipment:

4.1 Reagents

HBSS Lonza or equivalent
Cryoserv (DMSO) Bioniche or equivalent
Human AB+ serum (freeze in aliquots) Gemini or equivalent

4.2 Supplies

Plastic serological pipettes Falcon/BD or equivalent
Large Petri dishes or 24-well tissue culture plates Nunc or equivalent
1.8ml cryovials Nunc or equivalent
#21 disposable scalpels Feather Safety Razor
Isopropanol (for Mr Frosty)

4.3 Equipment

Biological safety cabinet (Class II, type 2A), certified
Pipette-aid Drummond or equivalent
Autoclaved forceps Fine Science Tools or equivalent
-80°C freezer
Liquid nitrogen storage tank
Mr. Frosty freezing container

4.4 Material Preparation

1. Prepare 1X Hank's Buffered Salt Solution (HBSS) by diluting at 10X stock solution in sterile water (1:9). Store HBSS at 4°C.
2. Prepare 10% DMSO freezing medium by diluting DMSO in HBSS (1:9). The freezing medium should be made fresh before cryopreservation and stored at 4°C until chilled.
3. Use a steel razor blade for the systematic chopping of tissue. Before use, sterilize the razor blade by submersing in 70% ethanol for 2 hr. Immediately before tissue processing, rinse the razor blade with sterile water 3 times. Avoid leaving the razor in sterile water for an excessive amount of time, as it is prone to oxidation.
4. Load a Mr. Frosty freezing container with cryovials (2.5 mL) and then place in a sterile biological cabinet. Add 1 mL of chilled freezing medium to each vial. Place the freezing container at 4°C for at least 2 hrs.

5.0 PROCEDURE

5.1 Snap Freezing of Tumor Tissue

5.1.1 Prepare materials and equipment, and label the required number of cryovials.

5.1.2 Freeze fresh tumor tissue as soon as possible. Optimally, freeze tissue within 30 minutes from resection.

5.1.3 Do not freeze the tissue directly on ice.

5.1.4 Ensure that the resected tissue never desiccates, nor is contaminated by surrounding tissue or other samples. Use clean scalpels and forceps between samples to avoid cross contamination between samples or between tumor and normal tissue.

5.1.5 Using clean forceps, place the specimen to be frozen into an empty screw capped cryovial in sterile HBSS.

5.1.6 Using sterile forceps, transfer tissue to a large Petri dish or an upturned lid of a 24-well tissue culture plate. Keep the tissue bathed in HBSS so it doesn't dry out.

5.1.7 Dissect away any unwanted portions of the tissue sample (e.g. fatty tissue, necrotic tissue) (generally not applicable for core needle biopsies)

5.1.8 Using a scalpel, gently cut the tissue into small fragments.

5.1.9 Using forceps, gently transfer fragments into 1.8ml labeled cryovials (for ~1x1x2mm fragments, put ~4 fragments/vial; for ~1x1x0.2cm fragments, put 1 piece/vial).

5.1.10 Immediately add 1ml of cryopreservation medium* into each cryovial. Ensure that tissue is immersed in the medium.

5.1.11 Transfer cryovials to Styrofoam box with dry ice and ship with serum samples to the ETCTN Biorepository.

* Cryopreservation medium: 10% DMSO/90% HAB serum (keeps for 1 week at 4°C)

5.1.12 Ship frozen on dry ice (Monday to Thursday) to the ETCTN Biorepository at Nationwide Children's Hospital.

APPENDIX 2E: Collection of Blood Samples from Patients in Streck Cell-Free DNA BCT Tubes

1.0 PURPOSE/SCOPE

This procedure describes the method for collection of patient blood in Streck Cell-Free DNA BCT tubes. The sites will use this protocol to collect the patient blood in Streck Cell-Free DNA BCT tubes. **This SOP is for research purposes only, and no clinical samples will be processed using this SOP. Any deviation from this SOP will be noted but will not be formally documented.**

2.0 REAGENTS/MATERIALS*

10ml Cell-Free DNA Streck BCT tube	Streck Cat# 218961 (6 tube pack) or Cat#218962 (100 tube pack) or Cat #218992 (1000 tube pack)
------------------------------------	--

3.0 PROCEDURE

3.1 Collect specimen by venipuncture according to CLSI H3-A66.

Prevention of Backflow - Since Cell-Free DNA BCT contains chemical additives, it is important to avoid possible backflow from the tube.

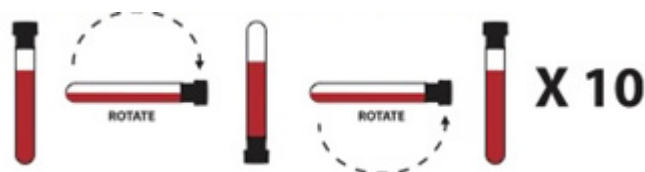
To guard against backflow, observe the following precautions:

- Keep patient's arm in the downward position during the collection procedure.
- Hold the tube with the stopper in the uppermost position so that the tube contents do not touch the stopper or the end of the needle during sample collection.
- Release tourniquet once blood starts to flow in the tube, or within 2 minutes of application.

Important: Follow recommendations for order of draw outlined in CLSI H3-A66. Cell-Free DNA BCT should be drawn after the EDTA tube and before the fluoride oxalate (glycolytic inhibitor) tube. If a Cell-Free DNA BCT tube immediately follows a heparin tube in the draw order, Streck recommends collecting a non-additive or EDTA tube as a waste tube prior to collection in the Cell-Free DNA BCT.

3.2 Fill tube completely.

3.3 Remove tube from adapter and immediately mix by gentle inversion 8 to 10 times. Inadequate or delayed mixing may result in incorrect analytical results or poor product performance. One inversion is a complete turn of the wrist, 180 degrees, and back per the figure below:



- 4.4. After collection, transport and store tubes within the recommended temperature range using the provided kit.

Note:

1. For best results, a 21G or 22G needle is advised. Slower fill times may be observed when using a smaller gauge needle.
2. When using a winged (butterfly) collection set for venipuncture and the Streck Cell-Free DNA BCT is the first tube drawn, a non-additive or EDTA discard tube should be partially drawn first in order to eliminate air or “dead space” from the tubing.

APPENDIX 3: SOPs for Biorepository

APPENDIX 3A: Processing Plasma from Whole Blood

Principle: The purpose of this procedure is to provide instructions for processing serum and plasma from fresh blood received in the Biopathology Center (BPC) and to outline the differences in these specimen types. Protocol instructions should be referred to in order to confirm processing instructions, when applicable. Streck tubes are must be stored at ambient temperature. **DO NOT REFRIGERATE BLOOD IN STRECK TUBES.**

I. Equipment

- Computer
- Centrifuge
- Biological Safety Cabinet
- Biological Fume Hood

II. Materials

- 15mL Conical Tubes
- Wire tube racks
- Sterile Transfer Pipettes
- 1000 μ L Pipette Tips
- 1.8mL red cap cryovials
- 1.8mL green cap cryovials
- Manual Digital Pipette
- Drummond/AquacueJet Pipetter
- 10mL serological pipette tips

V. Procedure

- 1.1.1 Place the anti-coagulated (heparinized, or EDTA or Streck) blood collection tube(s) on a rocker located in the biological safety cabinet until the specimen is visually mixed thoroughly.
- 1.1.2 Pre-label a new 15-mL conical tube
- 1.1.3 Place conical tubes upright in tube racks located in the biological safety cabinet.
- 1.1.4 Place the blood collection tube(s) in the centrifuge.
- 1.1.5 Centrifuge the blood collection tube(s)
 - a. Centrifuge the EDTA, NaHep or LiHep tubes at 1,200 x g for 10 minutes at room temperature with the brake on.
 - b. Centrifuge the Streck tube(s) at 1,500 x g for 10 minutes at room temperature with the brake off.
- 1.1.6 Remove the blood collection tube(s) from the centrifuge and place in a wire rack in the biological safety cabinet.
 - a. Distinct layers should be present after centrifugation.
 - i. The plasma layer will be clear and yellowish, and comprise the top layer.
 - ii. A thin, white, and cloudy buffy coat layer will be visible just below the plasma layer.
 - iii. The bottom layer will be red and contain other cell types and debris.

- 1.1.7 Line up the centrifuged collection tubes in the tube rack in the same order as the pre-labeled, empty 15mL conical tubes.
- 1.1.8 Working with one specimen at a time, verify the label on each centrifuged collection tube matches the label on the corresponding empty, pre-labeled 15-mL conical tube, and remove the cap from both.
- 1.1.9 Remove only the plasma layer from the centrifuged collection tube using a new sterile transfer pipette and place the plasma into the empty 15-mL conical tube.
 - 1.1.9.1 Avoid contact with the buffy coat layer.
 - 1.1.9.2 If the buffy coat layer is disturbed, the vial will need to be spun again.
- 1.1.10 Place cap on collection tube. The remaining specimen may be processed for peripheral blood mononuclear cells (PBMCs), white blood cells, or DNA.
- 1.1.11 FOR STRECK TUBES ONLY. If processing plasma from EDTA, NaHep or LiHep move on to 12.
 - 1.1.11.1 Obtain a second labeled 15 mL conical tube.
 - 1.1.11.2 Place the original 15mL conical tube with plasma in the centrifuge and balance.
 - 1.1.11.3 Centrifuge the original 15-mL conical tube with plasma at 3000 x g for 10 minutes at room temperature with the brake on.
 - 1.1.11.4 Remove the blood collection tube(s) from the centrifuge and place in a wire rack in the biological safety cabinet.
 - 1.1.11.5 Line up the centrifuged collection tubes in the tube rack in the same order as the pre-labeled, empty 15mL conical tubes.
 - 1.1.11.6 Working with one specimen at a time, verify the label on each centrifuged collection tube matches the label on the corresponding empty, pre-labeled 15-mL conical tube, and remove the cap from both.
 - 1.1.11.7 Remove only the plasma from the original conical tube using a sterile transfer pipette and place the plasma into the 15 -mL conical tube. Avoid contact with the debris layer on the bottom of the tube. If the debris layer is disturbed, the vial will need to be spun again.
- 1.1.12 Hold the 15mL conical tube at eye level and visually determine the amount of plasma obtained.
- 1.1.13 Record the total volume of plasma obtained.
- 1.1.14 Using a manual digital pipette and a new 1000 μ L pipette tip OR a Drummond/AquacueJet pipetter and new serological 10mL pipette tip, aliquot plasma into the corresponding, pre-labeled 1.8mL green cap cryovials.

APPENDIX 3B: H & E Staining

Title	H&E Staining
SOP	TVEC_ETCTN_6
Modified from	
Effective Date	

I. PRINCIPLE

This procedure is for H&E staining of FFPE slides.

III. REQUIRED EQUIPMENT, SUPPLIES, AND REAGENTS

Equipment

Microtome, water bath, oven, hotplate, cold plate, microscope, staining racks, Leica Multi stainer.

Supplies

Coverslips
Slides
Absorbent wipes

Reagents

Alcohol ACS/USP Grade
Americlear
Hematoxylin Shandon Instant
Eosin Y: Anatech
Hydrochloric Acid
Ammonium Hydroxide
Permaslip: Alban Scientific

V. REAGENT PREPARATION (INCLUDING STORAGE CONDITIONS)

Anatech Eosin comes ready to use in gallon containers. They are stored at room temperature under the fume hood with the H&E stainer.

Prepare Hematoxylin as stated on the package. Mix one bottle Part A and one bottle Part B in a 2L beaker. Add 1L of distilled water (DO NOT USE TAP WATER). Shake until dissolved. Wait at least one hour before using. Best results after 12 hours. Filter before use.

Acid Alcohol- 1 liter 70% ethanol + 2.5ml 12N HCl

Bluing solution: 1% ammonium hydroxide in dH₂O

VII. PROCEDURE – STEPWISE

1. Turn the machine on, toggle switch on right side of stainer.
2. Load the staining containers with the appropriate chemicals as per protocol (about 450mL/ container).

Station 1 – Citrus Clear

Station 2 – Citrus Clear

Station 3 – 100% Ethanol

Station 4 – 100% Ethanol

Station 5 – 95% Ethanol

Station 6 – Weigert's Hematoxylin (not needed for routine H&E)

Station 7 – DI H₂O (not needed for routine H&E)

Station 8 – water wash

Station 9 – water wash

Station 10 – water wash

Station 11 - water wash

Station 12 – water wash

Station 13 – Citrus Clear

Station 14 – 100% Ethanol

Station 15 – 100% Ethanol

Station 16 – 95% Ethanol

Station 17 – Eosin

Station 18 – Acetic Acid 1% (not needed for routine H&E)

Station 19 – 95% Ethanol (not needed for routine H&E)

Station 20 – Bluing

Station 21 – Acid Alcohol

Station 22 – Hematoxylin

Station 23 – Schiff Reagent (not needed for routine H&E)

Station 24 – Periodic Acid (not needed for routine H&E)

Station 25 – Exit station – no reagent

Station 26 – Biebrich Scarlet/ Acid Fuchsin (not needed for routine H&E)

Station 27 – DI H₂O (not needed for routine H&E)

Station 28 – Phosphomolybdic acid (not needed for routine H&E)

Station 29 – Aniline Blue (not needed for routine H&E)

Station 30 – DI H₂O (not needed for routine H&E)

Station 31- 70% (not needed for routine H&E)

Station 32- 0.01N HCl (not needed for routine H&E)

Station 33- Sirius Red (not needed for routine H&E)

Station 34- Phosphomolybdic Acid (not needed for routine H&E)

Station 35- Load station – no reagent

Station 36- Load station – no reagent

3. After assuring good quality of control slide, place slides in basket for autostainer.

4. Place basket on machine in container in drawer.
5. Choose program "H&E" (or use red clip for human tissue or a yellow clip for animal tissue. The run will start automatically.
6. Remove slide rack from last basket when finished and the machine beeps. Cover slip under hood. Coverslip with Permount and a #1.5 Glass Coverslip.
7. Cover all solutions on the stainer when finished at end of day. Turn off water to the machine and turn off stainer.

If need to run H&E by hand use the following times:

Heat fix on Slide warmer @ 60C for 30+ min, then dry in 60°C oven for 20-60min

Xylene or Citrus	3 min
Xylene or Citrus	3 min
100% Ethanol	2 min
100% Ethanol	2 min
95% Ethanol	2 min
Running Tap Water	2 min
Hematoxylin	4 min
Running Tap Water	2 min
Acid Alcohol	10 sec
Running Tap Water	2 min
Bluing	10-30 sec
Running Tap Water	1 min
95% Ethanol	30 sec
Eosin-Y	2 min
95% Ethanol	30 sec
100% Ethanol	1 min
100% Ethanol	1 min
Xylene or Citrus	2 min
Xylene or Citrus	2 min

Coverslip under the hood with Permount and a #1.5 Glass Coverslip

Do NOT permit the slide to dry out before coverslipping!

Proceed to macrodissection (**APPENDIX 3D**).

IX. REFERENCES

Carson Freida L.PhD, HT (ASCP) Histotechnology A Self-Instructional Text 2nd ED, 1996 pp. 90-102 ASCP Press

APPENDIX 3C: Procedure for Preparing RNase Free Slides or Scrolls (for both DNA and RNA Applications)

PRINCIPLE

This procedure details the steps needed to prepare paraffin slides or scrolls to maximize the RNA quality and quantity on extraction. RNA is susceptible to degradation from Rnases and other enzymes. Fixation and processing of tissues also damages RNA quality and quantity. Keeping samples cold and working quickly helps to maximize recovery.

REQUIRED EQUIPMENT, SUPPLIES, AND REAGENTS

Equipment:	Microtome Chill plate Water bath
Supplies:	Membrane coated slides or membrane coated coverslips Glass Slides Nitrile gloves disposable microtome blades 4x4 gauze kimwipes Sterile 1.8 mL cryovials Camel hair brush Glass dishes Forceps Ice cube trays
Reagents:	RNase Away or RNase Zap DEPC water

PROCEDURE – STEPWISE

RNase handling:

To avoid sample contamination, change blades between specimens. Change gloves between each sample. Wipe microtome paten (stage) with gauze soaked in RNase Away or RNase Zap. Wipe again with gauze soaked in DEPC treated water. Wipe all tools (brushes, forceps) with RNase Away and then with DEPC treated water. Clean glass dishes with RNase Away or RNase Zap and rinse with DEPC treated water. Wipe surface of water bath between samples to avoid cross contamination.

FOR SLIDES:

- a) Chill block on chill plate using small amount of DEPC treated water in clean glass dishes. Once well chilled, place block holder on microtome and orient so block face is parallel to knife edge. Optimum angle will vary for each microtome (usually between 5 and 15 degrees).
- b) Trim off any excess paraffin if necessary. Block face should be square or trapezoidal.
- c) Trim into block face until you get a complete section of the face of the tissue. Trim away at least 3-5 sections and discard these.
- d) Carefully advance block with flywheel to start taking sections.
- e) Take one 4-micron section and float on room temperature DEPC treated water bath to expand section and remove wrinkles. Using an uncharged slide, transfer to 40-45°C DEPC treated water bath briefly for final expansion of sections.
- f) Pick up expanded section on properly labeled, cleaned membrane slide. Center section on membrane, avoiding edges of the membrane. Place slide upright in ice cube tray to drain.
- g) Once drained, store slides in nuclease free box at 4°C until needed.
- h) Proceed to DNA/RNA isolation as per **APPENDIX 3E**.

FOR SCROLLS

- i) Chill block on chill plate using small amount of DEPC treated water in clean glass dishes. Once well chilled, place block holder on microtome and orient so block face is parallel to knife edge. Optimum angle will vary for each microtome (usually between 5 and 15 degrees).
- j) Trim into block face until you get a complete section of the face of the tissue.
- k) Trim off any excess paraffin if necessary. Nucleotide extraction is best if paraffin is minimized. Leave only a small amount of paraffin surrounding the tissue.
- l) Carefully advance block with flywheel to start taking sections.
- m) Take one 10 -15-micron scroll or thickness as requested.
- n) Pick up scroll with clean forceps and place in properly labeled cryotube. Continue until all scrolls are collected.
- o) Store cryovials at 4 degrees until needed.
- p) Proceed to DNA/RNA isolation as per **APPENDIX 3E**.

TISSUE	THICKNESS
For DNA/RNA All-Prep 9to be sent to MoCha lab)	~10 microns

APPENDIX 3D: Macrodissection

I. PRINCIPLE

Macrodissection is the gross manual dissection of a tissue sample (OCT embedded frozen tissue sample or a formalin fixed paraffin embedded (FFPE) tissue sample) guided by a histological section. It can be performed using two techniques: block trimming (when the target tissue makes up a significant part of the biospecimen) and target tissue dissection, (when the target tissue comprises a small focus or foci within the biospecimen). The technique used will be chosen by the review pathologist (who will examine the initial histological section and identify the target tissue areas to be dissected) based on the nature of the tumor sample, with the goal of maximally enriching the amount of usable tissue.

II. REQUIRED EQUIPMENT, SUPPLIES, AND REAGENTS

a. Equipment: paraffin tissue embedder

b. Supplies:

- Razor blades
- Gauze 4x4's
- Disposable scalpels
- RNase free vials
- Vial labels

c. Reagents

- 95% ethanol
- RNase Away
- DEPC water

III. PROCEDURE

1. For macrodissection of blocks:
 - i. Clean the work area (countertop, block holder), gloves, and all the tools (razor blades, scalpels) with 95% ethanol and allow to air dry. Followed by cleaning with RNase away. All areas and items will be wiped dry with sterile gauze and rinsed with DEPC H₂O and wiped dry again with sterile gauze.
 - ii. All tissue blocks should be accompanied by an annotated H&E stained tissue slide or virtual microscopy image
 - iii. QC the specimen data on the tissue block and slide to ensure that they match, and that the tissue block face matches the H&E stained tissue slide.
 - iv. The area designated for macrodissection should be marked by comparing the annotated H&E stained tissue slide to the tissue block face.

- v. Using a blade mounted on a scalpel, carefully scratch or score a shallow line on the surface of the tissue block. This line (or lines) should match the annotation on the H&E stained tissue slide or virtual microscopy image.
 - vi. Unwanted areas may be removed by carefully slicing into the tissue block along the scored surface with a blade mounted on a scalpel.
 - vii. Cut into the tissue block for the full thickness of the tissue.
 - viii. To fully remove the unwanted areas, make a horizontal slice under the tissue that is to be removed.
 - ix. This tissue chunk may be re-embedded if desired in a separate tissue block.
 - x. The macrodissected tissue block can now be used to make new slides that can be scraped and sent for nucleic acid extraction. Briefly, the tissue blocks will be sectioned using RNase free methods and tissue sections collected on uncharged glass slides.
2. For macrodissection of tissue slides (from previously-sectioned or freshly-sectioned unstained slides):
- i. Clean the work area, gloves, and all the tools (razor blades, scalpels) with 95% ethanol and allow to air dry. Followed by cleaning with RNase away. All areas and items will be wiped dry with sterile gauze and rinsed with DEPC H₂O and wiped dry again with sterile gauze.
 - ii. QC the specimen data on the H&E stained tissue slide (or tissue block) and the unstained tissue slide to ensure that they match, and that the tissue block face matches the H&E stained tissue slide.
 - iii. If banked slides are submitted, they may need to be cleaned
 - (a) Rinse slides in DEPC H₂O and allow to air dry overnight in a clean dust free environment.
 - iv. Tissue slides ready for macrodissection will be compared to the annotated H&E stained tissue slide from the same tissue block.
 - v. Tissue slides can be marked on the back with guidelines comparable to the annotated slide. Areas for macrodissection are then removed by carefully scraping the slide with a blade mounted on a scalpel.
 - vi. Once the unwanted tissue is removed, the slides may be scraped and forwarded for nucleic acid extraction.

APPENDIX 3E: DNA/RNA Isolation from FFPE Tissues using Qiagen AllPrep FFPE (DNA) and Roche Highpure miRNA (RNA) Kits

Note: This is the SOP to be used by Nationwide Children’s Hospital. The SOP to be used by MoCha is in the Appendix: SOPs for MoCha Reference Lab.

Title	Standard Operating Procedure (SOP) for FFPE RNA/DNA Co-Isolation Using the All Prep Kit
SOP	TACP_ETCTN_11
Modified from	Nationwide Children’s SOP, M027, Version 2
Effective Date	

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.

II. PROCEDURE

A. 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 μ L – 1000 μ L, single or multichannel
- 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; no substitution)
- c. 100% molecular grade ethanol (Sigma # E7023-6X500mL)
- d. RNase-Free DNase (Qiagen #79254)
- e. PureLink RNase A (Invitrogen #12091-021)
- f. Water, Molecular Biology Reagent (Sigma #W4502) Tris-EDTA Buffer, 100X (Sigma #T9285)
- g. Sodium Hydroxide, 5.0 M (Sigma #S8263)
- h.

B. 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 μ L 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 $2-8^{\circ}\text{C}$ for up to 6 weeks. Do not refreeze the aliquots after thawing.
3. 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.
4. 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.

5.

C. Deparaffinization and Cell Lysis

1. Add 8 x 10 μM scrolls with $\sim 150 \text{ mm}^2$ 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 $\sim 1,200 \text{ mm}^2$ (e.g., 16 scrolls with $\sim 75 \text{ mm}^2$ surface area or 12 scrolls with $\sim 100 \text{ mm}^2$ surface area).
 2. In the fume hood add 1.4 mL **heptane**, vortex for 10 seconds, and incubate at room temperature for 10 minutes.
 3. In the fume hood add 70 μL **methanol**, vortex for 10 seconds, and centrifuge for 2 minutes at 9,000 x g.
 4. 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.
 5. Add 1 mL 100% **ethanol** to each pellet and mix by brief vortexing.
 6. Centrifuge for 2 minutes at 14,000 x g.
 7. Remove the supernatant without disturbing the pellet. Retain the supernatant until after the isolation is complete.
 8. Leave the cap open and incubate the tissue pellet for 12 minutes at 37°C in the CentriVap.
 9. 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.
 10. Incubate the tubes on a dry block at 55°C for 45 minutes.
 11. Centrifuge the samples for 15 minutes at 16,000-20,000 x g. Remove from the centrifuge carefully, insuring the pellet is not disturbed.
 12. Transfer the supernatant to a new 2 mL safe-lock tube and continue with the **RNA Extraction** below with the supernatant.
 13. 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.
- D. 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.
 12. Add 700 μ L 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 μ L 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 μ L 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 μ L 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.
- E. RNA Extraction (HighPure miRNA reagents/columns)**
1. Take the supernatant from the Deparaffinization and Cell Lysis section.
 2. In the fume hood, add 800 μ L 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 μ L **Binding Enhancer** (Highpure miRNA kit) to each tube. Vortex 15 seconds. Briefly spin down the sample.
 4. Add 700 μ L 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 μ L 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 μ L 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 μ L 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.
 - a. 1st Dilution: 1 μ L DNase 1 (1500 Kunitz units) + 10 μ L 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 μ L of 1st dilution DNase 1 + 19 μ L 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 μ L 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 μ L of **Binding Buffer**, briefly vortex, and briefly spin down the sample.
14. In the fume hood, add 210 μ L **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 μ L of **Wash Buffer** working solution and centrifuge for 30 seconds at 13,000 x g. Discard the flow through.
18. Add 300 μ L 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 μ L 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.

F. 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 μ L of 50 mM sodium hydroxide to each tube.
2. Add 2 μ L 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.
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, "Hi

G. Sample Calculations

1. Example: Samples that need to be diluted (concentrations $>0.165 \mu\text{g}/\mu\text{L}$)

$$\begin{aligned} & [(current\ concentration/desired\ concentration) \times current\ volume] - current\ volume \\ & = [(0.21\ \mu\text{g}/\mu\text{L} / 0.165\ \mu\text{g}/\mu\text{L}) \times 18\ \mu\text{L}] - 18\ \mu\text{L} \\ & = 4.9\ \mu\text{L} \text{ (volume of diluent to add)} \end{aligned}$$

$$Final\ sample\ volume: 22.9\ \mu\text{L} \text{ (} 18\ \mu\text{L} + 4.9\ \mu\text{L)}$$

2. Example: Samples that need to be concentrated (concentrations $<0.165 \mu\text{g}/\mu\text{L}$)

$$\begin{aligned} & [(current\ concentration/desired\ concentration) \times current\ volume] - current\ volume \\ & = [(0.08\ \mu\text{g}/\mu\text{L} / 0.165\ \mu\text{g}/\mu\text{L}) \times 18\ \mu\text{L}] - 18\ \mu\text{L} \\ & = 9.3\ \mu\text{L} \text{ (volume to remove during concentration)} \end{aligned}$$

$$Final\ sample\ volume: 8.7\ \mu\text{L} \text{ (} 18\ \mu\text{L} - 9.3\ \mu\text{L)}$$

H. Quantification and Normalization: Perform QA/QC of DNA/RNA as per **APPENDIX 3H, 3I.**

I. Sample storage: Store the samples in the -80°C freezer.

APPENDIX 3F: DNA Isolation from Blood using Qiagen QIAamp Kit

I. SCOPE AND PURPOSE

Isolation of large quantities of DNA from blood presents a unique challenge. Blood contains few nucleated cells in a relatively large volume. Qiagen QIAamp Kits are designed to isolate high molecular weight DNA from up to 2 mL of blood or buffy coat. They provide a fast and simple method for the preparation of up to 60 µg of DNA. The purified DNA is ready for use in standard downstream applications such as DNA amplification and microarray.

Any deviation from the Standard Operating Procedure (SOP) will be noted in LabVantage and on the isolation worksheet; the number of the samples affected by the deviation will be noted as well.

II. PROCEDURE

A. Required Equipment, Supplies and Reagents

1. Biological Safety Cabinet or Splash Guard
2. 1.5 mL microcentrifuge tubes (Fisher, #05-408-137)
3. 15 mL Falcon tubes (Fisher, catalog# 14-959-49B)
4. Pipettes, 50 µL – 1,000 µL, single or multichannel
5. Filtered tips for pipettes
6. Absolute ethanol, molecular grade (Sigma, E7023)
7. QIAamp DNA Blood Midi Kit (no substitution, Qiagen, #51185). Do not use Protease supplied with kit.
8. Proteinase K (10mL) (Qiagen, #19133)
9. 70°C water bath
10. 37°C Water bath
11. RNase A, 10 mg/mL (Qiagen , #158924)
12. 1X Phosphate-buffered saline (PBS)(Invitrogen, #10010-031)
13. Tris-EDTA Buffer (100X) (Sigma, T9285)
14. Water nuclease-free (Sigma, # W4502-6X1L)
15. Centrifuges for 15 mL tubes (swinging bucket rotor)
16. Vortex Mixer
17. Capsule Centrifuge
18. Wet and dry ice
19. Insulating trays for dry ice
20. Racks to hold 15mL conical tubes and microtubes
21. Serological pipets for large volumes (5, 10, 25 mL)
22. Pipet-Aid (or equivalent) Colored labels: THT, B, WHT 1x0/5"W0.375"DIA: Blue (Y1439894), Red (Y1439892), Green (Y1439893), Yellow (Y1439895) (Brady)
23. Thermal Transfer Printer Labels (Freezerbondz, # THT-152-492-3)

B. Reagent Preparation (including storage conditions)

1. 0.1X TE is made by diluting a stock solution of 100X TE. To prepare 1 liter: add 1 mL of 100X TE to 999 mL of deionized water. This reagent may be stored at room temperature for up to one year.

C. Genomic DNA Extraction

1. Add up to 2 mL of sample (blood or buffy coat) to a 15 mL centrifuge tube. If less than 2 mL of sample is available, bring the volume of the sample up to 2 mL with 1X PBS.
2. Add 4 µL of RNase A and mix briefly by inversion (Note: do not vortex enzymes).
3. Add 200 µL of QIAGEN Proteinase K and mix thoroughly by repeated inversion.
4. Add 2.4 mL of Buffer AL and mix thoroughly by inverting the tube 15 times followed by additional vigorous shaking for at least 1 minute.
5. Incubate in a 70°C water bath for 10 minutes. (Incubation for longer than 10 minutes will not adversely affect sample quality/yield, but should not exceed 1 hour.)
6. Add 2 mL of ethanol (96% – 100%) to the sample and mix by inverting the tube 10 times followed by additional vigorous shaking for 10 seconds.
7. Carefully transfer one half of the solution from step II.F.7. onto the QIAamp Midi column by carefully dispensing the liquid into the column, taking care not to moisten the rim.
8. Close the cap and immediately centrifuge at 3,000 rpm (1610 x g) for 3 minutes (room temperature).
9. Remove the QIAamp Midi column and place the QIAamp Midi column into a new 15 mL centrifuge tube. Load the remainder of the solution from step II.F.7. onto the QIAamp Midi column. Close the cap and centrifuge again at 3,000 rpm (1610 x g) for 3 minutes (room temperature).
 - a) If necessary, repeat step to obtain a dry column.
10. Remove the QIAamp Midi column and place the QIAamp Midi column into a new, labeled 15 mL centrifuge tube.
11. Add 2 mL of Buffer AW1 to the QIAamp Midi column. Close the cap and centrifuge at 5,000 rpm (4472 x g) for 1 minute (room temperature). Do not discard the flow-through, but do not allow it to come in contact with the column.
12. Carefully, without moistening the rim, add 2 mL Buffer AW2 to the QIAamp Midi column. Close the cap and centrifuge at 5,000 rpm (4472 x g) for 15 min (room temperature).
13. Carefully transfer the QIAamp Midi column to a clean, labeled 15 mL centrifuge tube. Do not allow the flow-through to come in contact with the column during transfer.
14. Pipet 300 µL of 0.1X TE buffer directly onto the membrane of the QIAamp Midi column and close the cap. Incubate at room temperature for 5 minutes and centrifuge at 5,000 rpm (4472 x g) for 2 minutes (room temperature).

15. Transfer the flow-through from the 15 ml conical tube into a labeled 1.5 mL microcentrifuge tube. The label will contain the unique sample identifier of the DNA analyte.

D. **Sample Storage** – Store the samples in the -80°C freezer.

III. REFERENCES

A. QIAamp DNA Midi Blood Handbook June 2012

APPENDIX 3G: Picogreen DNA Quantification

I. SCOPE AND PURPOSE

PicoGreen is an ultra-sensitive fluorescent nucleic acid stain used for quantifying double-stranded DNA (dsDNA). Unlike many other DNA quantitation methods, PicoGreen is minimally affected by the presence of lipid, protein, carbohydrates and other cellular components, making it an ideal method to quantify dsDNA in cell or tissue lysates or other unfractionated matrices.

This SOP establishes a procedure for determining the concentration of double-stranded DNA in a sample and applies to all laboratory personnel. Any deviation from this SOP will be noted on the PicoGreen assay worksheet; the number of the samples affected by the deviation will be noted as well.

II. PROCEDURE

A. Reagents, Equipment, And Supplies

1. Single channel pipettes
2. Multi-channel pipettes (only to be used where noted)
3. Pipet-Aid
4. Pipette tips, assorted sizes
5. Serological pipettes, assorted sizes
6. Sample to be analyzed
7. Picogreen assay kit (Invitrogen, P7589)
 - a. Picogreen Reagent
 - b. Tris-EDTA Buffer (10 mM tris, 1 mM EDTA), pH8.0, 20X
 - c. 100 ng/ μ L of Lambda (λ) DNA, sheared by intensive vortexing
8. 50 mL conical tube
9. 1.5 mL micro-centrifuge tubes
10. Water nuclease-free (Sigma, W4502-6X1L)
11. FilterMax F5 Multi-Mode Microplate Reader (Molecular Devices)
12. Excitation Filter – EXP1 F535/25, F485/20, F485/20, F370/TRF
13. Emission Filter – EMP1 F595/35, F535/25, F535/25, F535/25, F625/35
14. Grenier Bio-one black flat bottom 96 well plate (Bio-Express, T-3025-16)
15. Foil seal (BioRad, MSF-1001)
16. ABgene 96-well PCR Plate, Non-Skirted (Fisher, AB-0600L)
17. Reservoirs, Sterile, 25 mL with divider (Diversified Biotech, RESE-2000)
18. CentriVap DNA Concentrator (Labconco, or equivalent)
19. Vortex Mixer
20. Minifuge Centrifuge
21. Centrifuge for spinning 96-well plates

B. Dilution of Reagents

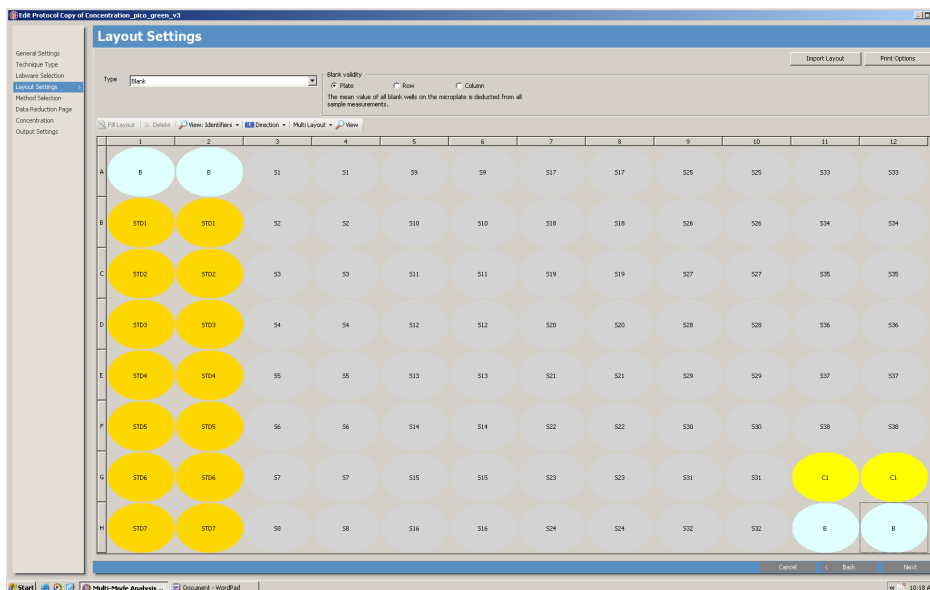
1. Preparing 1X TE from 20X TE Stock:
 - a. To make a 50 mL aliquot: add 2.5 mL 20X TE to 47.5 mL sterile water.

- b. To make 1 Liter: add 50 mL of 20X TE to 950 mL of sterile water.
 - c. 1X TE can be stored at room temperature and expires one year from date prepared. The container should be labeled with the Stock 20X TE lot number, date prepared, date of expiration, applicable hazards warnings, and preparer initials.
2. Dilute the PicoGreen reagent 1:200 by adding 5 μ L of stock dye per 1 mL 1X TE. PicoGreen is a light sensitive dye and should be protected from light when possible. Keep this reagent in the dark until ready for use.
 - a. Each well used will need 100 μ L of the diluted PicoGreen reagent; prepare enough 1:200 PicoGreen for number of samples to be analyzed (including controls and calibration standards).
 - b. The PicoGreen reagent is stored refrigerated and expires one year from date opened.
3. 100 ng/mL DNA standard is stored refrigerated and expires one year from date opened.

III. PROCEDURE STEPWISE

A. Preparation of Samples

1. Each sample is run in duplicate (two separate dilutions, both run once on the same plate). For example, sample #1 (S1) will be run in wells A3 and A4, sample 2 (S2) in wells B3 and B4, and so on throughout the plate up to 38 samples per plate. A duplicate reading of the control DNA used to make the standard curve is run in wells G11 and G12. This control comes with the kit at a concentration of 100 ng/ μ L. If the average value of these duplicates falls outside 15% of that (85-115 ng/ μ L) the plate must be repeated. A negative control is also read in wells H11 and H12, where no control DNA or sample is added. See the example below.



2. Make a DNA dilution plate using a 96-well PCR plate. Add 49 μL of 1X TE and 1 μL of stock sample DNA or control DNA for each dilution as appropriate. Control DNA is placed in positions G11 and G12. Pipet all dilutions up and down to mix.
3. Cover the plate with a foil seal, vortex and spin down.

B. Preparation of Standards

1. The first two columns of each plate are used to make a serial dilution in duplicate of the standard curve. Add 100 μL 1X TE into all wells of columns 1 and 2 (A1 through H1 and A2 through H2) of a black flat bottom Greiner plate (PicoGreen plate).
2. Add an additional 96 μL 1X TE into wells H1 and H2 of the same plate.
3. Pipette 4 μL of standard DNA (100 ng/ μL) into wells H1 and H2 and mix by pipetting. Wells H1 and H2 will be the highest points of the standard curve.
4. Serial dilutions are then prepared by transferring 100 μL of standard beginning from well H1 to G1, and H2 to G2 using a multichannel pipet. Pipet up and down to mix.
5. Continue the serial dilution up the plate transferring 100 μL from G1 to F1, F1 to E1, E1 to D1, D1 to C1 and C1 to B1 (same steps with column 2), mixing by pipetting in between each transfer. At this point take the extra 100 μL from wells B1 and B2 and discard, leaving A1 and A2 as blanks. Now all wells in the first two columns of the PicoDNA plate should have a volume of 100 μL .
6. This set of dilutions will give you a standard curve from 0-1,000 pg/ μL . Adding the subsequent dilutions made to the samples and plates, another 1:1,000 dilution (1:50 dilution for the stock DNA plate, 1:10 dilution when that stock DNA plate is added to the pico plate, and an additional 1:2 dilution when the pico green is added to the plate) is applied and adjusts the standard curve to read from 0-1000 ng/ μL .
 - a. H1/H2 – 1,000 ng/ μL
 - b. G1/G2 – 500 ng/ μL
 - c. F1/F2 – 250 ng/ μL
 - d. E1/E2 – 125 ng/ μL
 - e. D1/D2 – 62.5 ng/ μL
 - f. C1/C2 – 31.25 ng/ μL
 - g. B1/B2 – 15.625 ng/ μL
 - h. A1/A2 – blank

NOTE: The software used to analyze the data is programmed with the necessary factors to correct for concentration and dilutions applied to samples/controls on the bench.

C. Addition of Diluted DNA samples to prepared Black flat-bottom 96-well “PicoGreen” Plate

1. To the black flat bottom PicoGreen plate (containing prepared standard serial dilutions), add 90 μL of 1X TE to all wells that will have samples or controls.
2. Add 10 μL of the diluted DNA from the 96-well PCR plate to the black flat bottom PicoGreen plate according to the plate layout. Pipet up and down to mix.

3. Add 100 μL of the prepared PicoGreen reagent to all samples, controls, and standards. Pipet up and down to mix, avoiding the addition of bubbles.
4. Immediately proceed to Section D (assay is time sensitive once PicoGreen has been combined with DNA samples).

D. Load samples into the Fluorimeter plate

NOTE: the FilterMax should be turned on and the software opened prior to adding PicoGreen to samples so the incubation time of the PicoGreen plate can be controlled.

1. Preparing FilterMax 5 Software:
 - a. Samples will be measured using the excitation at 485 nm and the emission at 535 nm with the Molecular Devices FilterMax F5 Fluorimeter .
 - b. Turn on the power to the FilterMax F5.
 - c. Once the FilterMax F5 has completely powered on, double-click on the Multi-Mode Analysis Software.
 - d. Select the protocol "Concentration_pico_green_v3."
 - e. Click Run.
2. Click "Eject Plate Carrier."
3. Load prepared PicoGreen plate onto the FilterMax F5 to be read with position A1 in the upper right hand corner.
4. Click "Close Plate Carrier."
5. Click "Run." The program is designed to incubate the samples for 5 minutes before reading. (If the plate has already incubated for the appropriate time, this step can be skipped by clicking the next/skip step button in the software.)
6. Once the program is completed, record the "Correlation" and "R²" values on the worksheet. Verify that the standard curve fits a linear model. If not, the entire plate will need to be repeated.
7. Four files will appear after the run. Save the file labeled "Sheet 2" to a flash drive,. This file includes the concentration data for each sample. Use the flash drive to open the data file.

E. Data Analysis

1. Create standard curve to calculate the average concentration values, and normalization volume needed for each sample. If samples are outside the standard curve (concentration greater than 1,000 ng/ μL) they will need to be diluted and run again to obtain an accurate concentration.
2. This worksheet uses the average concentration value from the duplicate dilutions for each sample to normalize the sample to 160 ng/ μL \pm 15 ng/ μL using the following calculation:

$$\text{volume} = ((\text{current concentration} / \text{desired concentration}) \times \text{current volume}) - \text{current}$$

volume

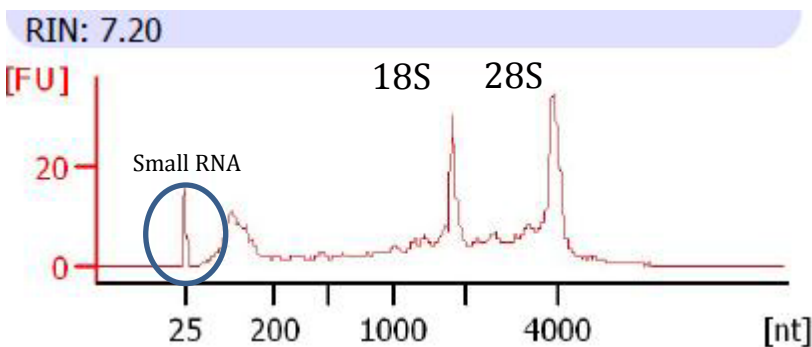
- a. This calculation will give the amount of diluent (0.1X TE) to be added for samples that are \geq 175 ng/ μL , or the target volume for concentrating samples that are \leq 145 ng/ μL .

- b. Concentration ranges may vary between projects, and as such the target concentration and range used for calculations can be adjusted if required.
3. After adjusting the volume to normalize the sample, record the adjusted volume. Repeat this procedure to confirm that the target concentration has been achieved.

APPENDIX 3H: RNA Nanoassay

II. SCOPE AND PURPOSE

RNA samples frequently contain low levels of degradation that are difficult to detect by standard gel electrophoresis methods, but which may hinder downstream applications. Such degradation can be detected and analyzed by the Agilent Bioanalyzer. This instrument estimates the concentration of RNA and calculates the amount of 18S and 28S ribosomal RNA in each sample. In addition, the Bioanalyzer calculates the RNA integrity number (RIN), from the electrophoretic trace of the RNA sample, which includes the presence of degradation products. The assigned RIN is independent of sample concentration, instrument, and analyst, therefore becoming a de facto standard for measuring RNA integrity. Small RNAs are apparent when using this kit, see diagram below. This assay should be used to determine the overall integrity of RNA following nucleic acid extraction.



III. PROCEDURE

(1)

B. Equipment and materials

1. Agilent RNA 6000 NanoChip kit (Agilent, 5067-1511)
 - a. 25 RNA Nano Chips
 - b. 2 electrode cleaners
 - c. 1 syringe
 - d. RNA Nano Dye Concentrate
 - e. RNA Nano Marker
 - f. RNA 6000 Nano Gel Matrix
 - g. Agilent RNA Nano 6000 molecular weight ladder
 - h. 4 spin filters
2. 0.5 mL RNase-free microcentrifuge tubes
3. Sterile, nuclease free water (Fisher, BP-2484-50)
4. RNase Zap (Ambion, AM9780)

5. Thermocycler or dry heat block
6. Microcentrifuge
7. Pipette man
8. Pipette tips, assorted sizes
9. Wet ice
10. Vortex mixer
11. Agilent 2100 Bioanalyzer
12. Agilent IKA vortex mixer
13. Universal Human Reference RNA (Agilent, 740000)

D. Preparing the RNA ladder

1. Upon receipt, thaw ladder on wet ice. Heat denature the ladder at 70°C for 2 minutes. Immediately cool the vial on wet ice and aliquot 5 µL of the RNA ladder into 0.5 mL RNase-free microcentrifuge tubes (provided by the Agilent kit) on wet ice. Store the aliquots in a -80°C freezer.
2. Before use, thaw an aliquot of the ladder on wet ice (avoid extensive warming of the ladder during the thawing process).

E. Preparing the gel

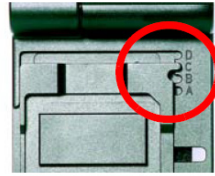
1. The Agilent LabChip kit reagents are stored refrigerated and used before the manufacturer's expiration date (the physical ships are stored at room temperature). Allow the RNA gel matrix to equilibrate to room temperature for 30 minutes.
2. Pipette 550 µL of RNA gel matrix to the spin filter (provided).
3. Place the spin filter in a microcentrifuge and spin for 10 minutes at room temperature at 1,500 x g.
4. Aliquot 65 µL of the filtered gel into 0.5 mL RNase-free microcentrifuge tubes (provided). Label the tube with the date of preparation, lot number, and date of expiration. Store the filtered gel in a refrigerator and use within 4 weeks of preparation.

F. Preparing the gel/dye mix

1. Allow the RNA dye concentrate (blue-capped tube) and RNA filtered gel to equilibrate to room temperature for 30 minutes. Protect the dye concentrate from light at all times. (Reference the kit instructions.)
2. Vortex the RNA dye concentrate for 10 seconds and centrifuge briefly.
3. Pipette 1 µL of the dye concentrate into a RNA filtered gel tube.
4. Cap the tube, vortex for 10 seconds, then centrifuge at 13,000 x g for 10min at room temperature. Visually inspect for homogenous mixing of gel and dye.
5. The prepared gel/dye mix can be stored for up to 8 hours in a refrigerator, and then must be discarded.

G. Preparing the priming station

1. Make sure the base plate is in position C as shown below. Using a screwdriver, open the screw at the underside of the base plate.



2. Make sure the syringe clip is at the top position by releasing the lever of the clip and sliding it up.




H. Heat denaturation of the RNA

1. Thaw the Agilent aliquot (test samples and controls) and a previously prepared aliquot of ladder on ice.
2. Place the tubes containing the ladder and each of the test samples and control in a thermocycler or heat block set to 70°C.
3. Heat the samples for 2 minutes while preparing the gel and chip.

I. Loading the gel/dye mix

1. Allow the gel/dye mix to equilibrate to room temperature for 30 minutes before use. Protect the gel/dye mix from light during this time. If the gel/dye mix is freshly prepared and has not been placed in the refrigerator, this step may be skipped.
2. Remove the RNA chip from its sealed bag.

3. Pipette 9.0 μ L of the gel/dye mix at the bottom of the well marked . Do not introduce air bubbles into the well when dispensing.
4. Place the RNA chip on the chip priming station. Set the timer for 30 seconds, make sure that the plunger is positioned at 1 mL, and then close the chip priming station. The lock of the latch will click when the chip priming station is closed properly.
5. Press the plunger of the syringe down until it is held by the clip.
6. Wait for exactly 30 seconds and then release the plunger with the clip release mechanism.
7. Visually inspect that the plunger moves back to at least the 0.8 mL mark.
8. Wait for 5 seconds and then slowly pull back the plunger to the 1 mL position.
9. Open the chip priming station.
10. Pipette 9.0 μ L of the gel/dye mix in each of the wells marked **G**.
11. Protect the gel/dye mix from light and store in the refrigerator when not in use for more than one hour. The gel/dye mix has a maximum shelf life of 8 hours.

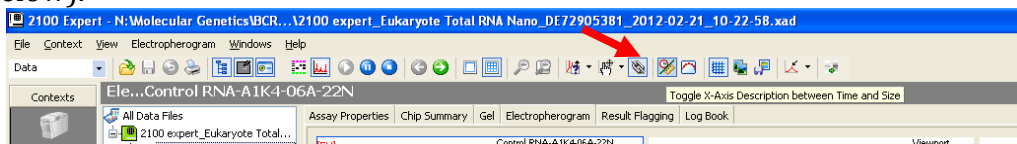
- J. Loading the Marker** - Pipette 5 μ L of the RNA 6000 Nano Marker into the well marked with the ladder symbol and into each of the 12 sample wells. If not running a full chip (12 samples), pipette 6 μ L of the RNA 6000 Nano Marker into any unused sample wells. Avoid introducing air bubbles into the wells when dispensing.

K. Loading the Ladder and Samples

1. Pipette 1 μ L of the heat-denatured RNA ladder into the well marked with the ladder symbol. Avoid introducing air bubbles into the wells when dispensing.
2. Add 1 μ L of heat-denatured test sample to the appropriate well. Avoid introducing air bubbles into the wells when dispensing.
3. Place the chip horizontally in the adapter of the IKA vortex mixer.
4. Vortex for 60 seconds at 2,400 rpm. NOTE: If the vortexing speed is too high, liquid may spill out of the channels and interfere with the analysis.
5. Run the chip in the Agilent 2100 Bioanalyzer within 5 minutes of loading.
6. Record the number of samples run on each machine on the equipment log.

L. Running the chip

1. Turn the Bioanalyzer unit on (switch is located on the back of the instrument).
2. Clean the Bioanalyzer probes (see section J) with RNase ZAP and Nuclease free water.
3. Place the chip in the Bioanalyzer. Close lid.
4. Start the software "2100 Expert".
5. Select Assay->RNA->"Eukaryote Total RNA Nano Series II"
6. Verify that the chip image displayed matches the chip being used.
7. Enter sample names on the instrument tab under the chip summary section.
8. Chip lot number and Reagent Kit lot number should also be recorded in this section.
9. Save file with the default date and time only.
10. Select the number of wells to be analyzed (e.g. 1-12).
11. Press "Run".
12. Select the pull down window labeled "Instrument" and select "Data".
13. After the run is complete, make sure the x-axis on the electropherogram is labeled with nt (nucleotide) units. If the x-axis is labeled with sec (seconds) click the Axis button at the top of the screen to change it back to nt.
14. If running the RNA Nano Assay for FFPE RNA samples, perform Smear Analysis (see below).



15. The file is automatically saved in a format that can be viewed only with Agilent software. To make a PDF copy, select file->print. Select "PDF", choose the correct path (e.g., external USB drive), verify that the following are checked: electropherogram, gel like, result tables, and include ladder, then press "save".

M. FFPE Smear Analysis

1. Highlight sample(s) to be analyzed.
2. At the right border of the software window, there are two tabs for additional options: Local and Global. Local is used for the individual sample selected. Global is used to apply the option to all samples.
3. Use the drop down menu to select "Advanced Settings."
4. Scroll down to "Smear Analysis" and check the box for "Smear Analysis."
5. Double click on row "Regions" to add table.

6. Add the following four smear reasons:
 - Low: base pair 35 – 235
 - Medium: base pair 500 – 1280
 - High: base pair 1800 – 3440
 - DV200: base pair 200 to max
7. Click “OK” to initiate calculation. The smear analysis will provide the total peak heights in the defined base pair ranges.

APPENDIX 3I: Shipping Procedures to MoCha Lab

All specimens that meet necessary quantity and quality will be sent for sequencing to the MoCha laboratory. DNA/RNA obtained from FFPE tissues and blood will be shipped frozen on dry ice.

The WES or TES on DNA/RNA will be performed by Dr. Mickey Williams' laboratory (MoCha) at the Frederick National Laboratory for Cancer Research. Samples labeled with unique patient IDs **only** will be sent directly to Dr. Mickey Williams' laboratory.

Please e-mail **Dr. Williams** (williamspm@mail.nih.gov), **Dr. Datta** (vivekananda.datta@nih.gov), and **Dr. Das** (dasbiswa@mail.nih.gov) 24 hours prior to shipment. A sample shipping manifest is included in [Appendix III](#). [The contact information of MoCha lab is as follows.](#)

Dr. P. Mickey Williams/Dr. Bishu Das/Dr. Anand Datta
MoCha Lab, Frederick National Laboratory for Cancer Research
Fort Detrick
Building 433 Room 302
Frederick, MD 21702
Leidos Biomedical Research, Inc.
Ph:301 620 3914
Email: vivekananda.datta@nih.gov

APPENDIX 4: SOPs for Sites or Biorepository

Note: The SOPs in this appendix may be used by adequately trained and equipped sites. Otherwise, the SOPs are to be used by Nationwide Children’s Hospital.

APPENDIX 4A: Processing of Biopsies for FFPE blocks

Title	Processing of Biopsies for FFPE blocks
SOP	TACP_ETCTN_5
Modified from	Nationwide Children’s SOP, CM-GL-54, LAB-058
Effective Date	

I. PURPOSE

This procedure explains how to process formalin-fixed tissue and load the cassette basket containing the tissue into the processor, program the processor, start the processor and unload the processor.

III. REQUIRED EQUIPMENT, SUPPLIES, AND REAGENTS

A. Equipment Leica ASP6025 tissue processor

B. Supplies Cassettes

C. Reagents Absolute ethanol, 95% ethanol, 80% ethanol, 70% ethanol, xylene, paraffin, deionized H₂O.

I. PROCEDURE – STEPWISE

A. Preparation of the Cassette:

1. Place the container of fixed tissue floating in formalin in the biological fume hood.
2. Place a few pieces of gauze in a stack the fume hood.
3. Visually estimate the number of embedding cassettes to make based upon the size of the tissue floating in formalin.
4. Obtain a “paraffin cassette” from the laboratory station.
5. Print a formalin cassette label for each embedding cassette to be used.
6. Verify the information on the printed label(s) is correct.
7. Place the label on the front (angled side) of the cassette.
8. Place the angled side of the cassette downwards in the label rivet puncher to adhere the label to the cassette.
9. Place the labeled embedding cassettes onto the stack of gauze previously placed in the biological fume hood.

10. Remove the piece(s) of fixed tissue floating in formalin from the container it arrived in using forceps.
11. Place the piece(s) of fixed tissue into the embedding cassette(s) and close the lid of the embedding cassette.
12. If the formalin-fixed tissue does not fit into one cassette, process as follows:
 - 12.1 Place a clean piece of gauze in the fume hood.
 - 12.2 Place a clean piece of 3" x 3" foil onto of the gauze.
 - 12.3 Place the tissue on top of the foil and anchor the tissue using clean forceps.
 - 12.4 Cut the tissue using a clean scalpel handle and a new blade.
 - 12.5 Cut pieces of formalin fixed tissue such that they will fit into an embedding cassette.
 - 12.6 Record the total number of embedding cassettes made (one embedding cassette per each piece cut).
 - 12.7 Place cut pieces of fixed tissue into the labeled embedding cassettes and close the lid of each cassette.

B. Processing of the Cassette:

2. Visually check the fluid and paraffin levels.
3. Open retort lid and load basket with cassettes into retort.
4. On smart screen select either "Favorites" or "Programs".
5. Select desired processing program from either the "Favorites " menu or the "Program" menu. Thick tissues and large embryos may need a longer run (choose 8 hour), very small tissues may use a shorter run (choose 4 hours). Tissues started on Friday evening must use a "program" run not a "favorite" run.
6. If a "favorite" program is selected, the run will start automatically once the number of cassettes has been entered.
7. Enter the total number of cassettes. Check the upper right corner to make sure that the end date and time are correct. Run will start. Proceed to step 10.
8. If a "program" run is selected touch the finish time button and enter the desired end time. On Friday runs, choose the next working day (usually Monday), and time and AM or PM (usually 7:00 AM.)
9. Press "start". The machine will prompt to add in the number of cassettes.
10. Enter the total number of cassettes. Check the upper right corner to make sure that the end date and time are correct. Run will start. Proceed to step 10.
11. When process is complete, a Drain retort message will show up on the screen.
12. Press button showing the completely drained Retort. It will take a few minutes to drain. A sound will alert you that the retort is drained.

13. Unlock the lid and remove the basket. Empty cassettes into the embedding station.
14. Check retort to be sure no blocks have been left in the machine.
15. If retort is clear, wipe out excess paraffin and replace empty basket.
16. Run "Standard Cleaning" program.
17. Remove basket when cleaning is completed.

18. For detailed programming steps and troubleshooting refer to the ASP6025 manual.
19. Proceed to paraffin embedding (**APPENDIX 4C**).

II. REFERENCES

Leica ASP6025 Instruction Manual, Version 1.8 RevC 07/2011.

Scheme used for processing biopsies

Reagents	Station	Time(H/Min)	Temp	P/V	Drain (secs)	Stir
Neutral Buffered Formalin	1	00:30	AMB	P/V	120	Off
ET-OH 70%	2	00:30	AMB	P/V	120	On
ET-OH 80%	3	00:30	AMB	P/V	80	On
ET-OH 95%	4	00:30	AMB	P/V	80	On
Abs ET-OH	5	00:30	AMB	P/V	80	On
Abs ET-OH	6	00:30	AMB	P/V	80	On
Abs ET-OH	7	00:40	AMB	P/V	80	On
Xylene	8	00:40	AMB	P/V	80	On
Xylene	9	00:55	AMB	P/V	80	On
Xylene	10	00:55	AMB	P/V	120	On
Paraffin Wax	Middle	00:55	60	P/V	140	On
Paraffin Wax	Left	00:55	60	P/V	140	On
Paraffin Wax	Right	01:00	60	P/V	140	On

APPENDIX 4B: Procedure for Cell Block Technique

1. Routine Cell Block¹

PURPOSE

Cellblock technique is used to concentrate small tissue fragments in a paraffin block. A cell block is a valuable addition to the preparation of many fluids, washings, or aspirates. Besides the advantage of capturing any tissue fragments, the cell block offers a concentration of cells in a small area that can be duplicated for ancillary stains and studies.

SCOPE

This procedure applies to all personnel in cytology.

SPECIMEN REQUIREMENT

Any specimen with sufficient cellularity can be prepared for cell block.

SUPPLIES/EQUIPMENT

1. Centrifuge
2. Ethanol 95%
3. Formalin 10% buffered
4. Conical tube (50 or 15 ml.)
5. Paper towel
6. Paper cup
7. Sharkskin filter paper (2X2 square)
8. Labeled tissue cassette with lid
9. Specimen spatula
10. Specimen cell block transport jar

SPECIAL SAFETY PRECAUTIONS

- Universal precautions are observed throughout the procedure
- Disposable gloves
- Disposable gowns

PROCEDURE

1. Centrifuge specimen at 1500 rpm for 10 minutes.
2. Decant supernatant
3. Add 5 ml. 95% alcohol

4. Add equal amount of 10% formalin
5. Centrifuge again (at 1500 RPM for 10 minutes)
6. Decant fixative
7. Carefully remove cell button from tube with spatula
8. Place button on sharkskin filter paper that is cut into a 2 in. square (If button is 1 to 2 cm cut in half so solutions will penetrate)
9. Fold paper using four overlapping edges forming a pocket so that specimen button is contained in the paper.
10. Place specimen in the tissue cassette labeled with the specimen number and barcode.
11. Lock the lid of the cassette in place and drop in formalin immediately.
12. Cell concentrates larger than 2 cm x 2 cm x 2 cm are divided accordingly and labeled "A-1" and "A-2"

2. Collodion Cell Block³ (used for scanty specimen)

PURPOSE

To retrieve diagnostic material from sparsely cellular specimens. The use of the Collodion bag cell block technique enables the retrieval of very small tissue fragments in a liquid specimen which is processed to form a paraffin block.

SPECIMEN REQUIREMENT

Specimen with limited cellularity

SUPPLIES/EQUIPMENT

1. Collodion
2. 10ml. glass centrifuge tube (do not use plastic)
3. Fume hood
4. Centrifuge
5. Alcohol 95%
6. Formalin 10% buffered
7. Paper towel
8. Paper cup
9. Wooden applicator stick
10. Metal forceps
11. Glass Pasteur pipette (do not use plastic)
12. Parafilm (white)
13. Scissors
14. Sharkskin filter paper (2X2 square)
15. Labeled tissue cassette with lid
16. Specimen cell block transport jar
17. Labeled with the specimen number and barcode

SPECIAL SAFETY PRECAUTIONS

- Disposable gloves
- Disposable gowns
- Always process under fume hood
- Do not expose bare skin to Collodion

PROCEDURE

1. Pour undiluted Collodion into a 10 ml. glass centrifuge tube under the fume hood.
2. Pour Collodion back into reagent bottle, rotating the tube while pouring.
3. Place tube upside down on paper towel to drain and dry for approximately 2 to 3 minutes.
4. Centrifuge 50 ml. tube containing the specimen for 10 minutes at 1,500 RPM in the centrifuge.
5. Decant the supernatant; add 5 ml. of 10% formalin and 5 ml. of 95% alcohol, vortex the mixture well, and centrifuge for 5 minutes at 1,500 RPM.
6. Pipette out the supernatant by using a glass pipette, leaving only a small amount. Vortex well, and add the remainder into a 10 ml. coated glass tube without touching the sides of the tube. Cover top with par film.
7. Centrifuge the 10 ml. glass tube for 7 minutes at maximum speed. (High)
8. Pour off supernatant and remove the bag by cutting around the top with a wooden applicator stick or forceps. Using forceps, twist the bag gently, carefully lifting and pulling bag away from the bottom of the tube.
9. Cut off excess collodion bag and place the bag containing the specimen onto 2 cm x 2 cm x 2 cm shark skin filter paper. Fold paper, place the specimen in the tissue cassette labeled with the specimen number and barcode
10. Lock the lid of the cassette in place and drop in 10% formalin immediately.
11. Deliver to histology section for processing.
12. A requisition form for histological stain must accompany the specimen.

3. Slide Scrape Cell Block

PURPOSE

To retrieve cellular material from a smear in order to use for multiple stains/ancillary studies.

SPECIMEN REQUIREMENT

Slide with thick diagnostic material

EQUIPMENT/SUPPLIES

1. Xylene
2. Ethanol 100%
3. Ethanol 95%
4. Formalin 10% buffered
5. Tap water in cup
6. Surgical knife blade
7. Sharkskin filter paper (2X2 square)
8. Labeled tissue cassette with lid
9. Specimen cell block transport jar
10. Papanicolaou Stain setup (see Papanicolaou Stain for instruction)

SPECIAL SAFETY PRECAUTIONS

- Disposable gloves
- Disposable gowns

PROCEDURE

1. Place slide in xylene to remove cover slip.
2. Soak slide in xylene until mounting media is removed.
3. Place slide in 100% alcohol then 95% alcohol for 10 dips each.
4. Dip the slide sufficient times in a cup of tap water to rehydrate the cells.
5. Blot the edge of the slide on paper towels to remove as much water as possible.
6. Using the blade carefully scrape off the thick cellular material and transfer it onto shark skin paper and return the slide to the cup of water.
7. Fold paper using four overlapping edges forming a pocket so that specimen button is contained in the paper.
8. Place specimen in the tissue cassette labeled with the specimen number and barcode.
9. Lock the lid of the cassette in place and drop in formalin immediately.
10. Dip the slide several times in water to rinse off floating cells.
11. Place the slide in 95% alcohol in the staining setup to dehydrate.
12. Stain in EA for 1 minute and complete the staining sequence to clear and recover slip.

REFERENCES

- 1 Koss, Diagnostic Cytopathology and its Histopathological Basis. Volume II.
- 2 Emory Medical Laboratories, Emory University Hospital.
- 3 Fahey, Collodion Bag: A cell block technique for enhanced cell collection. Lab Med 1993, 74:94-96.

APPENDIX 4C: Paraffin Embedding of Tissues

Title	Paraffin Embedding of Tissues
SOP	TACP_ETCTN_6
Modified from	Nationwide Children's SOP, CM-GL-22.2
Effective Date	

PRINCIPLE

Embedding involves placing properly fixed and processed tissue into the appropriately sized mold and surrounding that tissue with paraffin. Before the paraffin solidifies, the tissue is gently pushed flat so a whole representative section can be taken. Re-embedding involves melting previously embedded samples and placing them in fresh paraffin in an appropriate mold.

REQUIRED EQUIPMENT, SUPPLIES, AND REAGENTS

Equipment

Thermo Histocenter

Supplies

Cassettes Fisher order number 15-182-500

Embedding Molds Fisher order number 15-182-505

Forceps Fisher order number 10-316A

Reagents

Paraplast Plus –Fisher 12-646-111

Histoplast PE- Fisher cat# 8330

PROCEDURE – STEPWISE

1. For previously embedded samples, place tissue cut side down into an appropriately sized mold. Place mold into paraffin cassette well. Allow sample to melt before continuing on to step 4.
2. Using warmed forceps remove a processing cassette that contains the impregnated tissue from the paraffin cassette well. Open only one cassette at a time. Wipe forceps regularly to prevent cross contamination from other tissue samples.
3. Open the tissue-processing cassette in order to view the sample. Open and embed one tissue sample at a time.
4. Select a mold that best corresponds to the size of the tissue and partially fill with paraffin.
5. Rewarm the forceps, remove the tissue from the cassette and place it at the bottom center of the mold. Follow any specific instructions for orientation noted on the request. If there are no instructions, in general, the cut or flat side of the tissue goes down. Membranes, skin, and cyst walls should be embedded on edge. Gut should be

embedded in cross section so the lumen shows. If the processing cassette is also used as an embedding cassette the lid should be discarded and the bottom retained for embedding purposes.

6. Transfer the mold from the warm plate to the cold plate. Using the round wooden or metal tool, gently press the surface of the tissue to be sectioned so it will be flat against the bottom of the mold.
7. Place the embedding cassette on top of the mold. Fill the combined mold and embedding cassette with paraffin and cool immediately by placing on the cold plate.
8. When the paraffin solidifies the mold can be separated from the cassette.
9. The block may now be cleaned, trimmed and sectioned.
10. If multiple embedding was done (i.e. many pieces of tissue oriented in one block), be sure to record this on the request sheet.
11. **Proceed to storing the FFPE tissue or sections (APPENDIX 4C and 3B).**

REFERENCES

Carson Freida L.PhD, HT (ASCP) Histotechnology A Self-Instructional Text 2nd ED, 1996 pp.35-37, ASCP Press.

APPENDIX 5: SOPs for MoCha Reference Lab

APPENDIX 5A: DNA/RNA Isolation from FFPE Tissues using the AllPrep Kit

1.0 PURPOSE

The extraction of total RNA and DNA for downstream use in the ETCTN Biobanking and Molecular Characterization Initiative.

Purifying genomic DNA and total RNA from formalin-fixed, paraffin embedded (FFPE) tissue sections using the AllPrep FFPE Kit allows for extraction of both total RNA and genomic DNA from the same FFPE sample, thus reducing the amount of sample needed and increasing the possible yield per sample.

FFPE samples are deparaffinized using Hemo-De. Subsequently, the dissected tissue sections are then subjected to a rigorous proteinase K digestion with an incubation time tailored for recovery of RNA and DNA. Nucleic acids are purified using a silica membrane filter methodology that includes a nuclease treatment and elution into either nuclease-free water or low salt buffer.

This SOP is for research purposes only, and no clinical samples will be processed using this SOP. Any deviation from this SOP will be noted but will not be formally documented.

2.0 SCOPE

Extraction of DNA and RNA from FFPE samples: The AllPrep DNA/RNA FFPE kit is designed to purify genomic DNA and total RNA from formalin-fixed, paraffin embedded (FFPE) tissue sections. Following deparaffinization in Hemo-De and air drying, the tissue sections are incubated in a lysis buffer that contains Proteinase K. This will cause the RNA to be released into the solution while genomic DNA and other insoluble material are precipitated. Once the sample has been centrifuged, the supernatant will contain RNA while the pellet will contain DNA. The sample will then undergo separate purification procedures to yield either pure DNA or RNA.

3.0 EQUIPMENT*

Item	Source
Thermal Mixer set to 56°C	Eppendorf # 022670000
Thermal Mixer set to 80°C	Eppendorf # 022670000
Heat Block set to 90°C	Various (e.g. VWR #12621-088)
2.0ml Eppendorf Safe-Lock Tubes	Eppendorf # 0030 120.094
1.5ml Eppendorf Safe-Lock microfuge tube	Eppendorf # 0030 120.086
Gasketed screwcap tube	ThermoScientific # 363401
1000ul Pipet and tips	Rainin L1000
200ul Pipet and tips	Rainin L200
20 ul Pipet and tips	Rainin L20
2ul Pipet and tips	Rainin L2
Microcentrifuge capable of 20,000 x G	Various (e.g. Eppendorf #5424)
Vortexer	VWR #58816-121
Spectramax M2 or equivalent fluorometer	Molecular Diagnostics

4.0 REAGENTS/MATERIALS*

Item	Source
AllPrep DNA/RNA FFPE Kit (Qiagen #80234)	Qiagen

100% Isopropanol	Warehouse # 68101010
100% Ethanol	Warehouse # 68101025
RNase A, 100 mg/ml	Qiagen #19101
1.5 ml Eppendorf DNA/RNA LoBind Tubes	Eppendorf # 022431021
2.0 ml Eppendorf Safe Lock Tubes	Eppendorf # 022363352
1000ul Pipet and tips	Rainin L1000
Q33130 Quant-iT™ dsDNA Broad-Range (BR) Assay Kit	Molecular Probes
Q10213 Quant-iT™ RNA Broad-Range BR Assay Kit	Molecular Probes
96 well Black Flurotrac Plates ref#655076	Griener Bio-one

5.0 AUTOMATION METHODS

As per the protocol described.

6.0 SAFETY

- 6.1 Lab coats, safety glasses, and gloves must be worn at all times when handling hazardous or sensitive equipment, samples, reagents, and materials. These safety measures must also be followed when in close proximity to those who are working with these items.
- 6.2 Sample - Insulated gloves should be worn when handling any dry ice or liquid nitrogen vapor, both of which can cause severe burns to skin while handled
- 6.3 Sample - Dry ice and liquid nitrogen should be allowed to sublime or evaporate at room temperature in an appropriate cooler or dewar, ideally in a chemical fume hood, or in open air, away from ventilation sources at a minimum. Do not discard of either in trash or sink.

7.0 PREPARATIONS PRIOR TO STARTING

- 7.1 Turn on and warm up one thermal mixer to 56°C, one thermal mixer to 80°C, and one stationary heat block to 90°C. Press the start button on the thermal mixer in order for the unit to begin heating to the set point. This will also start the unit

shaking, and pressing stop will prevent unnecessary shaking without inhibiting the pre-warming of the unit.

- 7.2 **Buffer RLT** – check to see if any precipitate has formed. If necessary re-dissolve by warming with gentle agitation.
- 7.3 **Buffer FRN** – Add 42 ml of 100% isopropanol to the concentrate, and mix by shaking. Tick the check box on the bottle to indicate that the isopropanol has been added. Before use, mix the reconstituted buffer by shaking.
- 7.4 **DNase I Stock Solution** – Prepare a DNase I stock solution by adding 550 µl of nuclease-free water from the kit to the vial containing the lyophilized enzyme mix by gently inverting the tube several times. **Do not vortex or shake the solution to mix.**
- 7.5 **Buffer RPE** – Add 44 ml of 100% ethanol to the concentrate, and mix the buffer by shaking. Tick the check box on the bottle to indicate that the ethanol has been added. Before use, mix the reconstituted buffer by shaking.
- 7.6 **Buffer ATL** – Before starting the procedure, check to see if precipitate has formed in the buffer. If necessary, re-dissolve by heating to 70°C with gentle agitation.
- 7.7 **Buffer AL** - Before starting the procedure, check to see if precipitate has formed in the buffer. If necessary, re-dissolve by heating to 70°C with gentle agitation.
- 7.8 **Buffer AW1** – Add 25 ml of 100% ethanol to the concentrated buffer, and mix by shaking. Tick the check box on the bottle to indicate that the ethanol has been added. Before use, mix the reconstituted buffer by shaking.
- 7.9 **Buffer AW2** – Add 30 ml of 100% ethanol to the concentrated buffer, and mix by shaking. Tick the check box on the bottle to indicate that the ethanol has been added. Before use, mix the reconstituted buffer by shaking.

7.10

8.0 Procedure

8.1 Deparaffinization by Xylene

Safety Note: *Though FFPE samples have formalin fixed and paraffin embedded, specimens and materials should be considered to have potential to transmit infectious disease.*

The deparaffinization should be performed in the Biosafety cabinet (type II) with 100% exhausted ventilation in room 103 in order to contain vapors from the xylenes used. Tubes other than Safelock tubes have been shown to be leaky or cap popping open during deparaffinization.

- 8.2 Using forceps, move each FFPE ribbon to a clean, labeled SafeLock tube.
- 8.3 To each FFPE tissue in a tube, add 1 ml xylene, vortex vigorously for 10s, and centrifuge at full speed for 4 min.

- 8.4 Carefully remove all but ~150 μ l of supernatant with a P1000 pipet without disturbing the pellet.
- 8.5 Using a P200 pipet, carefully remove as much xylene as possible without disrupting the pellet.
- 8.6 Add 1 ml 100% ethanol to the pellet, mix by vortexing, and centrifuge at full speed for 2 min.
- 8.7 Carefully remove all but ~150 μ l of the supernatant with a P1000 pipet without disturbing the pellet.
- 8.8 Carefully remove any residual ethanol using a P200 pipet and discard.
- 8.9 Keeping the lid open, incubate at room temperature or a 37°C heat block for 10 min to dry the pellet.
- 8.10 Re-suspend the pellet by adding 150 μ l Buffer PKD and flicking the tube to loosen the pellet.
- 8.11 Add 10 μ l proteinase K, and mix by vortexing.
- 8.12 Incubate at 56°C for 15 min with agitation at 600 RPM.
- 8.13 Incubate on ice for 3 min.
- 8.14 Centrifuge for 15 min at 20,000 x g.
- 8.15 Carefully transfer the supernatant (RNA fraction, total volume = 160 μ l), without disturbing the pellet, to a new 2 ml Safe-Lock microcentrifuge tube for RNA purification. The pellet will be used for DNA purification.
- 8.16 The DNA-containing pellet can be stored for 2 hours at room temperature, for up to 1 day at 2–8°C, or for longer periods at –20°C if not processed immediately.
 - 8.16.1
- 8.17 Remove samples and spin 4 minutes full speed at 4° C.
 - 8.17.1 Transfer lysate to appropriately labeled 2.0 ml Safe Lock Eppendorf tubes.
 - 8.17.2 Samples may be stored @-80°C for processing at a later date or may be used immediately.

Purification of DNA Part 1

- 8.18 Resuspend the pellet in 180 μ l Buffer ATL, add 40 μ l proteinase K, and mix by vortexing.
- 8.19 Incubate at 56°C for 1 h with agitation at 600 RPM. Continue with the RNA extraction.

Purification of total RNA.

- 8.20 Incubate the supernatant from the deparaffinization step 13 at 80°C for 15 min.
- 8.21 Briefly centrifuge the tube to remove drops from the inside of the lid.

- 8.22 Add 320 μ l Buffer RLT to adjust binding conditions (total volume = 480 μ l), and mix by vortexing or pipetting.
- 8.23 Add 1120 μ l 100% ethanol (total volume = 1600 μ l), and mix well by vortexing.
- 8.24 Quick spin the tube to remove any liquid from the lid of the tube.
- 8.25 Transfer 700 μ l of the sample, including any precipitate that may have formed, to an RNeasy MinElute spin column (pink top) placed in a 2 ml collection tube.
- 8.26 Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm).
- 8.27 Discard the flow-through and return the column to the collection tube.
- 8.28 Repeat step R6 until the entire sample has passed through the RNeasy MinElute spin column. Ensure the labeling of columns and tubes is matched before transfer.
- 8.29 Add 350 μ l Buffer FRN to the RNeasy MinElute spin column.
- 8.30 Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm).
- 8.31 Discard the flow-through and return the column to the collection tube.
- 8.32 Prepare a DNase I reaction solution for the number of samples to be processed by mixing the reagents according to the table below (add 10% for pipet volume error):

Component	Per Sample (μ l)
DNase I Stock Solution	10
Buffer RDD	70
Total Volume	80

- 8.33 Mix by gently inverting the tube, and centrifuge briefly to collect residual liquid from the sides of the tube.
- 8.34 Note: DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube. Do not vortex.
- 8.35 Add the DNase I incubation mix (80 μ l) directly to the RNeasy MinElute spin column membrane, and incubate at room temperature for 15 min.
- Note: Be sure to add the DNase I incubation mix directly to the membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.
- 8.36 Add 500 μ l Buffer FRN to the RNeasy MinElute spin column.
- 8.37 Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm).
- 8.38 Save the flow-through for use in step 20. Do not discard the flow-through, as it contains RNA including small RNAs.
- 8.39 Place the RNeasy MinElute spin column in a new 2 ml collection tube.
- 8.40 Apply the flow-through from step R18 to the spin column.

- 8.41 Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm).
- 8.42 Discard the flow-through and return the column to the collection tube.
- 8.43 Add 500 µl Buffer RPE to the RNeasy MinElute spin column.
- 8.44 Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm) to wash the spin column membrane.
- 8.45 Discard the flow-through and return the column to the collection tube.
- 8.46 Add another 500 µl Buffer RPE to the RNeasy MinElute spin column.
- 8.47 Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm) to wash the spin column membrane.
- 8.48 Discard the collection tube with the flow-through.
- 8.49 Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied).
- 8.50 Open the lid, and centrifuge at full speed for 5 min.

- 8.51 Discard the collection tube with the flow-through.
- 8.52 Place the RNeasy MinElute spin column in a new 1.5 ml collection.
- 8.53 Add 14 µl RNase-free water directly to the spin column membrane.
- 8.54 Close the lid gently, and incubate for 1 min at room temperature (15–25°C).
- 8.55 Centrifuge at full speed for 1 min to elute the RNA.

Purification of genomic DNA Part II

- 8.56 Move DNA pellet lysate from 56°C to 90°C, and incubate for 2 h without agitation.
Note: Agitation during this incubation step leads to lower DNA yields. In addition, higher temperature or longer times will lead to more fragmented DNA
- 8.57 Briefly centrifuge the microcentrifuge tube to remove drops from the inside of the lid.
- 8.58 Allow the sample to cool to room temperature (15–25°C) and then add 4 µl RNase A (100 mg/ml) and mix well.
- 8.59 Incubate for 2 min at room temperature.
- 8.60 Add 200 µl Buffer AL (total volume = 380 µl), and mix thoroughly by vortexing.
- 8.61 Add 200 µl 100% ethanol (total volume = 580 µl), and mix thoroughly again by vortexing or pipetting.

Note: It is essential that the sample, Buffer AL, and ethanol are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution. A white precipitate may form on addition of Buffer AL and ethanol. This precipitate does not interfere with the AllPrep procedure.

8.62 Transfer the entire sample to a QIAamp MinElute spin column placed in a 2 ml collection tube (supplied).

8.63 Close the lid gently, and centrifuge for 1 min at 8000 x g (10,000 rpm).

8.64 Discard the collection tube with the flow-through.

Note: If the sample has not completely passed through the membrane after centrifugation, centrifuge again at a higher speed until the QIAamp MinElute spin column is empty.

8.65 Place the QIAamp MinElute spin column in a new 2 ml collection tube.

8.66 Add 700 µl Buffer AW1 to the spin column.

8.67 Close the lid gently, and centrifuge for 15 sec at 8000 x g (10,000 rpm) to wash the spin column membrane.

8.68 Discard the flow-through and return the column to the collection tube.

8.69 Add 700 µl Buffer AW2 to the QIAamp MinElute spin column.

8.70 Close the lid gently, and centrifuge for 15 sec at 8000 x g (10,000 rpm) to wash the spin column membrane.

8.71 Discard the flow-through and return the column to the collection tube.

8.72 Add 700 µl 100% ethanol to the QIAamp MinElute spin column.

8.73 Close the lid gently, and centrifuge for 15 sec at 8000 x g (10,000 rpm).

8.74 Discard the collection tube with the flow-through.

8.75 Place the QIAamp MinElute spin column in a new 2 ml collection tube.

8.76 Open the lid of the spin column, and centrifuge at full speed for 5 min.

Note: To avoid damage to their lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counterclockwise). It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during DNA elution.

8.77 Discard the collection tube with the flow-through.

8.78 Place the QIAamp MinElute spin column in a new 1.5 ml collection tube.

8.79 Add 30 µl Buffer ATE directly to the spin column membrane.

8.80 Close the lid gently, and incubate for 1 min at room temperature.

8.81 Centrifuge at full speed for 1 min to elute the DNA.

APPENDIX 5B: WES Library Preparation and Target Enrichment

1.0 PURPOSE/SCOPE

This Standard Operating Procedure (SOP) describes Automated DNA Library Preparation for Agilent SureSelect XT Target Enrichment System used/performed by the Molecular Characterization and Clinical Assay Development Laboratory (MoCha) at the Frederick National Laboratory for Cancer Research. This SOP applies to the ETCTN Project. **This SOP is for research purposes only and no clinical samples will be processed using this SOP. Any deviation from this SOP will be noted but will not be formally documented.**

2.0 REFERENCE DOCUMENTATION

Document Number	Title
G7530-90000	SureSelectXT Target Enrichment System for Illumina Paired-End Multiplexed Sequencing Library Protocol http://www.agilent.com/cs/library/usermanuals/Public/G7530-90000.pdf
N/A	Fragment Analyzer Automated CE System Quick Start Guide—96 Capillary DNF-473 Standard Sensitivity NGS Fragment Analysis Kit, (1 bp-6000 bp) http://www.aati-us.com/sites/default/files/DNF-473%20Quick%20Start%20Guide_96Capillary_2015NOV03.pdf
N/A	User's Guide: Agilent SureSelectXT Target Enrichment on the Sciclone NGS Workstation
N/A	Fragment Analyzer™ Automated CE System Quick Start Guide -96 Capillary DNF-474 High Sensitivity NGS Fragment Analysis Kit, (1 bp - 6,000 bp) http://www.aati-us.com/documents/quick-start-guides/dnf-474/dnf-474-quick-start-guide-96-capillary-11-03-2015.pdf
4375799 Rev. E	Applied Biosystems Veriti Thermal Cycler User Guide https://tools.thermofisher.com/content/sfs/manuals/cms_042832.pdf
N/A	exome_libraryprep_template_08162016 S:\Experiments\PDX\Library preps\templates\library_prep_template_files

3.0 BACKGROUND

This procedure is to be used to prepare whole exome libraries from DNA samples for use on the Illumina HiSeq Sequencing platform. The resulting DNA libraries are suitable for paired-end,

multiplexed sequencing applications.

4.0 EQUIPMENT*

Description	Model #	Vendor
Multi-Channel Pipettes (LTS 20,200,1000)	Variable	Rainin
Vortex	58816-121	VWR
MiniFuge	93000-196	VWR
Plate Centrifuge	022628203	Eppendorf
Veriti 96 well thermal cycler	4375786	ABI
Covaris Ultrasonicator	E220	Covaris
PCR Workstation	AC648LFUVC	AirClean Systems
Fragment Analyzer, 96 capillary electrophoresis system	Fragment Analyzer	Advanced Analytical
Vacuum concentrator	SpeedVac	Savant
Sciclone G3 Automated Workstation	Sciclone G3	Perkin Elmer
Argos Technologies Omega Single-Channel Pipet Controller	03-391-253	Fisher Scientific
Zephyr Automated Workstation	Zephyr	Perkin Elmer

*Or comparable/equivalent (include unless must use listed)

5.0 REAGENTS/MATERIALS*

Description	Product No.	Vendor
Standard Sensitivity NGS Fragment Analysis Kit (1 bp - 6,000 bp)	DNF-473-0500	Advanced Analytical
Herculase II Fusion DNA Polymerase	600679	Agilent
High Sensitivity NGS Fragment Analysis Kit (1 bp- 6000 bp)	DNF-474	Advanced Analytical
Nuclease-free Water	various	various
Agencourt AMPure XP Kit	A63881	Beckman Coulter

Description	Product No.	Vendor
1X Low TE Buffer	12090-015	Life Technologies
Ethanol, 100% for molecular biology	E7023	Sigma-Aldrich
Elution Buffer (EB)	19086	Qiagen
SureSelectXT Reagent Kit, HSQ	G9611B	Agilent
SureSelectXT Human All Exon V5	5190-6209	Agilent
Dynabeads MyOne Streptavidin T1	65602	Life Technologies
1.5-mL LoBind Tube	022431021	Eppendorf
Fisherbrand 96 DeepWell 1 mL plate	12-566-120	Fisher Scientific
Eppendorf™ 96-Well twin.tec™ PCR Plates	E951020346	Fisher Scientific
Disposable Standard Serological Pipets, 25 mL capacity	13-678-14B	Fisher Scientific
Disposable Standard Serological Pipets, 10 mL capacity	13-678-12E	Fisher Scientific
384 well U bottom plate	3672	Corning
Hard-Shell 96 well PCR plate	HSP9631	Bio-Rad
150 uL Barrier Sterile 96 Rack tips for Sciclone	111426	Perkin Elmer
Seahorse polypropylene reservoir, 170ml, 12 column	201250-100	Agilent Technologies
MicroAmp® Optical 96-Well Reaction Plate	4316813	ThermoFisher Scientific
Plate lids	600030	Perkin Elmer
96 well storage plates, 0.8 mL (MIDI)	AB-0859	Fisher Scientific
200-1000 µL Aerosol Barrier Pipette Tips	RT-L1000FLR	Rainin
20-200 µL Aerosol Barrier Pipette Tips	RT-L-200FLR	Rainin
0.2-20 µL Aerosol Barrier Pipette Tips	RT-L10FLR	Rainin
StorPlate 96V, 450uL capacity	6008290	Perkin Elmer

Description	Product No.	Vendor
96 Well Clear Round Bottom 1mL Polypropylene Deep Well Plate	3959	Corning
96-well polypropylene storage/reaction microplate, 2ml/square well, pyramid bottoms	201379-100	Agilent Technologies
NaCl PEG solution; 20% polyethylene glycol, 2.5M NaCl	P4136	Teknova
microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm Covaris tube	520045	Covaris, Inc.
Microamp Adhesive plate seals	4306311	Applied Biosystems

*Or comparable/equivalent (include unless must use listed items)

6.0 AUTOMATION METHODS

As per the described Sciclone protocol.

7.0 SAFETY

7.1 Lab coats, safety glasses, and gloves must be worn at all times when handling hazardous or sensitive equipment, samples, reagents, and materials. These safety measures must also be followed when in close proximity to those who are working with these items.

8.0 PROCEDURE

8.1 Shear DNA using Covaris E220 Ultrasonicator

Notes:

- *The volume of sample (DNA+1X Low TE) necessary for use with the microtubes is 130uL. If lower volumes are used, and air space may form in the fluid, partitioning the sample and resulting in a broad peak.*
- *Fragment size generation is affected by treatment duration. A longer duration will produce smaller fragments.*
- *Bath water should be clean distilled, deionized water as foreign materials such as algae and particulates may scatter the high frequency focused acoustic beam, resulting in a shift to larger mean fragment size.*
- *Insufficient degas levels within the bath may result in poor acoustic coupling and thereby shift the mean fragment size. System degas pumps should be run at least 60 minutes in advance of, as well as during, treatments.*
- *Warmer temperatures will cause a shift toward larger mean fragment size. Bath temperature should be closely monitored.*
- *Attention should be paid to maintaining a consistent water level.*

- *Do not remove the snap-cap of the microTUBE prior to sample processing.*

8.1.1 Prepare the Covaris for shearing

8.1.1.1 Open the Sonolab E220 program on the desktop of the computer attached to the E220 in 433 room 13.

8.1.1.2 Set the chiller to the correct temperature. When set at 3⁰C, the software temperature display should settle near 7⁰C.

8.1.1.3 Push the green door button and pull the handle to access the chamber of the E220.

8.1.1.4 Fill the water tank of the E220 to level 6 on the graduated fill line level with fresh deionized, distilled water. The microTUBE should be partially immersed in the water during shearing.

8.1.1.5 Select SureSelect from the Method Name pull down menu.

Setting	Value
Duty Cycle	10%
Intensity	5
Cycles per Burst	200
Time	6 cycles of 60 seconds each
Set Mode	Frequency sweeping
Temperature	4° to 7° C

8.1.1.6 Ensure that Degas On is selected. Water should be degased for a minimum of 60 minutes prior to the start of sample processing.

8.1.1.7 Select Method Editor and click and drag on the plate template to highlight the number of wells corresponding to the number and location of samples being processed.

8.1.2 Prepare DNA samples for shearing

8.1.2.1 Complete columns A through K on the 'shearing' sheet of the exome_libraryprep_template_08162016 file

8.1.2.2 Prepare all samples to shear 600 ng total DNA in 130ul total volume. Use 1X Low TE to normalize DNA volumes. Use 1.5 mL Lobind tubes to prepare samples.

8.1.2.3 Label one Covaris microTUBE for each DNA sample to be sheared. Label with a lab marker on the side of the lip of the snap cap portion of the tube.

8.1.2.4 Vortex sample tubes to mix.

8.1.2.5 Briefly centrifuge the sample tubes to remove drops from the walls of the tubes and insides of the lids.

8.1.2.6 Use a pipet tip to transfer each 130ul DNA+TE sample to the appropriately labeled microTUBE by inserting the pipette tip through the pre-split septa. With the pipette tip approximately halfway down the interior of the tube and alongside the interior wall, slowly discharge the fluid into the tube. Be careful not to introduce a bubble into the bottom of the tube. This may happen if the sample is loaded too quickly.

8.1.3 Load DNA samples on the Covaris and perform shearing

8.1.3.1 Load the microTUBES in the microTUBE rack. Attach the flat top portion of the tube rack.

8.1.3.2 Select Load Plate in the Sonolab E220 software.

8.1.3.3 Press the green door button and pull the handle to access the chamber of the E220.

Load the plate onto the black plate holder in the chamber. Close the door.

8.1.3.4 Select Start in the Sonolab E220 software.

8.1.3.5 When processing is complete remove plate with samples from the E220.

8.2 Perform post-shearing cleanup on the SciClone

8.2.1 Open Maestro software on the SciClone.

8.2.2 Open “SureSelect XT Workbook” worksheet in Excel.

8.2.3 Enter number of columns to be processed in each tab of the “SureSelect XT Workbook”, then save the file.

8.2.4 Open “SSXT Initial SPRI Cleanup” method within the Maestro software.

8.2.5 Let the AMPure XP beads come to room temperature for at least 30 minutes.

8.2.6 Mix the AMPure beads well so that the reagent appears homogeneous and consistent in color. Do not freeze.

8.2.7 Transfer sheared DNA from microTUBEs to plate and purify using Agencourt AMPureXP beads on the SciClone G3.

8.2.7.1 Add the sheared DNA library (~130 µL) to the 450ul/well sample plate, and place the plate onto the SciClone deck according to the deck layout.

8.2.8 Set up the SciClone according to reagent layouts listed on the workbook file and the prompts when the start button is pushing within the method.

8.2.8.1 Add 180 µL of homogeneous AMPure XP beads to each well of a 96 well BioRad hard shell plate, and place the plate onto the SciClone deck according to the deck layout.

8.2.8.2 Add 80% Ethanol to appropriate labware and place on the SciClone deck according to the deck layout.

8.2.8.3 Add Qiagen EB buffer to appropriate labware and place on the SciClone deck according to the deck layout.

8.2.8.4 Add tip boxes and additional labware to the SciClone deck according to the deck layout.

8.2.9 Press start to begin procedure.

8.2.10 When “SSXT Initial SPRI Cleanup” program is complete, cover sample plate with lid and place plate on ice while setting up for quality assessment.

Note: Stopping Point - If you do not continue to the next step, store the samples at -20°C.

8.3 Assess quality of sheared post-cleanup DNA samples using the Fragment Analyzer

8.3.1 Dilute 1 µl of sample with 1 µl Qiagen EB and load on an Advanced Analytical Fragment Analyzer using Standard Sensitivity NGS Fragment Analysis Kit.

8.3.2 Allow Inlet Buffer, Capillary Conditioning Solution, Gel, and Intercalating Dye to come to room temperature prior to mixing.

8.3.3 Allow standard sensitivity marker and ladder to come to room temperature

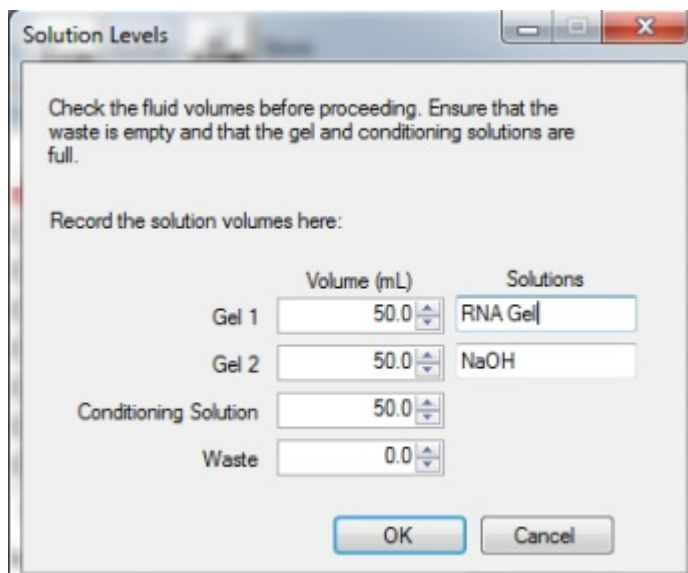
8.3.4 Mix appropriate volumes of Intercalating Dye and Separation Gel necessary for one day of operation. Use the supplied 50 mL conical centrifuge tube to allow a small minimum working volume. For larger volumes, use a 250 mL conical centrifuge tube and remove the collar of the tube holder in the instrument reagent compartment.

8.3.5 The volume of Separation Gel required per run on the 96-Capillary Fragment Analyzer™ system is summarized below.

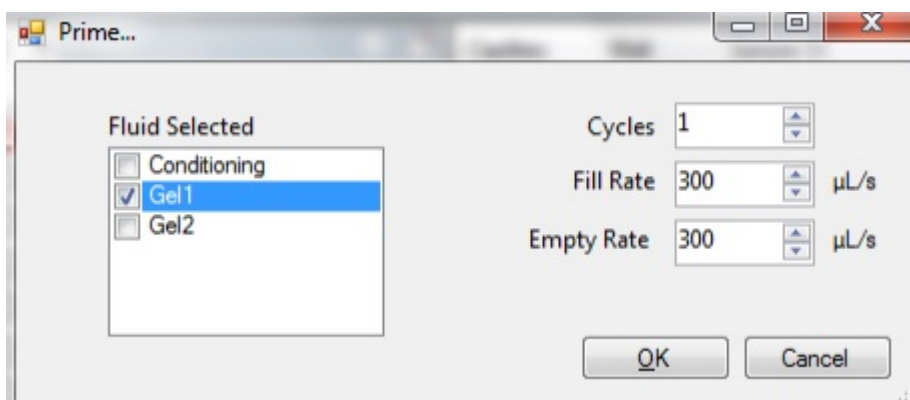
# of samples to be analyzed	Volume of Intercalating Dye	Volume of RNA Separation Gel
-----------------------------	-----------------------------	------------------------------

95	4.0 uL	40 mL
190	8.0 uL	80 mL
285	12.0 uL	120 mL
380	16.0 uL	160 mL
475	20.0 uL	200 mL

- 8.3.6 Place the Separation Gel/ Intercalating Dye mixture onto the instrument and insert into the desired gel fluid line (Gel 1 or Gel 2 pump position). Ensure the fluid line is positioned at the bottom of the conical tube to avoid introducing air bubbles, which can cause pressurization errors.
- 8.3.7 When adding Separation Gel to the instrument, update the solution levels in the Fragment Analyzer instrument control software. From the Main Menu, select Utilities – Solution Levels. A menu will be displayed to enter in the updated fluid levels.






- 8.3.8 When switching applications (e.g., between NGS and RNA kits), prime the appropriate gel fluid line after loading fresh gel/dye mixture. From the Main Menu of the Fragment Analyzer instrumental control software, select Utilities – Prime... Select the desired fluid line(s) (Conditioning, Gel 1, or Gel 2) and press OK to purge the fluid line with fresh gel.



- 8.3.9 In a clean container, add 20 mL of the 5X 930 dsDNA Inlet Buffer per 80 mL of deionized sub-micron filtered water. Agitate to mix. The entire bottle can be mixed to 1X concentration and stored at 4°C if desired.
- 8.3.10 In a clean container (e.g. 50 mL or 250 mL conical centrifuge tube), add 20 mL of the 5X Capillary Conditioning Solution per 80 mL of deionized sub-micron filtered water. Agitate to mix. The entire bottle can be mixed to 1X concentration and stored at room temperature if desired.
- 8.3.11 Once mixed, place the 1X Capillary Conditioning Solution onto the instrument and insert the CONDITIONING fluid line (Conditioning Solution pump position). Ensure the fluid line is positioned at the bottom of the conical tube to avoid introducing air bubbles, which can cause pressurization errors. Update the solutions levels in the Utilities section of the Fragment Analyzer software.
- 8.3.12 Check the fluid level of the waste bottle and waste tray daily and empty as needed.
- 8.3.13 Prepare a fresh 96 DeepWell 1mL Plate filled with 1.0 mL/well of 1X 930 dsDNA Inlet Buffer daily. Do NOT overfill the wells of the inlet buffer plate. Place buffer plate in Drawer "B".
- 8.3.14 Prepare a fresh sample plate filled with 200 µL/well of 0.6X TE Rinse Buffer daily. Place rinse plate in Drawer "M"
- 8.3.15 Using a fresh half-skirted 96-well sample plate, pipette 22 µL of the Standard Sensitivity NGS Diluent Marker to each well in a row that is to contain sample or NGS Ladder. Fill any unused wells within the row of the sample plate with 24 µL/well of BF-1 Blank Solution.
- 8.3.16 Pipette 2 µL of each diluted sample into the respective wells of the sample plate; mix the contents of the well using the pipette by aspiration/expulsion in the pipette tip.
- 8.3.17 After mixing sample/NGS Ladder and Diluent Marker in each well, centrifuge the plate to remove any air bubbles. Check the wells of the sample plate to ensure there are no air bubbles trapped in the bottom of the wells. The presence of trapped air bubbles can lead to injection failures.
- 8.3.18 For best results, run the plate as soon as possible. If the sample plate will not be used immediately, cover the sample plate with foil/seal, store at 4°C and use within the same day. Spin the plate again if any bubbles developed in the sample wells. *Be sure to remove the cover film before placing the plate into the instrument.*
- 8.3.19 In the Separation Setup pop-up form, left click the dropdown and select the appropriate

preloaded experimental Method file. The available methods are sorted by kit number and are linked to the directory containing methods for the currently installed capillary array length (e.g., 22cm, 33cm or 55cm). Select DNF-473-(33 or 55) - SS NGS Fragment 1-6000bp.mthds.

- 8.3.20 Select the appropriate Gel line being used for the experiment (Gel 1 or Gel 2) using the dropdown menu.
- 8.3.21 Prior to starting the experiment, verify all trays (buffer/storage, rinse, waste, sample, etc.) have been loaded into their respective drawer locations.
- 8.3.22 Press the Play icon () to start the sequence loaded into the queue. To Pause the queue after the currently running experiment is completed, press the  button. To Clear the run queue of all loaded runs press the  button.
- 8.3.23 Using ProSize 2.0, perform a smear analysis to quantify the amount of amplified library.
 - 8.3.23.1 Click Option
 - 8.3.23.1.1 Click Set Global Configuration
 - 8.3.23.2 Click on the Smear Analysis Tab
 - 8.3.23.2.1 Enter the Start size as 40 bp
 - 8.3.23.2.2 Enter the End size as 2000 bp
 - 8.3.23.2.3 Click Apply
 - 8.3.23.3 Click File
 - 8.3.23.3.1 Click Export Data
 - 8.3.23.3.1.1 Click Smear Analysis radio button
 - 8.3.23.3.2 Click Export
 - 8.3.23.3.2.1 Open the exported smear analysis csv file. The column labeled ng/uL contains the concentration information
- 8.3.24 Enter the concentration information in the 'shearing' tab of the library prep template file and calculate the total recovered amount and %.
- 8.3.25 Inspect the fragment analyzer traces to ensure that sheared samples have a peak size of 150 to 200 bp.

8.4 Library preparation using the SciClone

- 8.4.1 Open "SSXT Library Prep" program in Maestro software.
- 8.4.2 Prepare End Repair, A-Tailing, and Ligation reaction mixes (prepare on ice):
 - 8.4.2.1 Prepare the reaction mixes according to the "SureSelect XT Workbook", using tables found under the "SS XT Lib Prep" tab. Mix well on a vortex mixer.

End Repair Mix	1 Sample
10X End Repair Buffer	5
dNTP Mix	0.8
T4 DNA Polymerase	1
Klenow DNA Polymerase	2
T4 Polynucleotide Kinase	2.2
Water	10
Total	21
A-Tailing Mix	1 Sample
10X Klenow DNA Polymerase Buffer	5
dATP	1
Exo(-) Klenow DNA Polymerase	3
Water	11
Total	20
Adapter Ligation Mix	1 Sample
5X T4 DNA Ligase Buffer	10
InPE Adapter oligo mix (*added within progr	10
T4 DNA Ligase	1.5
Water	<u>15.5</u>
Total	37

8.4.2.2 Add appropriate amounts of each master mix to the Master Mix plate, according to the “SureSelect XT Workbook”.

8.4.3 Set up the SciClone for library preparation

8.4.3.1 Add appropriate amount of PEG solution to plate, according to the “SureSelect XT Workbook” and place on the SciClone deck according to the deck layout.

8.4.3.2 Add appropriate amount of SureSelect Adaptor Oligos to plate, according to the “SureSelect XT Workbook” and place on the SciClone deck according to the deck layout.

8.4.3.3 Add appropriate amount of AMPure XP beads to plate, according to the “SureSelect XT Workbook” and place on the SciClone deck according to the deck layout.

8.4.3.4 Place DNA sample plate (containing sheared, SPRI-purified samples from “SSXT Initial SPRI Cleanup” procedure) onto the SciClone deck according to the deck layout.

8.4.3.5 Place all additional labware onto SciClone deck according to the deck layout.

8.4.4 Press start to begin procedure.

8.4.5 During Ligation step, prepare PCR Enrichment Master Mix, according to the “SureSelect XT Workbook”, using tables found under the “SS XT Lib Prep” tab.

PCR Enrichment Mix	1 Sample
InPE Primer (Forward)	1.25
SureSelect Indexing Pre-Capture PCR (Reverse)	1.25
Herculase II 5X Reaction Buffer	10
dNTP Mix	0.5
Herculase II Polymerase	1
Water	21
Total	35

- 8.4.6 When prompted by the software, add appropriate amounts of each master mix to the Master Mix plate, according to the “SureSelect XT Workbook”.
- 8.4.7 After Ligation and SPRI cleanup, the software will prompt user to replace Master Mix plate, and a new PCR plate onto the deck. Resume procedure by clicking through the prompts.
- 8.4.8 When prompted by the software, move sample plate to Veriti Thermal Cycler and run the following program, programmed for 6 cycles:

Step	Temperature	Time
Step 1	98°C	2 minutes
Step 2	98°C	30 seconds
Step 3	65°C	30 seconds
Step 4	72°C	1 minute
Step 5		Repeat Step 2 through Step 4 for a total of 4 to 6 times.
Step 6	72°C	10 minutes
Step 7	4°C	Hold

- 8.4.9 Remove the plate containing remainder of ligated sample from the deck, seal with an adhesive plate seal, label, and store at -20°C.
- 8.4.10 While the plate is running on the thermal cycler prepare the SciClone for post-amplification cleanup
 - 8.4.10.1 Add 90 ul AMPure XP beads to a new BioRad hard shell plate, according to the “SureSelect XT Workbook”.
 - 8.4.10.2 Place bead plate onto the SciClone deck according to the deck layout.
 - 8.4.10.3 Replace Qiagen EB reservoir plate with one containing nuclease free water.
- 8.4.11 When PCR is complete, move the sample plate back to the SciClone deck, according to the software prompt.
- 8.4.12 Click “OK” on the software prompt to continue with AMPure cleanup.
- 8.4.13 When cleanup is complete, place sample plate on ice in preparation for quality assessment/quantitation using Fragment Analyzer.

Note: Stopping Point - If you do not continue to the next step, store the samples at -20°C.

8.5 Assess quality and quantity of prehybridization samples using the Fragment Analyzer

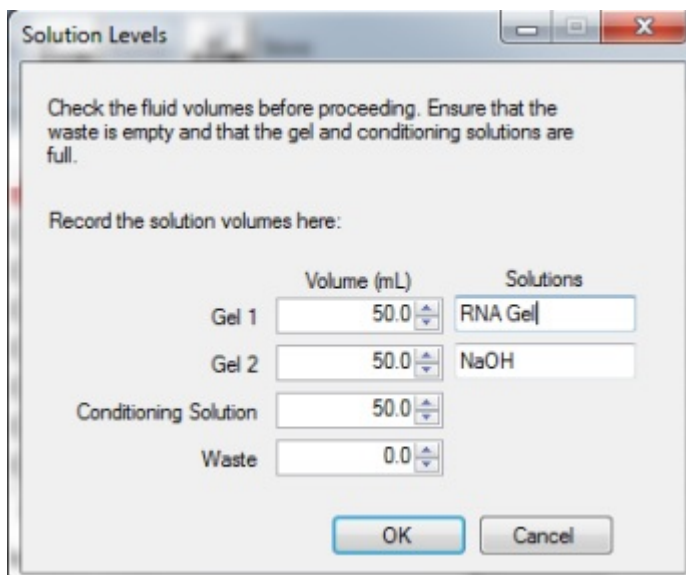
- 8.5.1 Dilute 1 µl of sample with 9 µl 1X Low TE and load on an Advanced Analytical Fragment

Analyzer using High Sensitivity NGS Fragment Analysis Kit.

- 8.5.2 Allow Inlet Buffer, Capillary Conditioning Solution, Gel, and Intercalating Dye to come to room temperature prior to mixing.
- 8.5.3 Allow high sensitivity marker and ladder to come to room temperature
- 8.5.4 Mix appropriate volumes of Intercalating Dye and Separation Gel necessary for one day of operation. Use the supplied 50 mL conical centrifuge tube to allow a small minimum working volume. For larger volumes, use a 250 mL conical centrifuge tube and remove the collar of the tube holder in the instrument reagent compartment.
- 8.5.5 The volume of Separation Gel required per run on the 96-Capillary Fragment Analyzer™ system is summarized below.

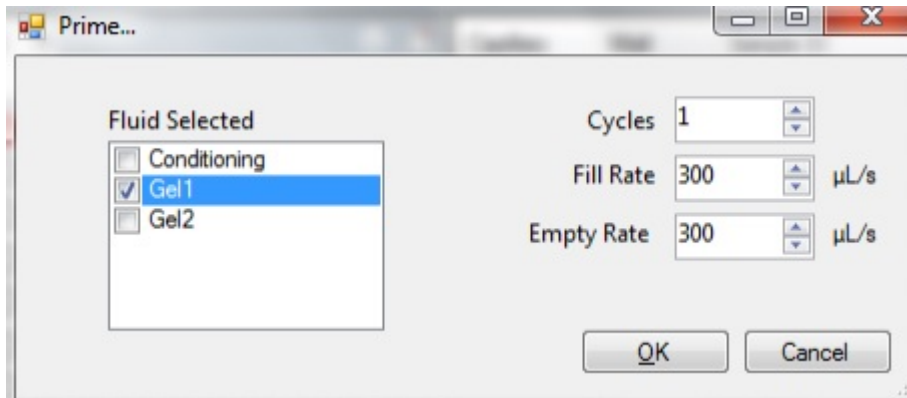
# of samples to be analyzed	Volume of Intercalating Dye	Volume of RNA Separation Gel
95	4.0 uL	40 mL
190	8.0 uL	80 mL
285	12.0 uL	120 mL
380	16.0 uL	160 mL
475	20.0 uL	200 mL

- 8.5.6 Place the Separation Gel/ Intercalating Dye mixture onto the instrument and insert into the desired gel fluid line (Gel 1 or Gel 2 pump position). Ensure the fluid line is positioned at the bottom of the conical tube to avoid introducing air bubbles, which can cause pressurization errors.
- 8.5.7 When adding Separation Gel to the instrument, update the solution levels in the Fragment Analyzer instrument control software. From the Main Menu, select Utilities – Solution Levels. A menu will be displayed to enter in the updated fluid levels.






- 8.5.8 When switching applications (e.g., between NGS and RNA kits), prime the appropriate gel fluid line after loading fresh gel/dye mixture. From the Main Menu of the Fragment

Analyzer instrumental control software, select Utilities — Prime... Select the desired fluid line(s) (Conditioning, Gel 1, or Gel 2) and press OK to purge the fluid line with fresh gel.



- 8.5.9 In a clean container, add 20 mL of the 5X 930 dsDNA Inlet Buffer per 80 mL of deionized sub-micron filtered water. Agitate to mix. The entire bottle can be mixed to 1X concentration and stored at 4°C if desired.
- 8.5.10 In a clean container (e.g. 50 mL or 250 mL conical centrifuge tube), add 20 mL of the 5X Capillary Conditioning Solution per 80 mL of deionized sub-micron filtered water. Agitate to mix. The entire bottle can be mixed to 1X concentration and stored at room temperature if desired.
- 8.5.11 Once mixed, place the 1X Capillary Conditioning Solution onto the instrument and insert the CONDITIONING fluid line (Conditioning Solution pump position). Ensure the fluid line is positioned at the bottom of the conical tube to avoid introducing air bubbles, which can cause pressurization errors. Update the solutions levels in the Utilities section of the Fragment Analyzer software.
- 8.5.12 Check the fluid level of the waste bottle and waste tray daily and empty as needed.
- 8.5.13 Prepare a fresh 96 DeepWell 1mL Plate filled with 1.0 mL/well of 1X 930 dsDNA Inlet Buffer daily. Do NOT overfill the wells of the inlet buffer plate. Place buffer plate in Drawer "B".
- 8.5.14 Prepare a fresh sample plate filled with 100 μL/well of 0.25X TE Rinse Buffer daily. Place rinse plate in Drawer "M"
- 8.5.15 Using a fresh half-skirted 96-well sample plate, pipette 22 μL of the High Sensitivity NGS Diluent Marker to each well in a row that is to contain sample or NGS Ladder. Fill any unused wells within the row of the sample plate with 24 μL/well of BF-25 Blank Solution.
- 8.5.16 Pipette 2 μL of each diluted sample into the respective wells of the sample plate; mix the contents of the well using the pipette by aspiration/expulsion in the pipette tip.
- 8.5.17 After mixing sample/NGS Ladder and Diluent Marker in each well, centrifuge the plate to remove any air bubbles. Check the wells of the sample plate to ensure there are no air bubbles trapped in the bottom of the wells. The presence of trapped air bubbles can lead to injection failures.
- 8.5.18 For best results, run the plate as soon as possible. If the sample plate will not be used immediately, cover the sample plate with foil/seal, store at 4°C and use within the same day. Spin the plate again if any bubbles developed in the sample wells. *Be sure to remove*

the cover film before placing the plate into the instrument.

- 8.5.19 In the Separation Setup pop-up form, left click the dropdown and select the appropriate preloaded experimental Method file. The available methods are sorted by number and are linked to the directory containing methods for the currently installed capillary array length (e.g., 22cm, 33cm or 55cm). Select DNF-474-033 - HS NGS Fragment 1-6000bp.mthds.
- 8.5.20 Select the appropriate Gel line being used for the experiment (Gel 1 or Gel 2) using the dropdown menu.
- 8.5.21 Prior to starting the experiment, verify all trays (buffer/storage, rinse, waste, sample, etc.) have been loaded into their respective drawer locations.
- 8.5.22 Press the Play icon () to start the sequence loaded into the queue. To Pause the queue after the currently running experiment is completed, press the  button. To Clear the run queue of all loaded runs press the  button.
- 8.5.23 Using ProSize 2.0, perform a smear analysis to quantify the amount of amplified library.
- 8.5.23.1 Click Option
 - 8.5.23.1.1 Click Set Global Configuration
 - 8.5.23.2 Click on the Smear Analysis Tab
 - 8.5.23.2.1 Enter the Start size as 40 bp
 - 8.5.23.2.2 Enter the End size as 2000 bp
 - 8.5.23.2.3 Click Apply
 - 8.5.23.3 Click File
 - 8.5.23.3.1 Click Export Data
 - 8.5.23.3.1.1 Click Smear Analysis radio button
 - 8.5.23.3.2 Click Export
 - 8.5.23.3.2.1 Open the exported smear analysis csv file. The column labeled ng/uL contains the concentration information. The data will be used to calculate normalization prior to hybridization.

Check that the Fragment Analyzer traces show a distribution with a peak height between 225 to 275 bp.

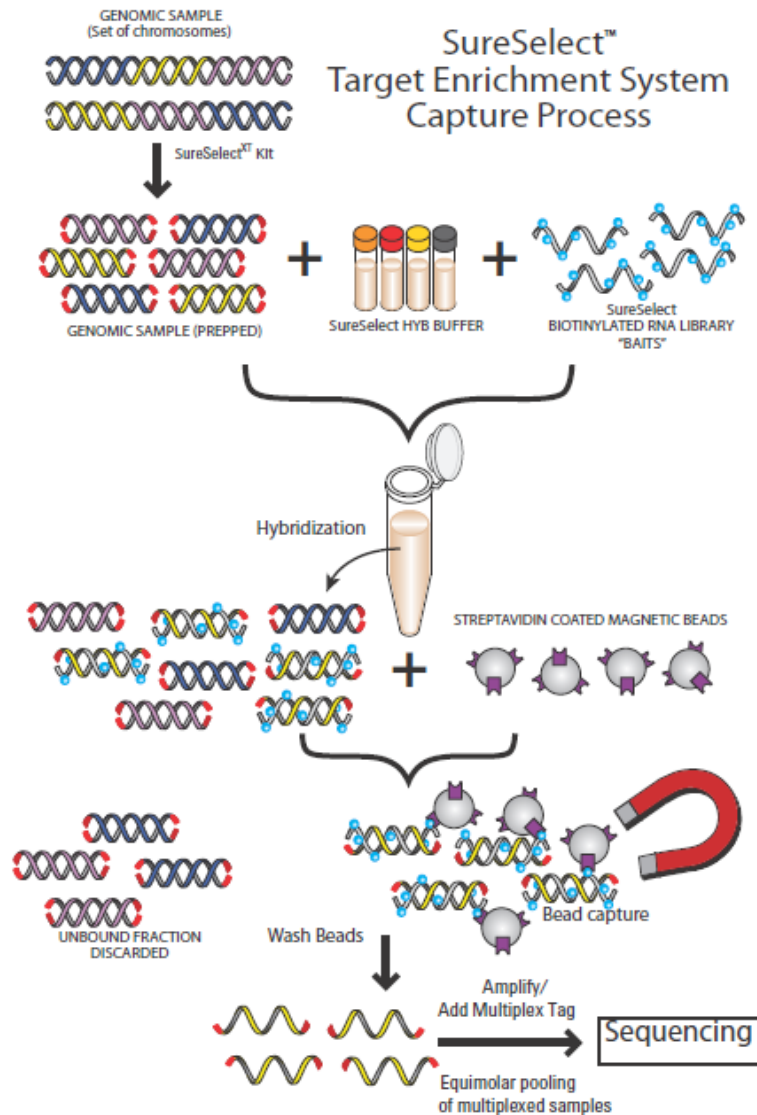
Note: Stopping Point - If you do not continue to the next step, seal the ligated DNA library sample plate and store at 4°C overnight or at -20°C for prolonged storage.

8.6 Normalization of samples for hybridization

- 8.6.1 Open the “Normalization_for_Hyb_Setup” excel spreadsheet on the Sciclone computer. Enter quantitation values from Fragment Analyzer run smear analysis.
- 8.6.2 Save the spreadsheet and close the file.
- 8.6.3 Open “SSXT Normalization for PreCapture Same Day Hyb” program in the Maestro control software.
- 8.6.4 Begin the procedure by clicking start button.
- 8.6.5 Software will prompt user to enter number of columns to be processed. Enter this number and click OK to continue.
- 8.6.6 Software will prompt user to select quantitation file. Select “Normalization_for_Hyb_Setup” file from the “Workbooks” Folder on the Desktop. Select file and click OK to continue.
- 8.6.7 Place a deep well reservoir containing nuclease-free water onto the SciClone deck

according to the deck layout.

- 8.6.8 Place DNA sample plate (containing amplified, ligated, SPRI-purified samples from “SSXT Library Prep” procedure) onto the SciClone deck according to the deck layout.
- 8.6.9 Place all additional labware onto SciClone deck according to the deck layout.
- 8.6.10 Press start to begin procedure.
- 8.6.11 When complete, the software will prompt user to place normalized DNA library sample plate on ice in preparation for hybridization procedure; it will also prompt user to seal the and store at -20 the sample plate containing the remaining DNA library.



SureSelect Target Enrichment System Capture Process

8.7 Hybridization

- 8.7.1 Remove SureSelect hybridization reagents from -20 storage to thaw on ice.
- 8.7.2 Remove SureSelect Human All Exon V5 capture library from -80 storage to thaw on ice.
- 8.7.3 Open “SureSelect XT Workbook” excel file, and enter the number of columns to be processed under the “SS Hyb Setup” tab. Save the file.

8.7.4 Open “SSXT Hyb Setup” method in the Maestro control software.

8.7.5 Press start to begin the procedure.

Note: There will be a 30 minute wait time to allow the heat blocks to come to the correct temperature. Use this time to prepare hyb reagent mixes.

8.7.6 Prepare SureSelect Block Mix, RNase Block Mix, Hybridization Buffer, SureSelect Capture Library mixes.

8.7.6.1 Prepare all mixes according to the “SureSelect XT Workbook”, using tables found under the “SSXT Hyb Setup” tab.

SureSelect Block Mix	
SureSelect Indexing Block #1 (green cap)	33.8
SureSelect Indexing Block #2 (blue cap)	33.8
SureSelect Indexing Block #3 (brown cap)	8.1
Total	75.7

Hybridization Buffer*	
SureSelect Hyb #1	61.0
SureSelect Hyb #2 (red cap)	2.4
SureSelect Hyb #3 (yellow cap)	24.4
SureSelect Hyb #4	31.7
Total	119.6

RNase Block Mix	
Nuclease Free Water	13.8
RNase Block (Purple Cap)	4.6
Total	18.4

SureSelect Capture Library	
SureSelect V5 Library	46
RNAse Block Mix (1:3)	18.4
Total	64.4

8.7.7 Prepare Capture Probes in Hyb Buffer according to the worksheet.

Capture Probes in Hyb Buffer	
Hybridization Buffer	119.6
SureSelect Capture Library	64.4
Total	184

8.7.8 Load the Block Mix and Hybridization Probe Plate with the SureSelect Block Mix and the Capture Probes in Hyb Buffer, according to the worksheet.

8.7.8.1 Place plate onto the SciClone deck according to the deck layout.

8.7.9 Place plate containing the Normalized DNA samples onto the SciClone deck according to the deck layout.

8.7.10 Click Start to begin procedure.

8.7.11 While the hybridization setup is proceeding, begin Veriti Thermal Cycler program set to 65 degrees, infinite hold, with a lid temperature of 105 degrees.

8.7.12 When prompted seal the hybridization plate using two plate seals, forming a double layer of seals.

8.7.13 Quickly move the hybridization plate onto the Veriti Thermal Cycler.

8.7.14 Incubate the hybridization mixture for 16 to 24 hours at 65°C with a heated lid at 105°C.

8.7.15 Return reagents to proper freezers. Be sure to store V5 Capture library at -80 degrees.

8.8 Post Hybridization Target Selection

8.8.1 Remove AMPure XP beads and Dynabeads MyOne Streptavidin T1 beads from refrigerator and allow to equilibrate to room temperature.

8.8.2 Load the following capture buffer reservoirs according to “SSXT Target Selection” tab of “SureSelect XT Workbook” excel spreadsheet:

- Binding Buffer
- Wash Buffer 1
- Wash Buffer 2
- Dynabead/10 mM Tris

8.8.3 Open “SSXT Target Selection” method using Maestro control software.

8.8.4 Load all reagent plates and additional labware onto the SciClone deck according to the deck layout.

8.8.5 Click Start to begin procedure.

- 8.8.6 When the Dynabead washing is complete, the software will prompt user to remove the hybridization plate from the thermal cycler, remove seals, and place onto the SciClone deck according to the prompt.
- 8.8.7 During the fifth and final Wash 2 Buffer cleanup, prepare PCR Master Mix and load a PCR plate according to the “SSXT Target Selection” tab of “SureSelect XT Workbook” excel spreadsheet.

PCR Enrichment Mix	1 Sample	10% extra
SureSelect Indexing Post-Capture PCR (Forward)	1	1.1
Herculase II 5X Reaction Buffer	10	11
dNTP Mix	0.5	0.55
Herculase II Polymerase	1	1.1
Water	7.5	8.25
Total	20	22

- 8.8.8 When prompted, place the PCR Master Mix plate, and the Index plate (found in the SureSelect Library Reagent Kit), onto the SciClone deck according to the deck layout.

NOTE: Captured DNA is retained on the streptavidin beads during the post-capture amplification step.

- 8.8.9 Click OK to continue PCR setup.

- 8.8.10 Program a Veriti Thermal Cycler with the following program:

Step	Temperature	Time
Step 1	98°C	2 minutes
Step 2	98°C	30 seconds
Step 3	57°C	30 seconds
Step 4	72°C	1 minute
Step 5		• Repeat Step 2 through Step 4 for a total of 12 to 16 times.
Step 6	72°C	10 minutes
Step 7	4°C	Hold

- 8.8.10.1 Program the thermal cycler to perform 11 cycles.

- 8.8.11 When washing is complete the Maestro software will prompt the user to seal the PCR plate and move it to the thermal cycler.

- 8.8.12 When the PCR program is complete, remove the PCR plate and place on ice in preparation for final AMPure cleanup on the Perkin Elmer Zephyr automated liquid handler.

8.9 Final Library AMPure cleanup on the Zephyr

- 8.9.1 Turn on SciClone Zephyr, Heating controller, and open Maestro software.

- 8.9.2 Open “Post_SPRI_Cleanup” method using the Maestro control software.

- 8.9.3 Click start to begin program.

- 8.9.4 Software will prompt user to enter the following:

- 8.9.4.1 Number of columns ___
- 8.9.4.2 Volume of starting sample: Enter “50”
- 8.9.4.3 Volume of AMPure beads: Enter “90”
- 8.9.4.4 Final volume upon elution: Enter “30”
- 8.9.5 Prepare all reagent plates/reservoirs according to the software prompts.
- 8.9.6 Place all reagents plates and additional labware onto the Zephyr deck according to the deck layout.
- 8.9.7 Place PCR Sample plate onto the Zephyr deck
- 8.9.8 Press Start to begin the procedure.
- 8.9.9 Software will prompt user to remove PCR Sample Plate when it has removed sample.
- 8.9.10 Click OK to proceed.
- 8.9.11 When process is completed, software will prompt user to remove Final Library Plate.
- 8.9.12 Proceed to Final Library Quality Assessment.

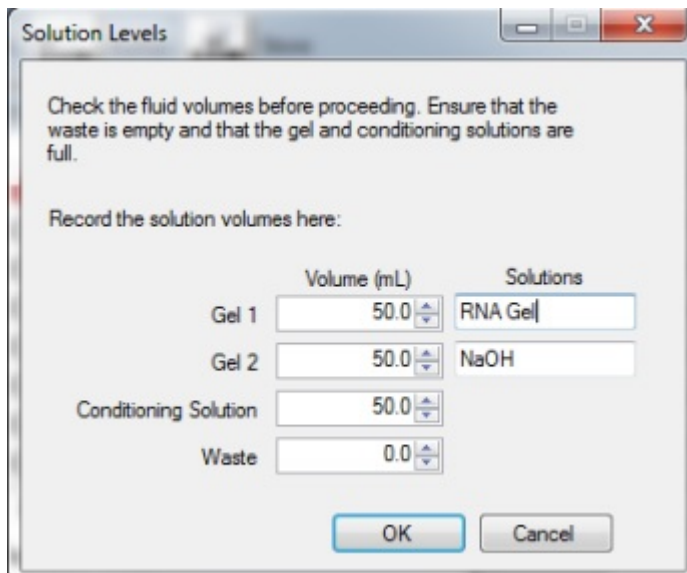
Note: Stopping Point - If you do not continue to the next step, store the samples at 4°C for up to a week, or at -20°C for longer periods.

8.10 Final Library Quality Assessment using the Fragment Analyzer

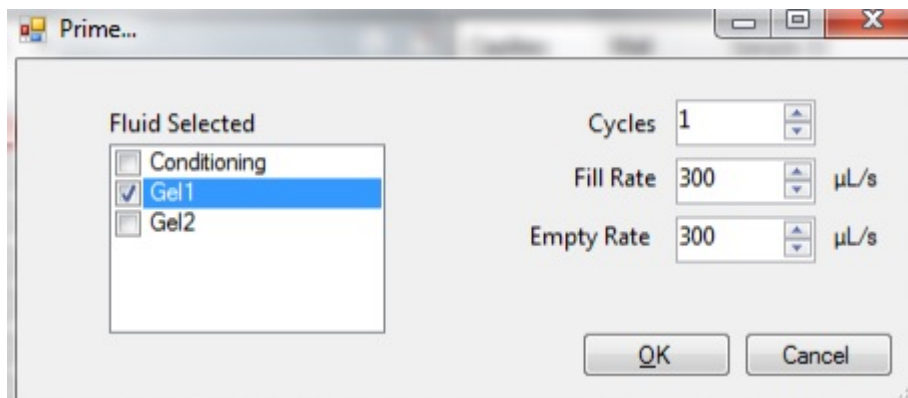
- 8.10.1 Dilute 1 µl of final library sample with 19 µl 1X Low TE and load on an Advanced Analytical Fragment Analyzer using High Sensitivity NGS Fragment Analysis Kit.
- 8.10.2 Allow Inlet Buffer, Capillary Conditioning Solution, Gel, and Intercalating Dye to come to room temperature prior to mixing.
- 8.10.3 Allow high sensitivity marker and ladder to come to room temperature
- 8.10.4 Mix appropriate volumes of Intercalating Dye and Separation Gel necessary for one day of operation. Use the supplied 50 mL conical centrifuge tube to allow a small minimum working volume. For larger volumes, use a 250 mL conical centrifuge tube and remove the collar of the tube holder in the instrument reagent compartment.
- 8.10.5 The volume of Separation Gel required per run on the 96-Capillary Fragment Analyzer™ system is summarized below.

# of samples to be analyzed	Volume of Intercalating Dye	Volume of RNA Separation Gel
95	4.0 uL	40 mL
190	8.0 uL	80 mL
285	12.0 uL	120 mL
380	16.0 uL	160 mL
475	20.0 uL	200 mL

- 8.10.6 Place the Separation Gel/ Intercalating Dye mixture onto the instrument and insert into the desired gel fluid line (Gel 1 or Gel 2 pump position). Ensure the fluid line is positioned at the bottom of the conical tube to avoid introducing air bubbles, which can cause pressurization errors.
- 8.10.7 When adding Separation Gel to the instrument, update the solution levels in the Fragment Analyzer instrument control software. From the Main Menu, select Utilities – Solution Levels. A menu will be displayed to enter in the updated fluid levels.



8.10.8 When switching applications (e.g., between NGS and RNA kits), prime the appropriate gel fluid line after loading fresh gel/dye mixture. From the Main Menu of the Fragment Analyzer instrumental control software, select Utilities – Prime... Select the desired fluid line(s) (Conditioning, Gel 1, or Gel 2) and press OK to purge the fluid line with fresh gel.






8.10.9 In a clean container, add 20 mL of the 5X 930 dsDNA Inlet Buffer per 80 mL of deionized sub-micron filtered water. Agitate to mix. The entire bottle can be mixed to 1X concentration and stored at 4°C if desired.

8.10.10 In a clean container (e.g. 50 mL or 250 mL conical centrifuge tube), add 20 mL of the 5X Capillary Conditioning Solution per 80 mL of deionized sub-micron filtered water. Agitate to mix. The entire bottle can be mixed to 1X concentration and stored at room temperature if desired.

8.10.11 Once mixed, place the 1X Capillary Conditioning Solution onto the instrument and insert the CONDITIONING fluid line (Conditioning Solution pump position). Ensure the fluid line is positioned at the bottom of the conical tube to avoid introducing air bubbles, which can cause pressurization errors. Update the solutions levels in the Utilities section of the Fragment Analyzer software.

8.10.12 Check the fluid level of the waste bottle and waste tray daily and empty as needed.

- 8.10.13 Prepare a fresh 96 DeepWell 1mL Plate filled with 1.0 mL/well of 1X 930 dsDNA Inlet Buffer daily. Do NOT overfill the wells of the inlet buffer plate. Place buffer plate in Drawer “B”.
- 8.10.14 Prepare a fresh sample plate filled with 100 µL/well of 0.25X TE Rinse Buffer daily. Place rinse plate in Drawer “M”
- 8.10.15 Using a fresh half-skirted 96-well sample plate, pipette 22 µL of the High Sensitivity NGS Diluent Marker to each well in a row that is to contain sample or NGS Ladder. Fill any unused wells within the row of the sample plate with 24 µL/well of BF-25 Blank Solution.
- 8.10.16 Pipette 2 µL of each 1:20 diluted sample into the respective wells of the sample plate; mix the contents of the well using the pipette by aspiration/expulsion in the pipette tip.
- 8.10.17 After mixing sample/NGS Ladder and Diluent Marker in each well, centrifuge the plate to remove any air bubbles. Check the wells of the sample plate to ensure there are no air bubbles trapped in the bottom of the wells. The presence of trapped air bubbles can lead to injection failures.
- 8.10.18 For best results, run the plate as soon as possible. If the sample plate will not be used immediately, cover the sample plate with foil/seal, store at 4°C and use within the same day. Spin the plate again if any bubbles developed in the sample wells. *Be sure to remove the cover film before placing the plate into the instrument.*
- 8.10.19 In the Separation Setup pop-up form, left click the dropdown and select the appropriate preloaded experimental Method file. The available methods are sorted by kit number and are linked to the directory containing methods for the currently installed capillary array length (e.g., 22cm, 33cm or 55cm). Select DNF-474-033 - HS NGS Fragment 1-6000bp.mthds.
- 8.10.20 Select the appropriate Gel line being used for the experiment (Gel 1 or Gel 2) using the dropdown menu.
- 8.10.21 Prior to starting the experiment, verify all trays (buffer/storage, rinse, waste, sample, etc.) have been loaded into their respective drawer locations.
- 8.10.22 Press the Play icon () to start the sequence loaded into the queue. To Pause the queue after the currently running experiment is completed, press the  button. To Clear the run queue of all loaded runs press the  button.
- 8.10.23 Using ProSize 2.0, perform a smear analysis to quantify the amount of amplified library.
- 8.10.23.1 Click Option
 - 8.10.23.1.1 Click Set Global Configuration
 - 8.10.23.2 Click on the Smear Analysis Tab
 - 8.10.23.2.1 Enter the Start size as 50 bp
 - 8.10.23.2.2 Enter the End size as 1000 bp
 - 8.10.23.2.3 Click Apply
 - 8.10.23.3 Click File
 - 8.10.23.3.1 Click Export Data
 - 8.10.23.3.1.1 Click Smear Analysis radio button
 - 8.10.23.3.2 Click Export

- 8.10.23.4 Open the exported smear analysis csv file. The column labeled nM/L indicates the concentration of the diluted final library that was loaded onto the Fragment Analyzer. The smear analysis data will be used to calculate dilutions required for digital PCR quantification.
- 8.10.24 Check that the Fragment Analyzer traces show a distribution with a peak size between 250 to 350 bp.
- 8.10.25 Using nuclease-free water, dilute the final libraries to a target concentration of ~6nM.
- 8.11 Quantify the individual Final Libraries using ddPCR**
- 8.11.1 Refer to ddPCR Library Quantitation SOP (**APPENDIX 5F**).
- 8.12 Pool the libraries**
- 8.12.1 Once all libraries have been quantified using digital PCR, pool samples into pools of 3 using quantitation data and Barcode Index ID information.
- 8.12.1.1 Samples should be pooled such that pools contain libraries with similar starting concentrations and unique indexes
- 8.12.1.2 Use the pool pairings sheet in the library prep template file to record pool information. The table will automatically calculate the volumes of each library and EBT necessary to create the pool at a target concentration of 3nM
- 8.13 Quantify the pooled libraries using ddPCR**
- 8.13.1 Refer to ddPCR Library Quantitation SOP (**APPENDIX 5F**).
- 8.14 Dilute final pools**
- 8.14.1 Final dilutions of each pool, prior to denaturing and clustering, should be diluted to a final concentration of 1.326 nM.
- 8.14.1.1 Use the flow cell sheets in the library prep template file to calculate how to dilute the pools to the desired concentration
- 8.15 Dilute, Denature, and Cluster Final Pools for Sequencing**
- 8.15.1 Refer to Denaturing and Clustering SOP (**APPENDIX 5G**).
- 8.15.1.1 Pools should be clustered at a concentration of 13.26 pM with a 1% phiX spike-in for each sample.
- 8.15.1.2 Each pool of 3 samples is sequenced in a single flow cell lane.

9.0 Detailed SciClone Automation Steps.

- 9.1.1 SureSelectXT Initial SPRI Cleanup
- 9.1.1.1 Load tips. Transfer 180 uL SPRI beads to sample plate. Mix. Dispose of bead plate.
- 9.1.1.2 Shake for 5 minutes
- 9.1.1.3 Move plate from shaker on D4 to magnet without spacer at B4. Incubate for 6 minutes
- 9.1.1.4 Remove supernatant from sample and dispose in waste deep-well. Eject tips.
- 9.1.1.5 Move sample plate from B4 to magnet with spacer at C4.
- 9.1.1.6 Load tips. Wash beads with 150 uL of 80% EtOH. Eject tips.
- 9.1.1.7 Move sample plate from C4 to D2 (37C). Dry EtOH 10 minutes.
- 9.1.1.8 Move sample plate from D2 to shaker at D4.
- 9.1.1.9 Load tips. Transfer 30 uL elution buffer to sample plate. Mix. Shake plate for 3 minutes. Mix again. Eject tips.

- 9.1.1.10 Load tips. Aspirate and dispense at D4, pool samples, mix, eject tips.
 - 9.1.1.11 Move sample plate from D4 to magnet without spacer at B4. Incubate 5 minutes.
 - 9.1.1.12 Move clean plate from A2 to A4.
 - 9.1.1.13 Transfer 30 uL eluted sample to new plate.
 - 9.1.1.14 Dispose of bead plate from B4.
 - 9.1.1.15 Application complete.
- 9.1.2 “SureSelectXT Library Prep” Application.
- 9.1.2.1 Step 1 – Prepare End Repair Plate
 - 9.1.2.2 Move lid from A3 to C2. Move lid from A4 to B2.
 - 9.1.2.3 Load tips. Transfer 20 uL End Repair mix from col1 of A3 to Q1 of 384-well plate at A4. Eject tips.
 - 9.1.2.4 Move lid from C2 to A3.
 - 9.1.2.5 Load tips. Transfer 20 uL End Repair mix from Q1 of 384-well plate at A4 to sample plate at B4. Mix. Eject tips.
 - 9.1.2.6 Step 2 – End Repair
 - 9.1.2.7 Move End Repair plate B4 to shaker on D4. Move lid on D3 deepwell to plate on shaker at D4.
 - 9.1.2.8 Incubate 30 minutes with shaking at 20C. During incubation:
 - 9.1.2.9 Move lid at A3 to C2.
 - 9.1.2.10 Load tips (single column). Transfer 20 uL of A-Tail mix from col 3/4 at A3 to Q2 of 384-well plate at A4. Eject tips.
 - 9.1.2.11 Load tips (single column). Transfer 27 uL of ligation mix from col 5/6 at A3 to Q3 of 384-well plate at A4. Eject tips.
 - 9.1.2.12 Move lid from C2 to A3. Move lid from B2 to A4.
 - 9.1.2.13 Move lid from D4 to D3. Move bead plate from C2 to D2.
 - 9.1.2.14 Load tips. Mix beads at D2.
 - 9.1.2.15 Transfer 90 uL beads from D2 to End Repair reaction on shaker at D4.
 - 9.1.2.16 Move bead plate from D2 to C2.
 - 9.1.2.17 Mix beads and sample at D4. Shake for 5 minutes.
 - 9.1.2.18 Move sample/bead plate from D4 to the magnet at B4. Incubate 5 minutes.
 - 9.1.2.19 Remove SN from beads at B4 to waste at A5. Eject tips.
 - 9.1.2.20 Move beads from B4 to magnet with spacer at C4. Move lid B5 to C5.
 - 9.1.2.21 Load tips. Wash beads with 150 uL of 80% EtOH. Eject tips. Repeat.
 - 9.1.2.22 Move lid from C5 to B5. Move plate from C4 to D2 CPAC at 37C. Incubate 5 minutes to dry EtOH.
 - 9.1.2.23 Move plate from D2 to D4. Move lid from D3 deepwell to C2.
 - 9.1.2.24 Load tips. Transfer 30 uL elution buffer from D3 deepwell to beads at D4.
 - 9.1.2.25 Move lid from C2 to D3.
 - 9.1.2.26 Mix at D4 to resuspend beads. Shake for 3 minutes. Eject tips.
 - 9.1.2.27 Wait for temp at D2 (20C). Move plate from D4 to D2
 - 9.1.2.28 Step 3 – A-Tailing
 - 9.1.2.29 Move lid from A4 to C2.
 - 9.1.2.30 Load tips. Transfer 20 uL A-Tail mix from Q2 384 at A4 to samples on D2. Mix. Eject

- tips
- 9.1.2.31 Move samples from D2 to D4 at 37C for A-Tailing incubation. Move lid from D3 to D4.
 - 9.1.2.32 Move lid from A2 to D3. Move Adapter Oligo plate from A2 to D2.
 - 9.1.2.33 Incubate A-Tailing reaction for 30 minutes at 37C with shaking. During incubation:
 - 9.1.2.34 Load tips (single column). Broadcast 10 uL of adapter oligos from plate at D2 to Q4 of 384-well plate at A4. Eject tips.
 - 9.1.2.35 Dispose of plate at D2. Move lid from C2 to A4.
 - 9.1.2.36 Move lid from D3 to A2. Move lid from D4 to D3.
 - 9.1.2.37 Move lid from B2 to C2. Move PEG plate from B2 to D2.
 - 9.1.2.38 Load tips. Transfer 90 uL PEG from D2 to D4. Move peg plate from D2 to B2.
 - 9.1.2.39 Move lid from C2 to B2.
 - 9.1.2.40 Mix samples/PEG at D4. Shake for 5 minutes.
 - 9.1.2.41 Move samples/beads from D4 to magnet without spacer at B4, incubate 5 min.
 - 9.1.2.42 Remove SN from B4 to waste at B5, eject tips.
 - 9.1.2.43 Move beads B4 to magnet with spacer C4. Move lid from B5 to C5.
 - 9.1.2.44 Load tips. Wash beads with 150 uL of 80% EtOH. Eject tips. Repeat.
 - 9.1.2.45 Move lid from C5 to B5. Move plate from C4 to D2 at 37C. Incubate for 5 minutes.
 - 9.1.2.46 Move plate from D2 to D4. Move lid from D3 deepwell to C2.
 - 9.1.2.47 Load tips. Transfer 15 uL elution buffer from D3 deepwell to beads at D4.
 - 9.1.2.48 Move lid from C2 to D3.
 - 9.1.2.49 Mix at D4 to resuspend beads. Shake for 3 minutes. Eject tips.
 - 9.1.2.50 Step 4 – Adapter Ligation
 - 9.1.2.51 Dispose of lid from 384 at A4.
 - 9.1.2.52 Load tips. Transfer 27 uL of ligation mix from Q3 and 10 uL adapter Q4 from A4 to D4. Mix. Eject tips.
 - 9.1.2.53 Move lid from D3 to D4. Incubate for 15 minutes at 20C. During incubation:
 - 9.1.2.54 Dispose of 384 plate from A4
 - 9.1.2.55 Move lid from B2 to C2. Move PEG plate from B2 to D2. Move lid from D4 to D3.
 - 9.1.2.56 Load tips. Transfer 90 uL PEG from D2 to D4.
 - 9.1.2.57 Move plate from D2 to B2. Move lid from C2 to B2.
 - 9.1.2.58 Mix at D4, shake for 5 minutes
 - 9.1.2.59 Transfer to magnet without spacer at B4. Incubate for 5 minutes.
 - 9.1.2.60 Remove SN from B4 to waste at B5, eject tips.
 - 9.1.2.61 Move beads B4 to magnet with spacer C4. Move lid from B5 to C5.
 - 9.1.2.62 Load tips. Wash beads with 150 uL of 80% EtOH. Eject tips. Repeat.
 - 9.1.2.63 Move lid from C5 to B5. Move plate from C4 to D2 at 37C. Incubate for 5 minutes.
 - 9.1.2.64 Move plate from D2 to D4. Move lid from D3 deepwell to C2.
 - 9.1.2.65 Load tips. Transfer 30 uL elution buffer from D3 deepwell to beads at D4.
 - 9.1.2.66 Move lid from C2 to D3.
 - 9.1.2.67 Mix at D4 to resuspend beads. Shake for 3 minutes. Eject tips.
 - 9.1.2.68 Step 5 – PCR Setup
 - 9.1.2.69 Move plate from D4 to magnet without spacer at B4. Incubate for 5 min.
 - 9.1.2.70 Prompt user to add PCR MM to col 7/8 of PCR plate on A3, re-lid, place again at A3.
 - 9.1.2.71 Prompt user to place empty PCR plate on D2 CPAC at 4C.

- 9.1.2.72 Dispose of lid from A3.
 - 9.1.2.73 Load tips (single column). Transfer 35 uL PCR mix from col 7/8 of A3 to clean plate on D2, eject tips.
 - 9.1.2.74 Move bead plate from C2 to waste. Move clean plate from C2 to D4.
 - 9.1.2.75 Load tips. Aspirate 30 uL sample from B4. Dispense 15 uL to clean plate at D4, 15 uL to PCR plate at D2. Mix at D2. Eject tips.
 - 9.1.2.76 Dispose of plate from B4.
 - 9.1.2.77 Prompt user to seal PCR plate, spin down, and run enrichment protocol on thermocycler.
 - 9.1.2.78 Prompt user to remove plate from D4, seal, spin down, and store at -20C for future use.
 - 9.1.2.79 Step 6 – Post PCR
 - 9.1.2.80 Prompt user to replace 10 mM Tris Elution buffer in deepwell A3 with water in deepwell at A3. Ensure clean, lidded plate on A2.
 - 9.1.2.81 Prompt user to place PCR/library plate at B4 and plate with beads at D4.
 - 9.1.2.82 Dispose of lid at B2. Move plate from B2 to C2.
 - 9.1.2.83 Transfer 50 uL PCR reactions from B4 to beads at D4.
 - 9.1.2.84 Mix sample and beads at D4. Incubate 5 minutes.
 - 9.1.2.85 Transfer to samples to magnet without spacer at B4. Incubate for 5 minutes.
 - 9.1.2.86 Remove SN from B4 to waste at B5, eject tips.
 - 9.1.2.87 Move beads B4 to magnet with spacer C4. Move lid from B5 to C5.
 - 9.1.2.88 Load tips. Wash beads with 150 uL of 80% EtOH. Eject tips. Repeat.
 - 9.1.2.89 Move lid from C5 to B5. Move plate from C4 to D2 at 37C. Incubate for 5 minutes.
 - 9.1.2.90 Move plate from D2 to D4. Move lid from D3 deepwell to C2.
 - 9.1.2.91 Load tips. Transfer 30 uL water from D3 deepwell to beads at D4.
 - 9.1.2.92 Move lid from C2 to D3.
 - 9.1.2.93 Mix at D4 to resuspend beads. Shake for 3 minutes. Eject tips.
 - 9.1.2.94 Dispose of plate from B4. Move lid from C2 to D4.
 - 9.1.2.95 Prompt user to remove final library plate from D4.
 - 9.1.2.96 Application complete.
- 9.1.3 “SureSelectXT Normalization” Application
- 9.1.3.1 Load tips (single column). Eject tips to empty tip box at C5.
 - 9.1.3.2 Load single tip from C5.
 - 9.1.3.3 Aspirate water (sample-specific volume) from A2 deepwell. Aspirate sample (sample specific volume) from A3 source plate.
 - 9.1.3.4 Dispense water and sample to destination plate at A4. Eject tip.
 - 9.1.3.5 Repeat steps 2-4 for remaining samples in column.
 - 9.1.3.6 Repeat steps 1-5 for remaining columns of samples.
 - 9.1.3.7 Prompt user to remove starting sample plate from A3 and seal for long-term storage.
 - 9.1.3.8 Prompt user to remove the normalized library plate from A4 and seal for short-term storage in preparation for hybridization setup.
 - 9.1.3.9 Prompt user to remove and discard the reservoir containing water from A2.
 - 9.1.3.10 Application complete.

9.1.4 “SureSelectXT Hyb Setup” Application

- 9.1.4.1 Set temperatures for INHECO blocks and equilibrate for 30 minutes.
- 9.1.4.2 Load tips (single column). Transfer 5.6 uL of block mix from C4 column 1 to library plate at B4. Mix. Eject tips. Repeat for additional columns.
- 9.1.4.3 Move library plate from B4 to CPAC on A3 (95C). Incubate for 5 minutes without lid. During incubation:
 - 9.1.4.4 Load tips (single column). Transfer 20 uL Capture Library in Hyb Buffer from C4 column 2/3 to plate on D4 (65C).
 - 9.1.4.5 Repeat for number of columns processed. Eject tips.
 - 9.1.4.6 Move denatured library plate from A3 to B4.
 - 9.1.4.7 Load tips. Transfer remaining volume denatured library to plate at D4. Mix. Eject tips.
 - 9.1.4.8 Prompt user to double seal the hybridization plate while on D4, then quickly transfer to pre-warmed thermocycler set to 65C for hybridization.
 - 9.1.4.9 Application complete.

9.1.5 “SureSelectXT Target Selection” Application

- 9.1.5.1 Step 1 – Prepare Magnetic Beads
- 9.1.5.2 Move lid from C3 to B4 (This is to position lid correctly on B4).
- 9.1.5.3 Move empty bead washing plate from A2 to D4. Move lid from A4 to A2.
- 9.1.5.4 Load tips (single column). Transfer 50 uL of Streptavidin beads from A4 column 1 to plate at D4. Repeat for number of columns processed. Eject tips.
- 9.1.5.5 Move lid from A2 to A4. Move lid B5 to C5.
- 9.1.5.6 Load tips. Transfer 100 uL binding buffer from B5 reservoir to beads at D4. Mix. Shake for 2 minutes.
- 9.1.5.7 Move beads from D4 to magnet with spacer at C2. Incubate for 3 minutes.
- 9.1.5.8 Remove SN from beads on magnet at C2 to waste deep-well at A5.
- 9.1.5.9 Move bead plate from C2 to D4. Eject tips.
- 9.1.5.10 Load tips. Transfer 150 uL of Binding Buffer from B5 to the bead plate at D4. Mix. Shake for 2 min.
- 9.1.5.11 Move beads from D4 to the magnet at C2. Incubate for 3 minutes.
- 9.1.5.12 Remove SN from beads on magnet at C2 to waste deep-well at A5.
- 9.1.5.13 Move beads from C2 to D4. Eject tips.
- 9.1.5.14 Repeat steps 9-12 two more times.
- 9.1.5.15 Load tips. Transfer 125 uL of Binding Buffer to bead plate at D4. Mix. Eject tips. Shake for 2 minutes.
- 9.1.5.16 Move lid from C5 to B5.
- 9.1.5.17 Move head out of way.
- 9.1.5.18 Step 2 – Capture DNA
- 9.1.5.19 Prompt user to place hybridized samples plate on magnet at C4.
- 9.1.5.20 Load tips. Transfer samples from C4 to bead plate at D4. Mix. Shake for 10 minutes at 22C. Repeat mix and shake.
- 9.1.5.21 Dispose of sample plate from C4.
- 9.1.5.22 Move sample/bead plate from D4 to magnet C2. Incubate for 2 minutes.
- 9.1.5.23 During incubation on magnet, move lid B4 to C3. Move Wash Buffer II deepwell plate

- B4 to A3 to warm at 65C. Move lid C3 to A3.
- 9.1.5.24 Remove SN from beads at C2 to waste deep-well at A5. Eject tips.
 - 9.1.5.25 Step 3 – Wash Buffer 1
 - 9.1.5.26 Move lid from D3 to B2.
 - 9.1.5.27 Load tips. Transfer 150 uL of Wash Buffer 1 from D3 to samples at D4. Mix. Shake at 22C for 10 min.
 - 9.1.5.28 Move sample plate from D4 to magnet at C2. Incubate 1 min.
 - 9.1.5.29 Remove SN from beads at C2 to waste deep-well at A5. Move plate from C2 to D4. Eject tips.
 - 9.1.5.30 Repeat wash (steps 2-4).
 - 9.1.5.31 Move lid from B2 to Wash Buffer 1 at D3.
 - 9.1.5.32 Move samples from D4 to A2.
 - 9.1.5.33 Step 4 – Wash Buffer 2
 - 9.1.5.34 Load tips. Pick lid from A3. Aspirate 150 uL of Wash Buffer 2 from A3 deep-well. Place lid at A3.
 - 9.1.5.35 Dispense 150 uL Wash Buffer 2 to sample plate at A2. Mix at A2.
 - 9.1.5.36 Move sample plate from A2 to D2 CPAC at 65C. Mix.
 - 9.1.5.37 Move lid from D3 to D2. Incubate for 5 minutes.
 - 9.1.5.38 Move lid from D2 to D3.
 - 9.1.5.39 Move plate to magnet at C2. Incubate for 1 minute.
 - 9.1.5.40 Remove SN from beads at C2 to waste at A5
 - 9.1.5.41 Move sample plate from C2 to A2. Eject tips.
 - 9.1.5.42 Repeat wash 5 times (steps 1-8)
 - 9.1.5.43 Move sample plate from A2 to D4.
 - 9.1.5.44 Step 5 – PCR Set-up
 - 9.1.5.45 Load tips (single column). Transfer 50 uL of TE Buffer from col 6 of deep-well A4 to sample plate at D4. Eject tips. Repeat for all sample columns.
 - 9.1.5.46 Wait for temp 4C for Inheco at D2.
 - 9.1.5.47 Prompt user to place PCR Master Mix plate at D2 and Pre-Arrayed Indexing Primers at C4.
 - 9.1.5.48 Load tips (single column). Broadcast 20 uL PCR MM across plate at D2. Eject tips.
 - 9.1.5.49 Load tips. Transfer indexed primers from C4 to D2 (total volume, 5 uL). Mix. Eject tips.
 - 9.1.5.50 Dispose of primer plate from C4.
 - 9.1.5.51 Load tips. Mix samples/beads at D4. Shake for 2 minutes. Mix again.
 - 9.1.5.52 Transfer 25 uL of sample/beads from D4 to PCR MM plate at D2. Mix. Eject tips.
 - 9.1.5.53 Prompt user to seal plate at D2 and place on thermocycler for Post-Capture PCR.
 - 9.1.5.54 Prompt user to store the remaining sample in plate at D4 for future use.

APPENDIX 5C: RNASeq Library Preparation

1.0 PURPOSE/SCOPE

This Standard Operating Procedure (SOP) describes Automated RNASeq Library Preparation using Illumina's RNA Access Library Prep kit performed by the Molecular Characterization Laboratory (MoCha) at the Frederick National Laboratory for Cancer Research. This SOP is intended for processing up to 94 samples with two positive controls (Universal Human Reference RNA (UHR) and Human Brain Reference RNA (HBR)). **This SOP is for research purposes only and no clinical samples will be processed using this SOP. Any deviation from this SOP will be noted but will not be formally documented.**

2.0 REFERENCE DOCUMENTATION

Document Number	Title
RS-301-9001DOC	TruSeq RNA Access Library Prep Guide http://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/samplepreps_truseq/truseqrnaaccess/truseq-rna-access-library-prep-guide-15049525-b.pdf
N/A	Fragment Analyzer Automated CE System Quick Start Guide—96 Capillary DNF-473 Standard Sensitivity NGS Fragment Analysis Kit, (1 bp-6000 bp) http://www.aati-us.com/sites/default/files/DNF-473%20Quick%20Start%20Guide_96Capillary_2015NOV03.pdf
N/A	Fragment Analyzer™ Automated CE System Quick Start Guide 96 Capillary DNF-472 High Sensitivity RNA Analysis Kit, 15 nt http://www.aati-us.com/sites/default/files/DNF-472%20Quick%20Start%20Guide_96Capillary_2015NOV03.pdf
4375799 Rev. E	Applied Biosystems Veriti Thermal Cycler User Guide https://tools.thermofisher.com/content/sfs/manuals/cms_042832.pdf
15042173	TruSeq Sample Preparation Pooling Guide

Document Number	Title
470-2014-001	Technical Note: Evaluating RNA Quality from FFPE Samples http://www.illumina.com/content/dam/illumina-marketing/documents/products/technotes/evaluating-rna-quality-from-ffpe-samples-technical-note-470-2014-001.pdf
N/A	PDXRNAAccesstemplate20160815 S:\Experiments\PDX\Library preps\templates\library_prep_template_files

3.0 BACKGROUND

This procedure is to be used to process RNA samples for sequencing on the Illumina HiSeq Sequencing platform. The resulting libraries are suitable for paired-end, multiplexed sequencing applications.

4.0 DEFINITIONS

Abbreviation/Term	Definition
UHR	Universal Human Reference RNA
HBR	Human Brain Reference RNA
PCR	Polymerase Chain Reaction
RSB	Resuspension Buffer

5.0 EQUIPMENT*

Description	Model #	Vendor
Multi Channel Pipettes (LTS 20,200,1000)	Variable	Rainin
Vortex	58816-121	VWR
MiniFuge	93000-196	VWR
Plate Centrifuge	022628203	Eppendorf
Veriti 96 well thermal cycler	4375786	ABI

Description	Model #	Vendor
PCR Workstation	AC648LFUVC	AirClean Systems
Vacuum concentrator	SpeedVac	Savant
Argos Technologies Omega Single-Channel Pipet Controller	03-391-253	Fisher Scientific
Microplate shaker	VX2500	VWR
Sciclone G3 Automated Workstation	Sciclone G3	Perkin Elmer
Fragment Analyzer, 96 capillary electrophoresis system	Fragment Analyzer	Advanced Analytical

*Or comparable/equivalent

6.0 REAGENTS/MATERIALS*

Description	Product No.	Vendor
High Sensitivity RNA Analysis Kit (15nt), 500 Samples	DNF-472-0500	Advanced Analytical
Microseal 'B' adhesive seal	MSB1001	Bio-Rad
Standard Sensitivity NGS Fragment Analysis Kit (1 bp - 6,000 bp)	DNF-473-0500	Advanced Analytical
Nuclease-free Water	various	various
Agencourt AMPure XP Kit	A63881	Beckman Coulter
Ethanol, 100% for molecular biology	E7023	Sigma-Aldrich
TruSeq RNA Access Library Prep Kit – Set A or B	RS-301-2001 or RS-301-2002	Illumina
1.5-mL LoBind Tube	022431021	Eppendorf
Fisherbrand 96 DeepWell 1 mL plate	12-566-120	Fisher Scientific
Eppendorf™ 96-Well twin.tec™ PCR Plates	E951020346	Fisher Scientific
0.2 mL PCR tube with lid	Various	various
Centrifuge tubes, 250 mL	430776	Corning
Disposable Standard Serological Pipets, 25 mL capacity	13-678-14B	Fisher Scientific
Disposable Standard Serological Pipets, 10 mL capacity	13-678-12E	Fisher Scientific

Description	Product No.	Vendor
Centrifuge tubes, 50 mL	43082	Corning
DEPC-treated water	CG480	Hardy Diagnostics
Hard-Shell 96 well PCR plate	HSP9631	Bio-Rad
150 uL Barrier Sterile 96 Rack tips for Sciclone	111426	Perkin Elmer
NaCL PEG solution; 20% polyethylene glycol, 2.5M NaCL	P4136	Teknova
Seahorse single cavity polypropylene reservoir, 170ml, 12 column	200686-100	Agilent Technologies
MicroAmp® Optical 96-Well Reaction Plate	4316813	ThermoFisher Scientific
384 well U bottom plate	6008890	Perkin Elmer
Plate lids	600030	Perkin Elmer
96 well storage plates, 0.8 mL (MIDI)	AB-0859	Fisher Scientific
200-1000 µL Aerosol Barrier Pipette Tips	RT-L1000FLR	Rainin
20-200 µL Aerosol Barrier Pipette Tips	RT-L-200FLR	Rainin
0.2-20 µL Aerosol Barrier Pipette Tips	RT-L10FLR	Rainin

*Or comparable/equivalent

7.0 SAFETY

- 7.1 Lab coats, safety glasses, and gloves must be worn at all times when handling hazardous or sensitive equipment, samples, reagents, and materials. These safety measures must also be followed when in close proximity to those who are working with these items.
- 7.2 First Strand Synthesis Act D mix contains Actinomycin D, a toxin. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear appropriate personal protective equipment when handling.

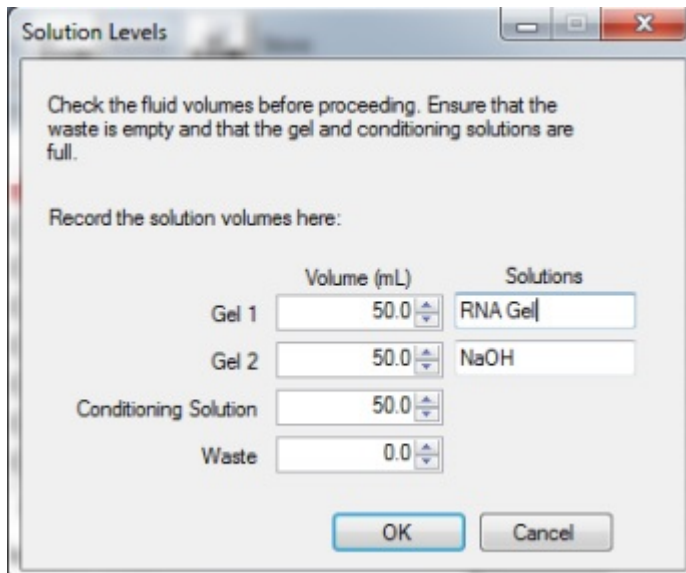
8.0 PROCEDURE

8.1 Quantify and assess quality of RNA samples using Advanced Analytical Technologies' High Sensitivity RNA Analysis Kit for the Fragment Analyzer.

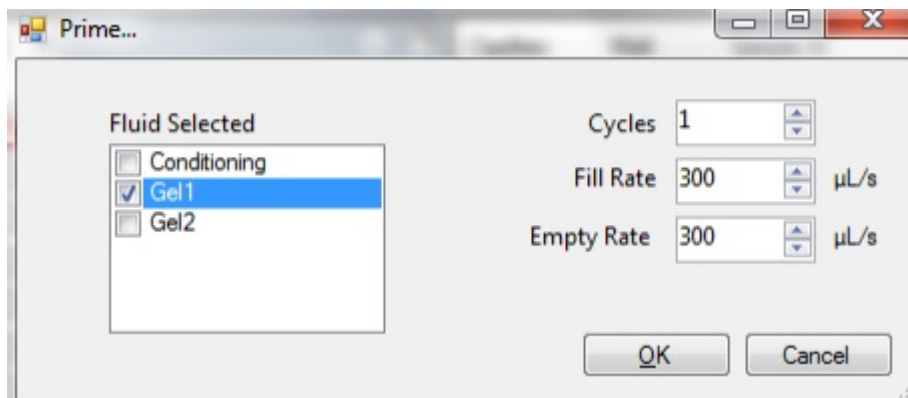
- 8.1.1 Allow Inlet Buffer, Capillary Conditioning Solution, Gel, and Intercalating Dye to come to room temperature prior to mixing.
- 8.1.2 Mix appropriate volumes of Intercalating Dye and RNA Separation Gel necessary for one day of operation. For small volumes use the supplied 50 mL conical centrifuge tube. For larger volumes, use a 250 mL conical centrifuge tube and remove the collar of the tube holder in the instrument reagent compartment.
- 8.1.3 The volume of RNA Separation Gel required per run on the 96-Capillary Fragment Analyzer™ system is summarized below.

Number of Samples to be Analyzed	Volume of Intercalating Dye	Volume of RNA Separation Gel
95	4.0 uL	40 mL
190	8.0 uL	80 mL
285	12.0 uL	120 mL
380	16.0 uL	160 mL
475	20.0 uL	200 mL

- 8.1.4 Place the RNA Separation Gel/ Intercalating Dye mixture onto the instrument and insert into the desired gel fluid line (Gel 1 or Gel 2 pump position). Ensure the fluid line is positioned at the bottom of the tube to avoid introducing air bubbles, which can cause pressurization errors.
- 8.1.5 After adding RNA Separation Gel to the instrument, update the solution levels in the Fragment Analyzer instrument control software. From the Main Menu, select Utilities – Solution Levels. A menu will be displayed to enter in the updated fluid levels.



- 8.1.6 When switching applications (e.g., between NGS and RNA kits), prime the appropriate gel fluid line after loading fresh gel/dye mixture. From the Main Menu of the Fragment Analyzer instrumental control software, select Utilities – Prime... Select the desired fluid line(s) (Conditioning, Gel 1, or Gel 2) and press OK to purge the fluid line with fresh gel.



- 8.1.7 In a clean container, add 20 mL of the 5X 930 dsDNA Inlet Buffer per 80 mL of deionized sub-micron filtered water. Agitate to mix. The entire bottle of 5x Inlet Buffer can be mixed to 1X concentration and stored at 4°C if desired.
- 8.1.8 In a clean container (e.g. 50 mL or 250 mL conical centrifuge tube), add 20 mL of the 5X Capillary Conditioning Solution per 80 mL of deionized sub-micron filtered water. Agitate to mix. The entire bottle of 5x Capillary Conditioning Solution can be mixed to 1X concentration and stored at room temperature if desired.
- 8.1.9 Once mixed, place the 1X Capillary Conditioning Solution onto the instrument and insert the CONDITIONING fluid line (Conditioning Solution pump position). Ensure the




fluid line is positioned at the bottom of the conical tube to avoid introducing air bubbles, which can cause pressurization errors. Update the solutions levels in the Utilities section of the Fragment Analyzer software.

- 8.1.10 Check the fluid level of the waste bottle and waste tray daily and empty as needed.
- 8.1.11 Prepare a fresh 96 DeepWell 1mL Plate filled with 1.0 mL/well of 1X 930 dsDNA Inlet Buffer daily. Do NOT overfill the wells of the inlet buffer plate. Place buffer plate in Drawer "B".
- 8.1.12 Prepare a fresh 96 well sample plate using an Eppendorf™ 96-Well twin.tec™ PCR Plate filled with 240 µL/well of 0.25X TE Rinse Buffer daily. Place rinse plate in Drawer "M".
- 8.1.13 Remove the High Sensitivity RNA Diluent Marker (15nt) or an a 3 uL 25 ng/uL aliquot from -20°C and keep it on ice before use. Vortex the tube briefly to mix the content. Spin the tube after mixing to ensure liquid is at the bottom of the tube.
- 8.1.14 Transfer 2 µL of the 25 ng/µL Ladder to a 0.2 mL PCR tube. Heat-denature the ladder at 70°C for 2 min, immediately cool to 4°C and keep on ice.
- 8.1.15 Dilute the ladder solution to a working concentration of 2 ng/µL by adding 23 µL of RNase-free water and mixing well. Divide the diluted ladder solution into aliquots with working volume typical for one day use or one sample plate. Store aliquots in the provided Eppendorf LoBind® 0.5 mL tubes at -70°C or below.
- 8.1.16 Heat-denature the RNA samples to be analyzed at 70°C for 2 minutes. Immediately cool to 4°C and keep on ice before use.
- 8.1.17 Using a fresh RNase-free 96-well Eppendorf twin.tec sample plate, pipette 18 µL of the High Sensitivity RNA Diluent Marker (15nt) (DM) Solution to each well that is to contain sample or RNA Ladder. Fill any unused wells of the sample plate with 20 µL/well of BF-1 Blank Solution.
- 8.1.18 Pipette 2 µL of each denatured RNA sample into the respective wells of the sample plate. Mix the contents of the well using the pipette by aspiration/expulsion in the pipette tip.
- 8.1.19 After mixing sample or RNA Ladder with Diluent Marker (15nt) Solution in each well, centrifuge the plate to remove any air bubbles. Check the wells of the sample plate to ensure there are no air bubbles trapped in the bottom of the wells. The presence of trapped air bubbles can lead to injection failures.
- 8.1.20 For best results, run the plate as soon as possible. If the sample plate will not be used immediately, cover the sample plate with RNase-free cover film, store at 4°C and use within the same day. Spin the plate again if any bubbles developed in the sample wells. *Be sure to remove the cover film before placing the plate into the instrument.*
- 8.1.21 In the Separation Setup pop-up form, left click the dropdown and select the appropriate preloaded experimental Method file. The available methods are sorted by kit number and are linked to the directory containing methods for the currently installed capillary array length (e.g., 22cm, 33cm or 55cm). Select DNF-472T22 - HS Total RNA

15nt.mthds when the 22 cm effective, 47 cm total “ultra-short” capillary array is installed (for Total RNA).

8.1.22 Select the appropriate Gel line being used for the experiment (Gel 1 or Gel 2) using the dropdown.

8.1.23 Prior to starting the experiment, verify all trays (buffer/storage, rinse, waste, sample, etc.) have been loaded into their respective drawer locations.

8.1.24 Press the Play icon () to start the sequence loaded into the queue. To Pause the queue after the currently running experiment is completed, press the  button. To Clear the run queue of all loaded runs press the  button.

8.1.25 Using ProSize 2.0 software, perform a smear analysis to determine the level of degradation.

8.1.25.1 Click Option

8.1.25.1.1 Click Set Global Configuration

8.1.25.2 Click on the Smear Analysis Tab

8.1.25.2.1 Enter the Start size as 200 bp

8.1.25.2.2 Enter the End size as 6000 bp

8.1.25.2.3 Click Apply

8.1.25.3 Click File

8.1.25.3.1 Click Export Data

8.1.25.3.1.1 Click Smear Analysis radio button

8.1.25.3.2 Click Export

8.1.25.3.2.1 Open the exported smear analysis csv file. The column labeled % total contains the DV200 RNA quality information

8.2 Pre-program the Thermal Cyclers

8.2.1 Pre-program the thermal cycler with the following program and save as **Elution 2 - Frag - Prime**:

Choose the pre-heat lid option and set to 100°C

Pre-heat to 94°C & hold

94°C for 8 minutes

4°C hold

8.2.2 Pre-program the thermal cycler with the following program and save as **Synthesize 1st Strand**:

Choose the pre-heat lid option and set to 100°C

Pre-heat to 25°C & hold

25°C for 10 minutes

42°C for 15 minutes
 70°C for 15 minutes
 4°C hold

8.2.3 Pre-program the thermal cycler with the following program and save as **Synthesize 2nd Strand:**

Choose the pre-heat lid option and set to 100°C
 Pre-heat to 16°C & hold
 16°C for 60 minutes
 16°C hold

8.2.4 Pre-program the thermal cycler with the following program and save as **First PCR Amplification:**

Choose the pre-heat lid option and set to 100°C
 Hold at 98°C
 98°C for 30 seconds
 15 cycles of:
 98°C for 10 seconds
 60°C for 30 seconds
 72°C for 30 seconds
 72°C for 5 minutes
 Hold at 4°C

8.3 RNA Sample Preparation

8.3.1 Fill in the information for columns A through H of the Initial Quant Setup Data sheet within the PDXRNAAccessstemplate20160815 file.

8.3.2 RNA sample input amount is based on sample quality. Illumina recommends using the percentage of RNA fragments > 200nt DV200 as a reliable determinant of RNA quality.

8.3.2.1 Use the table below with DV200 data from the Fragment Analyzer smear analysis to determine RNA input amount for each sample.

8.3.2.2 Copy DV200 data into the DV200 column of the Initial Quant Setup Data sheet within the PDXRNAAccessstemplate20160815 file. The RNA Access Recommended Input, ng column will be auto-filled to indicate the recommended RNA input for each sample.

RNA Quality	DV200	Input Requirement per Library Prep
High	>70	10 ng
Medium	50-70%	20 to 40 ng
Low	0-50%	40 to 100 ng

8.3.3 Normalize the RNA samples to a final volume of 8.5 uL using DEPC-treated water in each well of the 96-well plate.

8.4 Automated cDNA Library Preparation on the SciClone

Note: Fragment size generation is affected by treatment duration. A longer duration will produce smaller fragments.

8.4.1 Allow the AMPure XP beads come to room temperature for at least 30 minutes.

8.4.2 Open Maestro software on the SciClone computer.

8.4.3 Open “Illumina RNA Access Workbook” worksheet in Excel (shortcut is available on the desktop).

8.4.4 Enter number of columns to be processed in tabs ‘1 cDNA Library Prep’ and ‘2 PostPCRSPPRI’ of the “Illumina RNA Access Workbook”, then save the file.

8.4.4.1 Entering the number of columns will update the volumes for the reagent recipes

8.4.5 Open “1 RNA Access cDNA Library Preparation” method in the Illumina RNA Access folder.

8.4.6 cDNA Library Preparation

8.4.6.1 Prepare indexing setup

8.4.6.1.1 Determine pooling strategy. Refer to the TruSeq Library Preparation Pooling Guide and the table below for details.

Note: RNAseq samples will be pooled 4 samples per pool.

Table 17 Single-Indexed Pooling Strategies for 2–4 Samples with RNA and ChIP Kits

Plexity	Option	Set A Only	Set B Only
2	1	AR006 and AR012	Not recommended
	2	AR005 and AR019	
3	1	AR002 and AR007 and AR019	AR001 and AR010 and AR020
	2	AR005 and AR006 and AR015	AR003 and AR009 and AR025
	3	2-plex options with any other adapter	AR008 and AR011 and AR022
4	1	AR005 and AR006 and AR012 and AR019	AR001 and AR008 and AR010 and AR011
	2	AR002 and AR004 and AR007 and AR016	AR003 and AR009 and AR022 and AR027
	3	3-plex options with any other adapter	3-plex options with any other adapter

8.4.6.1.2 Click on the ‘Indexing’ sheet within the “Illumina RNA Access Workbook”

8.4.6.1.2.1 In the Index well column (column B) located to the right of the Sample well column, enter in the well location of the desired index for the sample using the index well corresponding to the index names located in columns H and I.

8.4.6.1.2.2 The workbook will automatically calculate the necessary volumes of indexes and autofill these values in the '1 cDNA library prep' sheet.

8.4.6.1.3 Fill in the index information for each sample in the PDXRNAAccesstemplate20160815 file.

8.4.6.2 Prepare 1st Strand, 2nd Strand, A-Tailing, and Ligation reaction mixes as well as the index aliquots according to the "Illumina RNA Access Workbook" with the reagents on ice. Use the tables found under the "1 cDNA Library Prep" tab.

The values below illustrate an example recipe for 1 column of samples (8 samples) and may not reflect those required for your specific run.

8.4.6.2.1 Mix well using a P1000 pipette. **

1st Strand Master Mix	per well	total
1st Strand Master Mix	9	87.1 uL
SuperScriptII	1	9.7 uL
Total	10	96.8 uL

2nd Strand Master Mix	per well	total
2nd Strand Marker Mix	20	190.1 uL
Resuspension Buffer (RSB)	5	47.5 uL
Total	25	237.6 uL

A-Tailing Mix	Per Reaction	Total
A-Tailing Mix (ATL)	12.5	132.0 uL
Resuspension Buffer (RSB)	2.5	26.4 uL
Total	15	158.4 uL

Ligation Mix	Per Reaction	Total
Ligase Mix	2.5	57.2 uL
Resuspension Buffer	2.5	57.2 uL
Total	5	114.4 uL

PCR MIX	Per Reaction	Total
PCR Master Mix (PMM)	25	238.0 uL
PCR Primer Mix (PPC)	5	47.6 uL
Total	30	285.6 uL

8.4.6.3 Place unused cDNA preparation reagents in -20°C storage. Maintain PCR reagents on ice until prompted.

Combine the PCR mix components during the ligation step and add to plate when prompted.

8.4.6.4 Prepare approximately 8 mL of 80% ethanol per column of library prep samples using nuclease-free water (plus a dead volume of 2 mL)

8.4.6.5 Prepare reagent plates with aliquots of the mixes in the plate type specified in the top left of the workbook table.

The values below illustrate an example for 1 column of samples (8 samples) and may not reflect those required for your specific run.

Master Mixes in PCR Plate (Bio-Rad HSP-96 PCR Plate)				
Sciclone Deck Location: A4				
	1st Strand Master Mix (w/SSII added)	2nd Strand Master Mix	2nd Strand Master Mix	EPH
	1	2	3	4
A	62	152	27	66
B	62	152	27	66
C	62	152	27	66
D	62	152	27	66
E	62	152	27	66
F	62	152	27	66
G	62	152	27	66
H	62	152	27	66

Reagent Plate (Bio-Rad HSP-96 PCR Plate)											
Sciclone Deck Location: D2											
	A-Tailing Mix	Ligation Mix (Ligase + RSE)	Stop Ligation Buffer	ID001-008	ID009-016	ID017-024	ID025-032			PCR Master Mix	PCR Master Mix
	1	2	3	4	5	6	7			11	12
A	108	61	52	26ul ID001	26ul ID009	0	0			185	35
B	108	61	52	26ul ID002	26ul ID010	0				185	35
C	108	61	52	26ul ID003	26ul ID011	26ul ID019	26ul ID027			185	35
D	108	61	52	26ul ID004	26ul ID012	0				185	35
E	108	61	52	26ul ID005	0	0				185	35
F	108	61	52	26ul ID006	0	26ul ID022				185	35
G	108	61	52	26ul ID007	0	0				185	35
H	108	61	52	26ul ID008	26ul ID016					185	35

- 8.4.6.6 Broadcast reagents to match the number of sample columns for Ampure beads, PEG buffer, and Resuspension Buffer.
- 8.4.6.7 Begin the '**Elution 2 -Frag - Prime**' program on the thermocycler.
- 8.4.7 Prepare the SciClone automation deck with boxes of tips and the reagent plates previously prepared.
 - 8.4.7.1 Press the play button and follow prompts for placement of consumables and reagent plates.
 - 8.4.7.2 Once completed, place RNA samples on the deck.
 - 8.4.7.3 Click finish to complete prep and begin automation.

Note: Only perform fragmentation of samples with DV200 values greater than 70. Remove lower quality samples from the reaction plate following the adding of EPH and store on ice during the EPF incubation. Return samples to the library preparation plate after the EPF step.

8.5 Elute Prime and Fragment RNA

- 8.5.1 The SciClone will broadcast 8.5 uL of EPH to each column of sample.
- 8.5.2 Once prompted, seal the plate with a Microseal 'B' adhesive seal.
- 8.5.3 Shake the plate on a microplate shaker at 1600 rpm for 20 seconds.
- 8.5.4 Place the sealed plate on the pre-programmed thermal cycler. Close the lid and select 'skip step' to continue the Elution 2 - Frag - Prime program and fragment the RNA.
- 8.5.5 Remove the sealed plate from the thermal cycler when it reaches 4°C and centrifuge briefly.
- 8.5.6 Carefully unseal the plate and place it on the Sciclone deck as prompted.

8.6 First Strand Synthesis

- 8.6.1 Begin the '**Synthesize 1st Strand**' program on the thermocycler.
 - 8.6.1.1 Automation will add 8 uL of First Strand Synthesis and SuperScript II combination and mix thoroughly.
 - 8.6.1.2 Once prompted, seal the plate with a Microseal 'B' adhesive seal.
 - 8.6.1.3 Shake the plate on a microplate shaker at 1600 rpm for 20 seconds.
 - 8.6.1.4 Place the sealed plate on the pre-programmed thermal cycler. Close the lid and select 'skip step' to continue Synthesize 1st Strand program.
 - 8.6.1.5 When the thermal cycler reaches 4°C, remove the sealed plate from the thermal cycler and centrifuge briefly.
 - 8.6.1.6 Carefully unseal the plate and place it on the deck as prompted and proceed immediately with Synthesize Second Strand.

8.7 Second Strand Synthesis

- 8.7.1 Begin the '**Synthesize 2nd Strand**' program on the thermocycler.
 - 8.7.1.1 Automation will add 25 uL of Second Strand Synthesis mix thoroughly.
 - 8.7.1.2 Once prompted, seal the plate with a Microseal 'B' adhesive seal.
 - 8.7.1.3 Shake the plate on a microplate shaker at 1600 rpm for 20 seconds.
 - 8.7.1.4 Place the sealed plate on the pre-programmed thermal cycler. Close the lid and select 'skip step' to continue Synthesize 2nd Strand program.
 - 8.7.1.5 After the hour long incubation, remove the sealed plate from the thermal cycler and centrifuge briefly.
 - 8.7.1.6 Carefully unseal the plate and place it on the deck as prompted and proceed immediately with SPRI Clean Up.

8.8 SPRI Clean Up

- 8.8.1 Automation will proceed with the following:
 - 8.8.1.1 Add 90 uL of Ampure to each sample.
 - 8.8.1.2 Shake and incubate at room temperature for 5 minutes.
 - 8.8.1.3 Place on magnet and incubate for 2 minutes.
 - 8.8.1.4 Remove 135 uL of supernatant.
 - 8.8.1.5 Add 150 uL of 80% EtOH to each well.
 - 8.8.1.6 Incubate for 30 seconds.
 - 8.8.1.7 Remove ethanol.
 - 8.8.1.8 Repeat steps 5 through 7.
 - 8.8.1.9 Let the plate stand at 37°C for 3 minutes.
 - 8.8.1.10 Add 15 uL of Resuspension Buffer.
 - 8.8.1.11 Shake and incubate at room temperature for 5 minutes.

Note: This double stranded cDNA will remain in bead solution through library preparation.

8.9 A-Tailing

- 8.9.1 Automation will proceed with the following:
 - 8.9.1.1 Add 15 uL of the A-Tailing and Resuspension Buffer pre-mix.
 - 8.9.1.2 Incubate sample plate at 37°C for 30 minutes, then 70°C for 5 minutes.

8.10 Ligation

- 8.10.1 Automation will proceed with the following:

- 8.10.1.1 Broadcast indices from pre-mix plate to 384 well plate on deck.
- 8.10.1.2 Broadcast ligation pre-mix to the 384 well plate on deck.
- 8.10.1.3 Add 5 uL of the ligation pre-mix and 2.5 uL of the respective adapter to each well. Pipette mix.
- 8.10.1.4 Incubate sample plate at 30°C for 10 minutes
- 8.10.1.5 Add 5 uL of Stop Ligation buffer to each well. Pipette mix.
- 8.10.1.6 Shake and incubate at room temperature for 2 minutes.

8.11 **SPRI Clean-Up**

8.11.1 Automation will proceed with the following:

- 8.11.1.1 Add 25 uL of PEG Buffer.
- 8.11.1.2 Shake and incubate at room temperature for 5 minutes.
- 8.11.1.3 Place on magnet and incubate for 2 minutes.
- 8.11.1.4 Remove 79.5 uL of supernatant.
- 8.11.1.5 Add 150 uL of 80% EtOH to each well.
- 8.11.1.6 Incubate for 30 seconds.
- 8.11.1.7 Remove ethanol.
- 8.11.1.8 Repeat steps 5 through 7.
- 8.11.1.9 Let the plate stand at 37°C for 3 minutes.
- 8.11.1.10 Add 52.5 uL of Resuspension Buffer.
- 8.11.1.11 Shake and incubate at room temperature for 5 minutes.
- 8.11.1.12 Place plate on magnet and incubate for 5 minutes.
- 8.11.1.13 Transfer 50 uL of supernatant to a clean plate.
- 8.11.1.14 Add 50 uL of Ampure XP beads for a second clean up.
- 8.11.1.15 Place on magnet and incubate for 2 minutes.
- 8.11.1.16 Remove 95 uL of supernatant from each well.
- 8.11.1.17 Repeat steps 5 through 8 for a total of 2 ethanol washes.
- 8.11.1.18 Let the plate stand at 37°C for 3 minutes.
- 8.11.1.19 Add 22.5 uL of Resuspension Buffer.
- 8.11.1.20 Shake and incubate at room temperature for 5 minutes.
- 8.11.1.21 Place plate on magnet and incubate for 5 minutes.

8.12 **First PCR Amplification**

8.12.1 Begin the **'First PCR Amplification'** program on the thermocycler to allow it to come to temperature.

8.12.2 Automation will proceed with the following:

8.12.2.1 Broadcast the PCR pre-mix

8.12.2.2 Transfer 20 μ L of purified sample from the plate on the magnet directly to the PCR plate and mix.

8.12.3 Seal the plate and centrifuge for 1 minute at 280 g.

8.12.4 Place the plate in the thermocycler and press 'skip step' to continue the First PCR Amplification program.

8.12.5 While cycling, prepare the deck by closing the '1 RNA Access cDNA Library Preparation' method and opening '2 RNA Access Post Library Amp Clean' method. Remove any partially used tip boxes and clear any used reagent plates.

8.13 **Purify the sample using Agencourt AMPure XP beads on the SciClone G3 NGS.**

8.13.1 Bring Ampure XP beads and RSB to room temperature.

8.13.2 Enter number of columns to be processed in '2 PostPCRSPI' tab of the "Illumina RNA Access Workbook", then save the file.

8.13.3 Mix the Ampure beads well so that the reagent appears homogeneous and consistent in color. Do not freeze.

8.13.4 Add 55 μ L of homogeneous AMPure XP beads per well to each column being processed of a 96 well BioRad hard shell plate, and place the plate onto the SciClone deck according to the deck layout.

8.13.5 Add 80% Ethanol to 12 well column plate as directed by the Sciclone software and place on the SciClone deck according to the deck layout.

8.13.6 Add RSB to appropriate labware and place on the SciClone deck according to the deck layout.

8.13.7 Add tip boxes and additional labware to the SciClone deck according to the deck layout.

8.13.8 Press start to begin procedure.

8.13.9 When "2 RNA Access Post Library Amp Cleanup" method is complete, cover sample plate with lid and place plate on ice while setting up for quality assessment.

Note: Stopping Point - If you do not continue to the next step, store the samples at -20°C.

8.14 **Quality Assessment using the Fragment Analyzer**

8.14.1 Dilute 1 μ L of library sample with 1 μ L Resuspension Buffer and load on an Advanced Analytical Fragment Analyzer using Standard Sensitivity NGS Fragment Analysis Kit.

8.14.2 Allow Inlet Buffer, Capillary Conditioning Solution, Gel, and Intercalating Dye to come to room temperature prior to mixing.

8.14.3 Allow standard sensitivity marker and ladder to come to room temperature

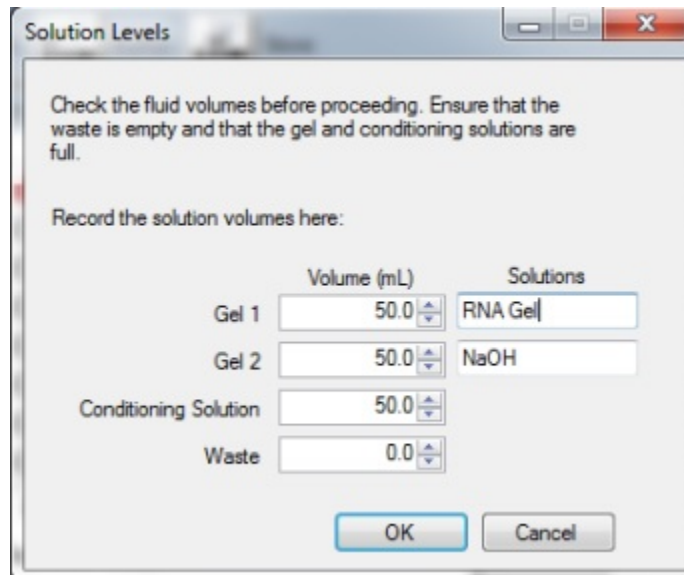
8.14.4 Mix appropriate volumes of Intercalating Dye and Separation Gel necessary for one day of operation. Use the supplied 50 mL conical centrifuge tube to allow a small minimum working volume. For larger volumes, use a 250 mL conical centrifuge tube and remove the collar of the tube holder in the instrument reagent compartment.

8.14.5 The volume of Separation Gel required per run on the 96-Capillary Fragment Analyzer™ system is summarized below.

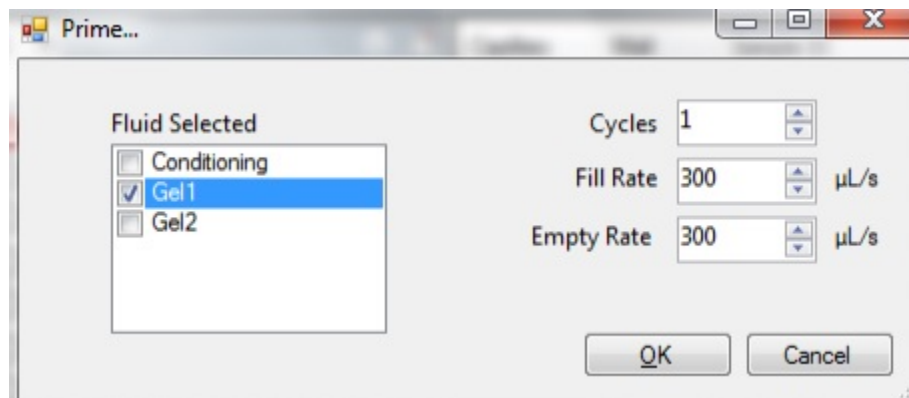
# of samples to be analyzed	Volume of Intercalating Dye	Volume of RNA Separation Gel
95	4.0 uL	40 mL
190	8.0 uL	80 mL
285	12.0 uL	120 mL
380	16.0 uL	160 mL
475	20.0 uL	200 mL

8.14.6 Place the Separation Gel/ Intercalating Dye mixture onto the instrument and insert into the desired gel fluid line (Gel 1 or Gel 2 pump position). Ensure the fluid line is positioned at the bottom of the conical tube to avoid introducing air bubbles, which can cause pressurization errors.

8.14.7 When adding Separation Gel to the instrument, update the solution levels in the Fragment Analyzer instrument control software. From the Main Menu, select Utilities – Solution Levels. A menu will be displayed to enter in the updated fluid levels.



8.14.8 When switching applications (e.g., between NGS and RNA kits), prime the appropriate gel fluid line after loading fresh gel/dye mixture. From the Main Menu of the Fragment Analyzer instrumental control software, select Utilities — Prime... Select the desired fluid line(s) (Conditioning, Gel 1, or Gel 2) and press OK to purge the fluid line with fresh gel.






8.14.9 In a clean container, add 20 mL of the 5X 930 dsDNA Inlet Buffer per 80 mL of deionized sub-micron filtered water. Agitate to mix. The entire bottle can be mixed to 1X concentration and stored at 4°C if desired.

8.14.10 In a clean container (e.g. 50 mL or 250 mL conical centrifuge tube), add 20 mL of the 5X Capillary Conditioning Solution per 80 mL of deionized sub-micron filtered water. Agitate to mix. The entire bottle can be mixed to 1X concentration and stored at room temperature if desired.

8.14.11 Once mixed, place the 1X Capillary Conditioning Solution onto the instrument and insert the CONDITIONING fluid line (Conditioning Solution pump position). Ensure

the fluid line is positioned at the bottom of the conical tube to avoid introducing air bubbles, which can cause pressurization errors. Update the solutions levels in the Utilities section of the Fragment Analyzer software.

- 8.14.12 Check the fluid level of the waste bottle and waste tray daily and empty as needed.
- 8.14.13 Prepare a fresh 96 DeepWell 1mL Plate filled with 1.0 mL/well of 1X 930 dsDNA Inlet Buffer daily. Do NOT overfill the wells of the inlet buffer plate. Place buffer plate in Drawer “B”.
- 8.14.14 Prepare a fresh twin.tec 96-well plate filled with 200 μ L/well of 0.6X TE Rinse Buffer daily. Place rinse plate in Drawer “M”
- 8.14.15 Using a fresh twin.tec 96-well sample plate, pipette 22 μ L of the Standard Sensitivity NGS Diluent Marker to each well in a row that is to contain sample or NGS Ladder. Fill any unused wells within the row of the sample plate with 24 μ L/well of BF-1 Blank Solution.
- 8.14.16 Pipette 2 μ L of each diluted sample into the respective wells of the sample plate. Mix the contents of the well using the pipette by aspiration/expulsion in the pipette tip.
- 8.14.17 After mixing sample/NGS Ladder and Diluent Marker in each well, centrifuge the plate to remove any air bubbles. Check the wells of the sample plate to ensure there are no air bubbles trapped in the bottom of the wells. The presence of trapped air bubbles can lead to injection failures.
- 8.14.18 For best results, run the plate as soon as possible. If the sample plate will not be used immediately, cover the sample plate with foil/seal, store at 4°C and use within the same day. Spin the plate again if any bubbles developed in the sample wells. *Be sure to remove the cover film before placing the plate into the instrument.*
- 8.14.19 In the Separation Setup pop-up form, left click the dropdown and select the appropriate preloaded experimental Method file. The available methods are sorted by kit number and are linked to the directory containing methods for the currently installed capillary array length (e.g., 22cm, 33cm or 55cm). Select DNF-473-(33 or 55) - SS NGS Fragment 1-6000bp.mthds.
- 8.14.20 Select the appropriate Gel line being used for the experiment (Gel 1 or Gel 2) using the dropdown menu.
- 8.14.21 Prior to starting the experiment, verify all trays (buffer/storage, rinse, waste, sample, etc.) have been loaded into their respective drawer locations.
- 8.14.22 Press the Play icon () to start the sequence loaded into the queue. To Pause the queue after the currently running experiment is completed, press the  button. To Clear the run queue of all loaded runs press the  button.
- 8.14.23 Using ProSize 2.0, perform a smear analysis to quantify the amount of amplified library.

8.14.23.1 Click Option

8.14.23.1.1 Click Set Global Configuration

8.14.23.2 Click on the Smear Analysis Tab

8.14.23.2.1 Enter the Start size as 150 bp

8.14.23.2.2 Enter the End size as 600 bp

8.14.23.2.3 Click Apply

8.14.23.3 Click File

8.14.23.3.1 Click Export Data

8.14.23.3.1.1 Click Smear Analysis radio button

8.14.23.3.2 Click Export

8.14.23.3.3 Open the exported smear analysis csv file. The column labeled ng/uL contains the library concentration data.

8.14.23.4 Check library quality. Peak sizes should be ~260 bp.

8.15 Pool and Normalize samples

8.15.1 Pool 200 ng of 4 samples with different adapters together with RSB up to a maximum volume of 45 uL. If the total volume is greater than 45 uL, concentrate the pooled sample using a vacuum concentrator with no heat and medium drying rate.

8.15.2 Enter information into the '3 Normalize and Pool Libraries' tab on the 'Illumina RNA Access Workbook'. Save file.

8.15.2.1 Copy and paste sample names into column B.

8.15.2.2 Copy and paste concentration information from the smear analysis into column F.

8.15.2.3 Ensure that the desired samples will be pooled together. Consult the pooling guidelines publication for more information.

8.15.2.4 If the total sample volume listed in column N is greater than 45 uL sample will need to be dried down using the speedvac.

8.15.3 Open the Maestro method 'RNA Access Normalize and Pool Library

8.15.3.1 Click OK when Normalization with Pooling? Pop-up window appears

8.15.3.2 Normalization Confirmation screen will appear. Confirm that the pooling setup is correct.

8.15.4 Follow the prompts within the Maestro software to set up the deck with the appropriate reagents and consumables to complete the normalization.

Note: Stopping Point - If you do not continue to the next step, store the samples at -20°C.

8.16 First Hybridization

Note: A 90 minute incubation is optimal for hybridization. Hybridizing longer than 2 hours results in a high degree of non-specific binding

8.16.1 Remove the following from -25°C to -15°C storage and thaw them at room temperature:

- Capture Target Buffer 3
- Coding Exome Oligos

8.16.2 Remove the plate of pooled samples from -25°C to -15°C storage, if it was stored at the conclusion of Normalization and Pooling and thaw on ice.

8.16.2.1 Centrifuge the thawed plate at 280 × g for 1 minute.

8.16.2.2 Remove the adhesive seal from the thawed plate.

8.16.3 Pre-program the thermal cycler with the following program and save as RNA HYB:

Choose the pre-heat lid option and set to 100°C

95°C for 10 minutes

18 cycles of 1 minute incubations, starting at 94°C, then decreasing 2°C per cycle

58°C for 90 minutes

58°C hold

8.16.4 Begin the RNA HYB program to pre-heat the thermocycler.

8.16.5 Thoroughly vortex the Capture Target Buffer 3 tube until the solution is completely resuspended. Visually make sure that no crystal structures are present.

Note: If crystals and cloudiness are observed, vortex the Capture Target Buffer 3 tube until it is clear.

8.16.6 Open Maestro software on the SciClone.

8.16.7 Open “Illumina RNA Access Workbook” worksheet in Excel (shortcut is available on the desktop).

8.16.8 Enter the new number of columns to be processed in tabs ‘4 Hyb Setup’, ‘5&6 Target Capture’, and ‘7 Final Post-PCR SPRI’ of the “Illumina RNA Access Workbook”, then save the file.

8.16.9 Open “4 RNA Access Hyb Setup” method in the Illumina RNA Access folder.

8.16.10 Prepare Enrichment Hyb Solution mix according to the “Illumina RNA Access Workbook”. Use the tables found under the “4 Hyb Setup” tab. Mix well using a P1000 pipette.

Note: Prepare Enrichment Hyb Solution immediately before use.

8.16.11 Click play in the Hyb method and set up the deck with consumables, reagents, and samples as directed.

8.16.11.1 Automation will then proceed with the following:

8.16.11.1.1 Addition of 55 uL of Enrichment Hyb Solution to 45 uL of sample

8.16.11.1.2 Pipette mixing

8.16.12 After hybridization setup is complete, immediately seal the plate with two Microseal 'B' adhesive seals and place in the pre-heated thermocycler.

8.16.13 Press the skip step button to continue the RNA HYB program.

8.17 First Capture and Second Hybridization

8.17.1 Remove the 2N NaOH, Enrichment Elution Buffer 1, and Enrichment Wash Solution from -25°C to -15°C storage and thaw at room temperature.

8.17.2 Remove the Elute Target Buffer 2 and Streptavidin Magnetic Beads from 2°C to 8°C storage and let stand at room temperature.

8.17.3 Open the Illumina RNA Access Workbook and click on the 5&6 Target Capture sheet.

8.17.3.1 Update the number of columns to be processed and save the file.

8.17.3.2 Prepare reagent mixes and reagent plates according to information in the tables.

8.17.4 Open Maestro software on the SciClone.

8.17.5 Open the "5&6 Target Capture" method in the Illumina RNA Access folder.

8.17.5.1 Press play and prepare the SciClone automation deck with consumables, plates, and samples as directed.

8.17.5.2 With approximately 5 minutes remaining in the hybridization program before the 58°C hold, click finish on the SciClone prompt to complete preparation and begin automated washing of beads.

8.17.5.3 When prompted, immediately transfer hybridization sample plate to the magnet on deck and carefully remove the seals.

8.17.5.4 Automation will proceed with the following:

8.17.5.4.1 Transfer the entire contents (~100 µl) from each well of the RAH1 plate to the corresponding well of the new 96-well MIDI plate labeled RAW1.

8.17.5.4.2 Vortex the Streptavidin Magnetic Beads tube until the beads are well dispersed, then add 250 µl well-mixed Streptavidin Magnetic Beads to the wells of the RAW1 plate.

8.17.5.4.3 Shake the RAW1 plate on a microplate shaker at 1200 rpm for 5 minutes.

8.17.5.4.4 Let the RAW1 plate stand at room temperature for 25 minutes.

8.17.5.4.5 Place the RAW1 plate on the magnetic stand for 2 minutes at room temperature or until the liquid is clear.

- 8.17.5.4.6 Carefully remove and discard all of the supernatant from each well of the RAW1 plate without disturbing the beads.
- 8.17.5.4.7 Remove the RAW1 plate from the magnetic stand.
- 8.17.5.4.8 Add 200 μ l Enrichment Wash Solution to each well of the RAW1 plate.
- 8.17.5.4.9 Shake the RAW1 plate on a microplate shaker at 1800 rpm for 4 minutes.
- 8.17.5.4.10 Pipette the entire volume of each well up and down to ensure complete resuspension of the sample.
- 8.17.5.4.11 Incubate at 50°C for 20 minutes.
- 8.17.5.4.12 Place plate on magnetic stand for 2 minutes or until the liquid is clear.
- 8.17.5.4.13 Immediately remove and discard all of the supernatant from each well of the RAW1 plate.
- 8.17.5.4.14 Remove the RAW1 plate from the magnetic stand.
- 8.17.5.4.15 Repeat steps 8-14 one time for a total of two Enrichment Wash Solution washes.

8.17.6 While target capture is being performed on the Sciclone, prepare the thermocycler for the second hybridization.

Choose the pre-heat lid option and set to 100°C

95°C for 10 minutes

18 cycles of 1 minute incubations, starting at 94°C, then decreasing 2°C per cycle

58°C for 90 minutes

58°C hold

8.17.7 Automation will proceed with the following:

8.17.7.1 Add 23 μ l of the mix to each well of the RAW1 plate.

8.17.7.2 Shake the RAW1 plate on a microplate shaker at 1800 rpm for 2 minutes.

8.17.7.3 Let the RAW1 plate stand at room temperature for 2 minutes.

8.17.7.4 Place the RAW1 plate on the magnetic stand for 2 minutes or until the liquid is clear.

8.17.7.5 Transfer 21 μ l of clear supernatant from each well of the RAW1 plate to the corresponding well of the new unskirted PCR plate.

8.17.7.6 Add 4 μ l Elute Target Buffer 2 to each well of the RAH2 plate containing samples to neutralize the elution.

8.17.7.7 Add 75 μ l of secondary Hyb Solution to each sample.

8.17.8 Immediately seal the plate with two Microseal 'B' adhesive seals and place in the pre-heated thermocycler.

8.17.9 Press the skip step button to continue the RNA HYB program.

8.18 Second Capture and PCR Setup

8.18.1 Prepare the SciClone automation deck with fresh tip boxes and the reagent plates.

8.18.1.1 Press the play button to begin prompts on specific placement.

8.18.1.2 With approximately 5 minutes remaining before the 58°C hold, click finish to complete preparation and begin automated washing of binding beads

8.18.1.3 When prompted, immediately transfer plate to the magnet on deck and carefully remove seals.

8.18.2 Automation will proceed with the following:

8.18.2.1 Transfer the entire contents (~100 µl) from each well of the RAH1 plate to the corresponding well of the new 96-well MIDI plate labeled RAW1.

8.18.2.2 Vortex the Streptavidin Magnetic Beads tube until the beads are well dispersed, then add 250 µl well-mixed Streptavidin Magnetic Beads to the wells of the RAW1 plate.

8.18.2.3 Shake the RAW1 plate on a microplate shaker at 1200 rpm for 5 minutes.

8.18.2.4 Let the RAW1 plate stand at room temperature for 25 minutes.

8.18.2.5 Place the RAW1 plate on the magnetic stand for 2 minutes at room temperature or until the liquid is clear.

8.18.2.6 Carefully remove and discard all of the supernatant from each well of the RAW1 plate without disturbing the beads.

8.18.2.7 Remove the RAW1 plate from the magnetic stand.

8.18.2.8 Add 200 µl Enrichment Wash Solution to each well of the RAW1 plate.

8.18.2.9 Shake the RAW1 plate on a microplate shaker at 1800 rpm for 4 minutes.

8.18.2.10 Pipette the entire volume of each well up and down to ensure complete resuspension of the sample.

8.18.2.11 Incubate at 50°C for 20 minutes.

8.18.2.12 Place plate on magnetic stand for 2 minutes or until the liquid is clear.

8.18.2.13 Immediately remove and discard all of the supernatant from each well of the RAW1 plate.

8.18.2.14 Remove the RAW1 plate from the magnetic stand.

8.18.2.15 Repeat steps 8-14 one time for a total of two Enrichment Wash Solution washes.

8.18.3 When prompted, place plate containing Elution Prep Mix, Elute Target Buffer 2, Enhanced PCR mix, and PCR Primer Cocktail on deck as instructed.

8.18.4 Prepare the thermocycler for the PCR with **EPM AMP**.

Choose the pre-heat lid option and set to 100°C

Preheat to 98°C

98°C for 30 seconds

10 cycles of:

 98°C for 10 seconds

 60°C for 30 seconds

 72°C for 30 seconds

72°C for 5 minutes

Hold at 10°C

8.18.5 Automation will proceed with the following:

8.18.5.1 Add 23 µl of the mix to each well of the RAW1 plate.

8.18.5.2 Shake the RAW1 plate on a microplate shaker at 1800 rpm for 2 minutes.

8.18.5.3 Let the RAW1 plate stand at room temperature for 2 minutes.

8.18.5.4 Place the RAW1 plate on the magnetic stand for 2 minutes or until the liquid is clear.

8.18.5.5 Transfer 21 µl of clear supernatant from each well of the RAW1 plate to the corresponding well of the new unskirted PCR plate.

8.18.5.6 Add 4 µl Elute Target Buffer 2 to each well of the RAH2 plate containing samples to neutralize the elution.

8.18.5.7 Add 45 µl well-mixed AMPure XP beads to each well of the RAC1 plate.

8.18.5.8 Shake the plate on a microplate shaker at 1800 rpm for 1 minute.

8.18.5.9 Incubate the RAC1 plate at room temperature for 5 minutes.

8.18.5.10 Place the RAC1 plate on the magnetic stand for 2 minutes or until the liquid is clear.

8.18.5.11 Remove and discard all of the supernatant from each well of the RAC1 plate.

8.18.5.12 With the RAC1 plate on the magnetic stand, slowly add 150 µl freshly made 80% EtOH to each well without disturbing the beads.

8.18.5.13 Let the RAC1 plate stand at room temperature for 30 seconds.

8.18.5.14 Remove and discard the 80% EtOH from each well of the RAC1 plate.

8.18.5.15 Repeat steps 12-14 one time for a total of two 80% EtOH washes.

8.18.5.16 Let the RAC1 plate stand at room temperature for 5 minutes to dry at 37°C.

8.18.5.17 Add 27.5 µl Resuspension Buffer to each well of the RAC1 plate.

8.18.5.18 Shake the RAC1 plate on a microplate shaker at 1800 rpm for 1 minute.

8.18.5.19 Incubate the RAC1 plate at room temperature for 2 minutes.

8.18.5.20 Place the RAC1 plate on the magnetic stand for 2 minutes or until the liquid is clear.

8.18.5.21 Transfer 25 µl of clear supernatant from each well of the RAC1 plate to the corresponding well of the new unskirted PCR plate with 20 uL of EPM and 5 uL of PPC

8.18.6 Seal and place the plate in the preheated thermocycler.

8.18.7 Press 'skip step' to continue with amplification.

8.19 **Purify the sample using Agencourt AMPure XP beads on the SciClone G3 NGS.**

8.19.1 Bring Ampure XP beads to room temperature and RSB.

8.19.2 In the Illumina RNA Access Workbook enter number of columns to be processed in '7 Final Post-PCR SPRI' tab of the "Illumina RNA Access Workbook", then save the file.

8.19.3 In the Maestro Software open "7 Final Post-PCR SPRI" method.

8.19.4 Press Start to begin procedure.

8.19.5 Mix the Ampure beads well so that the reagent appears homogeneous and consistent in color. Do not freeze.

8.19.6 Add 90 µL of homogeneous AMPure XP beads to each well of a 96 well BioRad hard shell plate for the wells being processed, and place the plate onto the SciClone deck according to the deck layout.

8.19.7 Add 80% Ethanol to appropriate labware and place on the SciClone deck according to the deck layout.

8.19.8 Add RSB to appropriate labware and place on the SciClone deck according to the deck layout.

8.19.9 Add tip boxes and additional labware to the SciClone deck according to the deck layout.

8.19.10 When "7 Final Post-PCR SPRI" program is complete, cover sample plate with lid and place plate on ice while setting up for quality assessment.

Note: Stopping Point - If you do not continue to the next step, store the samples at -20°C.

8.20 **Assess Library Quality**

8.20.1 Dilute 3 µl of final library pools with 21 µl Resuspension Buffer and load on an Advanced Analytical Fragment Analyzer using High Sensitivity NGS Fragment Analysis Kit.

8.20.2 Allow Inlet Buffer, Capillary Conditioning Solution, Gel, and Intercalating Dye to come to room temperature prior to mixing.

8.20.3 Allow high sensitivity marker and ladder to come to room temperature

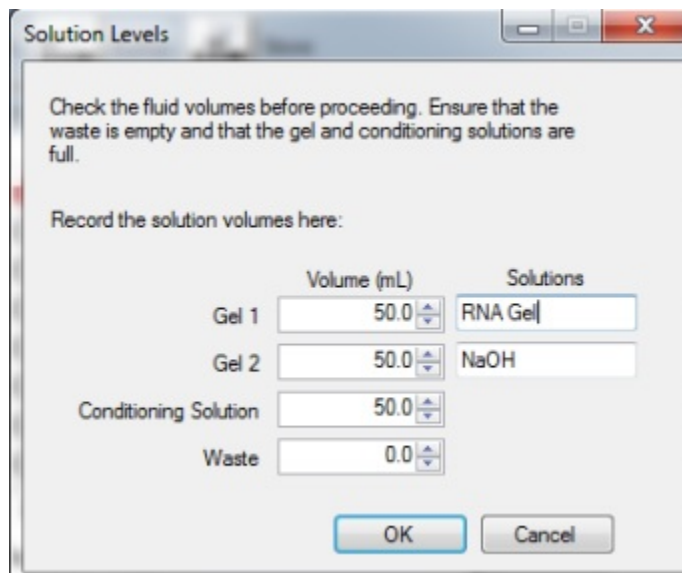
8.20.4 Mix appropriate volumes of Intercalating Dye and Separation Gel necessary for one day of operation. Use the supplied 50 mL conical centrifuge tube to allow a small minimum working volume. For larger volumes, use a 250 mL conical centrifuge tube and remove the collar of the tube holder in the instrument reagent compartment.

8.20.5 The volume of Separation Gel required per run on the 96-Capillary Fragment Analyzer™ system is summarized below.

# of samples to be analyzed	Volume of Intercalating Dye	Volume of RNA Separation Gel
95	4.0 uL	40 mL
190	8.0 uL	80 mL
285	12.0 uL	120 mL
380	16.0 uL	160 mL
475	20.0 uL	200 mL

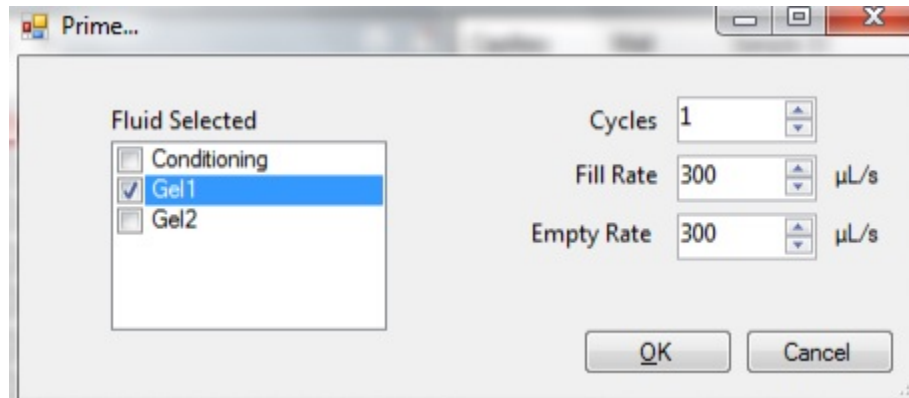
8.20.6 Place the Separation Gel/ Intercalating Dye mixture onto the instrument and insert into the desired gel fluid line (Gel 1 or Gel 2 pump position). Ensure the fluid line is positioned at the bottom of the conical tube to avoid introducing air bubbles, which can cause pressurization errors.

8.20.7 When adding Separation Gel to the instrument, update the solution levels in the Fragment Analyzer instrument control software. From the Main Menu, select Utilities – Solution Levels. A menu will be displayed to enter in the updated fluid levels.



8.20.8 When switching applications (e.g., between NGS and RNA kits), prime the appropriate gel fluid line after loading fresh gel/dye mixture. From the Main Menu of the Fragment Analyzer instrumental control software, select Utilities – Prime... Select the desired

fluid line(s) (Conditioning, Gel 1, or Gel 2) and press OK to purge the fluid line with fresh gel.



8.20.9 In a clean container, add 20 mL of the 5X 930 dsDNA Inlet Buffer per 80 mL of deionized sub-micron filtered water. Agitate to mix. The entire bottle can be mixed to 1X concentration and stored at 4°C if desired.

8.20.10 In a clean container (e.g. 50 mL or 250 mL conical centrifuge tube), add 20 mL of the 5X Capillary Conditioning Solution per 80 mL of deionized sub-micron filtered water. Agitate to mix. The entire bottle can be mixed to 1X concentration and stored at room temperature if desired.

8.20.11 Once mixed, place the 1X Capillary Conditioning Solution onto the instrument and insert the CONDITIONING fluid line (Conditioning Solution pump position). Ensure the fluid line is positioned at the bottom of the conical tube to avoid introducing air bubbles, which can cause pressurization errors. Update the solutions levels in the Utilities section of the Fragment Analyzer software.




8.20.12 Check the fluid level of the waste bottle and waste tray daily and empty as needed.

8.20.13 Prepare a fresh 96 DeepWell 1mL Plate filled with 1.0 mL/well of 1X 930 dsDNA Inlet Buffer daily. Do NOT overfill the wells of the inlet buffer plate. Place buffer plate in Drawer "B".

8.20.14 Prepare a fresh sample plate filled with 200 µL/well of 0.6X TE Rinse Buffer daily. Place rinse plate in Drawer "M"

8.20.15 Using a fresh half-skirted 96-well sample plate, pipette 22 µL of the High Sensitivity NGS Diluent Marker to each well in a row that is to contain sample or NGS Ladder. Fill any unused wells within the row of the sample plate with 24 µL/well of BF-1 Blank Solution.

8.20.16 Pipette 2 µL of each diluted sample into the respective wells of the sample plate; mix the contents of the well using the pipette by aspiration/expulsion in the pipette tip.

- 8.20.17 After mixing sample/NGS Ladder and Diluent Marker in each well, centrifuge the plate to remove any air bubbles. Check the wells of the sample plate to ensure there are no air bubbles trapped in the bottom of the wells. The presence of trapped air bubbles can lead to injection failures.
- 8.20.18 For best results, run the plate as soon as possible. If the sample plate will not be used immediately, cover the sample plate with foil/seal, store at 4°C and use within the same day. Spin the plate again if any bubbles developed in the sample wells. *Be sure to remove the cover film before placing the plate into the instrument.*
- 8.20.19 In the Separation Setup pop-up form, left click the dropdown and select the appropriate preloaded experimental Method file. The available methods are sorted by kit number and are linked to the directory containing methods for the currently installed capillary array length (e.g., 22cm, 33cm or 55cm). Select DNF-474-(33 or 55) - HS NGS Fragment 1-6000bp.mthds.
- 8.20.20 Select the appropriate Gel line being used for the experiment (Gel 1 or Gel 2) using the dropdown menu.
- 8.20.21 Prior to starting the experiment, verify all trays (buffer/storage, rinse, waste, sample, etc.) have been loaded into their respective drawer locations.
- 8.20.22 Press the Play icon () to start the sequence loaded into the queue. To Pause the queue after the currently running experiment is completed, press the  button. To Clear the run queue of all loaded runs press the  button.
- 8.20.23 Using ProSize 2.0, perform a smear analysis to quantify the amount of the final library pools.
- 8.20.23.1 Click Option
- 8.20.23.1.1 Click Set Global Configuration
- 8.20.23.2 Click on the Smear Analysis Tab
- 8.20.23.2.1 Enter the Start size as 150 bp
- 8.20.23.2.2 Enter the End size as 1000 bp
- 8.20.23.2.3 Click Apply
- 8.20.23.3 Click File
- 8.20.23.3.1 Click Export Data
- 8.20.23.3.1.1 Click Smear Analysis radio button
- 8.20.23.3.2 Click Export
- 8.20.23.3.3 Open the exported smear analysis csv file. The column labeled nM/L contains the library concentration data.
- 8.20.23.4 Check library quality. Libraries should show a distribution of approximately 200 to 1000 bp

APPENDIX 5D: WGS Library Preparation using PCR Amplification

1.0 PURPOSE/SCOPE

This Standing Operating Procedure (SOP) describes the KAPA HyperPlus Kit library construction for shallow whole genome sequencing (WGS) used/performed by the Molecular Characterization and Clinical Assay Development Laboratory (MoCha) at the Frederick National Laboratory for Cancer Research. **This SOP is for research purposes only and no clinical samples will be processed using this SOP. Any deviation from this SOP will be noted but will not be formally documented.**

2.0 REFERENCE DOCUMENTATION

Document Number	Title
N/A	KAPA HyperPlus Kit Protocol file:///C:/Users/peachal/Downloads/KAPA_HyperPlus_TDS_KR1145_v3.16_A4%20(8).pdf
N/A	Agilent High Sensitivity D1000 ScreenTape Assay Quick Guide for 4200 TapeStation System http://www.agilent.com/cs/library/usermanuals/Public/4200-TapeStation_HS-D1000_QG.pdf
N/A	C1000 Touch Thermal Cycler and CFX Real-Time PCR Detection Systems http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10000068706.pdf
N/A	Whole genome_libraryprep_template_ S:\Experiments\PDX\Library preps\templates\library_prep_template_files

3.0 BACKGROUND

This procedure is to be used to prepare whole genome libraries from DNA samples for use on the Illumina HiSeq Sequencing platform. The resulting DNA libraries are suitable for paired-end, multiplexed sequencing applications.

4.0 EQUIPMENT*

Description	Model #	Vendor
Multi-Channel Pipettes (LTS 20,200,1000)	Variable	Rainin
Vortex	58816-121	VWR
MiniFuge	93000-196	VWR
Plate Centrifuge	022628203	Eppendorf
C1000 96 well thermal cycler	4375786	BioRad
PCR Workstation	AC648LFUVC	AirClean Systems
4200 TapeStation	G2991AA	Agilent Technologies

*Or comparable/equivalent (include unless must use listed)

5.0 REAGENTS/MATERIALS*

Description	Product No.	Vendor
KAPA HyperPlus Kit	KK8514	Roche
High Sensitivity D1000 Screen Tape	5067-5584	Agilent Technologies
High Sensitivity D1000 Reagents	5067-5585	Agilent Technologies
Nuclease-free Water	various	various
Agencourt AMPure XP Kit	A63881	Beckman Coulter
Ethanol, 100% for molecular biology	E7023	Sigma-Aldrich
Elution Buffer (EB)	19086	Qiagen
SureSelectXT2 Adaptors/Indices 96 well plate	931019	Agilent Technologies
Eppendorf™ 96-Well twin.tec™ PCR Plates	E951020346	Fisher Scientific
Hard-Shell 96 well PCR plate	HSP9631	Bio-Rad
200-1000 µL Aerosol Barrier Pipette Tips	RT-L1000FLR	Rainin

Description	Product No.	Vendor
20-200 µL Aerosol Barrier Pipette Tips	RT-L-200FLR	Rainin
0.2-20 µL Aerosol Barrier Pipette Tips	RT-L10FLR	Rainin
Microamp Adhesive plate seals	4306311	Applied Biosystems

*Or comparable/equivalent (include unless must use listed items)

6.0 SAFETY

- 6.1 Lab coats, safety glasses, and gloves must be worn at all times when handling hazardous or sensitive equipment, samples, reagents, and materials. These safety measures must also be followed when in close proximity to those who are working with these items.

7.0 PROCEDURE

7.1 Dilute DNA to appropriate concentration

7.1.1 Dilute DNA to 50ng with EB buffer.

7.2 Enzymatic Fragmentation of DNA

Notes:

Fragment size generation is affected by treatment duration. A longer duration will produce smaller fragments.

7.2.1 Prepare the fragmentation reaction mix on ice

Component	Volume
KAPA Frag Buffer (10x)	5 ul
KAPA Frag Enzyme	10 ul
Total Voume	15 ul

7.2.2 Add 15 ul of the fragmentation reaction mix to each well of a BioRad Hard Shell plate containing 35 ul of 50ng of dsDNA to get a total volume of 50 ul.

7.2.3 Mix thoroughly and centrifuge briefly.

7.2.4 Incubate fragmentation mix and dsDNA mix in a C1000 thermal cycler using the following conditions:

Step	Temp	Time
Pre-cool block	4°C	∞
Fragmentation	37°C	10 min
HOLD	4°C	∞

Notes: Set the heated lid temperature to 50°C and volume 50 ul.

7.3 End Repair and A-Tailing

7.3.1 Prepare the End Repair and A-tailing reaction mix on ice.

Component	Volume
End Repair and A-Tailing Buffer	7 ul
End Repair and A-Tailing Enzyme Mix	3 ul
Total Volume	10 ul

7.3.2 Add 10 ul of the End Repair and A-Tailing reaction mix to each well of the plate containing the fragmented samples.

7.3.3 Mix thoroughly and centrifuge briefly.

7.3.4 Incubate the reaction in a thermal cycler using the following conditions:

Step	Temp	Time
End Repair and A-Tailing	65°C	30 min
HOLD	4°C	∞

Notes: Set the heated lid to 85°C and volume 60 ul.

7.4 Adapter Ligation

7.4.1 Prepare the Ligation reaction mix on ice.

Component	Volume
Nuclease-Free Water	5 ul
Ligation Buffer	30 ul
DNA Ligase	10 ul
Total Volume	45 ul

7.4.2 Add 5 ul of SureSelect XT2 adapters to each well of the end repair/a-tailing reaction plate.

7.4.3 Add 45 ul of ligation mix to each well of the reaction plate.

7.4.4 Mix thoroughly and centrifuge briefly.

7.4.5 Incubate at 20°C for 15 minutes.

7.5 Post Ligation Clean Up

7.5.1 Add 88 ul of Ampure XP beads to each well of the adaptor ligation reaction product.

7.5.2 Mix thoroughly by pipetting up and down several times.

7.5.3 Incubate the plate at room temperature for 15 minutes to bind the DNA to the beads.

- 7.5.4 Place the plate on a magnet to capture the beads, incubate until the liquid is clear.
- 7.5.5 Carefully remove and discard the supernatant.
- 7.5.6 Keep the plate on the magnet and add 200 ul of 80% ethanol.
- 7.5.7 Incubate the plate on the magnet at room temperature for 30 seconds.
- 7.5.8 Carefully remove and discard the ethanol.
- 7.5.9 Keep the plate on the magnet and add 200 ul of 80% ethanol.
- 7.5.10 Incubate the plate on the magnet at room temperature for 30 seconds.
- 7.5.11 Carefully remove and discard the ethanol.
- 7.5.12 Seal the plate with an adhesive seal and centrifuge briefly.
- 7.5.13 Remove any remaining ethanol.
- 7.5.14 Dry the beads at room temperature for 5 minutes or until all of the ethanol has evaporated.
- 7.5.15 Remove the plate from the magnet.
- 7.5.16 Resuspend the beads in 25 ul of elution buffer, pipette up and down to mix.
- 7.5.17 Incubate the plate at room temperature for 2 minutes to elute the DNA off the beads.
- 7.5.18 Capture the beads by placing the plate on the magnet and incubate until the liquid is clear.
- 7.5.19 Transfer 20 ul of the clear supernatant to a new BioRad Hard Shell plate.

7.6 Library Amplification

- 7.6.1 Prepare the library amplification mix on ice.

Component	Volume
KAPA HiFi HotStart Ready Mix (2x)	25 ul
Library Amplification Mix (10x)	5 ul
Total Volume	30 ul

- 7.6.2 Add 30 ul of library amplification reaction mix to 20 ul of the adapter-ligated library.
- 7.6.3 Mix thoroughly and centrifuge briefly.
- 7.6.4 Amplify using the following conditions:

Step	Temp	Duration	Cycles
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Initial Denaturation	98°C	45 sec	1
Denaturation	98°C	15 sec	4
Annealing	60°C	30 sec	
Extension	72°C	30 sec	
Final Extension	72°C	1 min	1
HOLD	4°C	∞	1

7.7 Post-Amplification Clean Up

- 7.7.1 Add 50 ul of Ampure XP beads to each well of the amplified product.
- 7.7.2 Mix thoroughly by pipetting up and down several times.
- 7.7.3 Incubate the plate at room temperature for 15 minutes to bind the DNA to the beads.
- 7.7.4 Place the plate on a magnet to capture the beads, incubate until the liquid is clear.
- 7.7.5 Carefully remove and discard the supernatant.
- 7.7.6 Keep the plate on the magnet and add 200 ul of 80% ethanol.
- 7.7.7 Incubate the plate on the magnet at room temperature for 30 seconds.
- 7.7.8 Carefully remove and discard the ethanol.
- 7.7.9 Keep the plate on the magnet and add 200 ul of 80% ethanol.
- 7.7.10 Incubate the plate on the magnet at room temperature for 30 seconds.
- 7.7.11 Carefully remove and discard the ethanol.
- 7.7.12 Seal the plate with an adhesive seal and centrifuge briefly.
- 7.7.13 Remove any remaining ethanol.
- 7.7.14 Dry the beads at room temperature for 5 minutes or until all of the ethanol has evaporated.
- 7.7.15 Remove the plate from the magnet.
- 7.7.16 Resuspend the beads in 25 ul of elution buffer, pipette up and down to mix.
- 7.7.17 Incubate the plate at room temperature for 2 minutes to elute the DNA off the beads.
- 7.7.18 Capture the beads by placing the plate on the magnet and incubate until the liquid is clear.

7.7.19 Transfer 20 ul of the clear supernatant to a new BioRad Hard Shell plate

7.8 Pool Samples (pool samples according to the requirements needed for mean depth of sequencing requested)

7.8.1 Pool xx samples into 1 pool: yy ul of each sample in 1 Eppendorf tube. (for example: for 0.2X mean of depth of sequencing required, pool 48 samples (xx=48) with 5 ul of each sample (yy=5) in a pool; for 5x mean of depth of sequencing required, pool 3 samples (xx=3) in a pool; and for 20x mean of depth of sequencing required, only 1 sample will represent an entire pool and will be sequenced in a single lane of HiSeq 2500).

7.9 Size Selection Double Cut

7.9.1 Perform the first (0.7x) size cut (to exclude library molecules larger than ~450 bp) by taking 50 ul of the pooled samples and adding 35 ul of the Ampure XP beads.

7.9.2 Mix thoroughly by pipetting up and down several times.

7.9.3 Incubate the plate at room temperature for 15 minutes to bind the library molecules larger than ~450 bp to the beads.

7.9.4 Place the plate on a magnet to capture the beads, incubate until the liquid is clear.

7.9.5 Carefully transfer 80 ul of the clear supernatant containing the library molecules smaller than ~450 bp to a new plate.

7.9.6 Perform the second size cut (0.9x) to retain library molecules >250 bp by adding 10 ul of Ampure XP beads to the 80 ul of supernatant from the first cut.

7.9.7 Mix thoroughly by pipetting up and down several times.

7.9.8 Incubate the plate at room temperature for 15 minutes to bind the library molecules larger than ~250 bp to the beads.

7.9.9 Place the plate on the magnet to capture the beads, incubate until the liquid is clear.

7.9.10 Carefully remove and discard the supernatant containing library molecules smaller than ~250 bp.

7.9.11 Keep the plate on the magnet and add 200 ul of 80% ethanol.

7.9.12 Incubate the plate on the magnet at room temperature for 30 seconds.

7.9.13 Carefully removed and discard the ethanol.

7.9.14 Keep the plate on the magnet and add 200 ul of 80% ethanol.

7.9.15 Incubate the plate on the magnet at room temperature for 30 seconds.

7.9.16 Carefully removed and discard the ethanol.

7.9.17 Seal the plate with an adhesive seal and centrifuge briefly.

7.9.18 Remove any remaining ethanol.

7.9.19 Dry the beads for 5 minutes at room temperature or until all of the ethanol has evaporated.

7.9.20 Remove the plate from the magnet.

- 7.9.21 Resuspend the beads in 25 μ l of elution buffer, pipette up and down to mix well.
- 7.9.22 Incubate the plate at room temperature for 2 minutes to elute DNA off the beads.
- 7.9.23 Capture the beads by placing the plate on the magnet and incubate until the liquid is clear.
- 7.9.24 Transfer 20 μ l of the clear supernatant to a new plate/tube.

7.10 Final Library Quality Assessment using the Tape Station

- 7.10.1 Allow High Sensitivity D1000 ScreenTapes, High Sensitivity D1000 sample buffer, and High Sensitivity D1000 ladder to equilibrate to room temperature for 30 minutes prior to use.
- 7.10.2 Launch TapeStation software. Select well positions to be run on the virtual plate set-up. This will calculate the necessary number of tapes and amount of ladder necessary.
- 7.10.3 Flick the required number of D1000 ScreenTapes to eliminate bubbles in the separation channel. Bubbles in the separation chambers can interfere with sample loading.
- 7.10.4 Load a High Sensitivity D1000 ScreenTape in the processing bucket and provide tips. Load the required number of additional High Sensitivity D1000 ScreenTapes in the holding rack.
- 7.10.5 Vortex and spin down the ScreenTape reagents prior to use.
- 7.10.6 If running 15 samples or fewer, prepare 4 μ l of ladder by mixing 2 μ l D1000 Sample Buffer with 2 μ l D1000 ladder and place in a strip tube. If running more than 15 samples, dilute 15 μ l of D1000 ladder (yellow cap) with 15 μ l of D1000 sample buffer (green cap) and place in a strip tube.
 - Cap strip and vortex mix using the IKA vortexer and adapter at 2000 rpm for 1 minute.
 - Spin the plate down.
- 7.10.7 Dilute 2 μ l of each final library pool sample with 2 μ l of D1000 sample buffer and load on an Eppendorf skirted PCR plate.
 - Seal the plate with pierceable foil.
 - Mix sample plate on the IKA vortexer with adapter at 2000 rpm for 1 minute.
 - Spin the plate down.
- 7.10.8 Load the sample plate into the TapeStation, close the lid, and click *Start*.

7.11 Quantify the pooled libraries using ddPCR

- 7.11.1 Refer to ddPCR Library Quantitation SOP (**APPENDIX 5F**).

7.12 Dilute, Denature, and Cluster Final Pools for Sequencing

- 7.12.1 Refer to Illumina Sequencing SOP(**APPENDIX 5H**).

- Pools should be clustered at a concentration of 13.26 pM with a 1% phiX spike-in for each sample.

APPENDIX 5E: WGS Library Preparation (PCR-Free)

1.0 PURPOSE/SCOPE

This Standing Operating Procedure (SOP) describes the KAPA HyperPlus Kit library construction for shallow whole genome sequencing used/performed by the Molecular Characterization and Clinical Assay Development Laboratory (MoCha) at the Frederick National Laboratory for Cancer Research. **This SOP is for research purposes only and no clinical samples will be processed using this SOP. Any deviation from this SOP will be noted but will not be formally documented.**

2.0 REFERENCE DOCUMENTATION

Document Number	Title
N/A	KAPA HyperPlus Kit Protocol file:///C:/Users/peachal/Downloads/KAPA_HyperPlus_TDS_KR1145_v3.16_A4%20(8).pdf
N/A	Agilent High Sensitivity D1000 ScreenTape Assay Quick Guide for 4200 TapeStation System http://www.agilent.com/cs/library/usermanuals/Public/4200-TapeStation_HS-D1000_QG.pdf
N/A	C1000 Touch Thermal Cycler and CFX Real-Time PCR Detection Systems http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10000068706.pdf
N/A	Whole genome_libraryprep_template_ S:\Experiments\PDX\Library preps\templates\library_prep_template_files

3.0 BACKGROUND

This procedure is to be used to prepare whole genome libraries from DNA samples for use on the Illumina HiSeq Sequencing platform. The resulting DNA libraries are suitable for paired-end, multiplexed sequencing applications. These resulting DNA libraries are prepared without amplification and shown to have more uniform coverage but requires more input DNA.

4.0 EQUIPMENT*

Description	Model #	Vendor
Multi-Channel Pipettes (LTS 20,200,1000)	Variable	Rainin
Vortex	58816-121	VWR
MiniFuge	93000-196	VWR
Plate Centrifuge	022628203	Eppendorf
C1000 96 well thermal cycler	4375786	BioRad
PCR Workstation	AC648LFUVC	AirClean Systems
4200 TapeStation	G2991AA	Agilent Technologies

*Or comparable/equivalent (include unless must use listed)

5.0 REAGENTS/MATERIALS*

Description	Product No.	Vendor
KAPA HyperPlus Kit	KK8514	Roche
High Sensitivity D1000 Screen Tape	5067-5584	Agilent Technologies
High Sensitivity D1000 Reagents	5067-5585	Agilent Technologies
Nuclease-free Water	various	various
Agencourt AMPure XP Kit	A63881	Beckman Coulter
Ethanol, 100% for molecular biology	E7023	Sigma-Aldrich
Elution Buffer (EB)	19086	Qiagen
SureSelectXT2 Adaptors/Indices 96 well plate	931019	Agilent Technologies
Eppendorf™ 96-Well twin.tec™ PCR Plates	E951020346	Fisher Scientific
Hard-Shell 96 well PCR plate	HSP9631	Bio-Rad
200-1000 µL Aerosol Barrier Pipette Tips	RT-L1000FLR	Rainin

Description	Product No.	Vendor
20-200 µL Aerosol Barrier Pipette Tips	RT-L-200FLR	Rainin
0.2-20 µL Aerosol Barrier Pipette Tips	RT-L10FLR	Rainin
Microamp Adhesive plate seals	4306311	Applied Biosystems

*Or comparable/equivalent (include unless must use listed items)

6.0 SAFETY

- 6.1 Lab coats, safety glasses, and gloves must be worn at all times when handling hazardous or sensitive equipment, samples, reagents, and materials. These safety measures must also be followed when in close proximity to those who are working with these items.

7.0 PROCEDURE

7.1 Dilute DNA to appropriate concentration

- 7.1.1 Dilute DNA to 250 ng with EB buffer.

7.2 Enzymatic Fragmentation of DNA

Notes:

Fragment size generation is affected by treatment duration. A longer duration will produce smaller fragments.

- 7.2.1 Prepare the fragmentation reaction mix on ice

Component	Volume
KAPA Frag Buffer (10x)	5 ul
KAPA Frag Enzyme	10 ul
Total Voume	15 ul

- 7.2.2 Add 15 ul of the fragmentation reaction mix to each well of a BioRad Hard Shell plate containing 35 ul of 250ng of dsDNA to get a total volume of 50 ul.
- 7.2.3 Mix thoroughly and centrifuge briefly.
- 7.2.4 Incubate fragmentation mix and dsDNA mix in a C1000 thermal cycler using the following conditions:

Step	Temp	Time
Pre-cool block	4°C	∞
Fragmentation	37°C	10 min

HOLD	4°C	∞
------	-----	---

Notes: Set the heated lid temperature to 50°C and volume 50 ul.

7.3 End Repair and A-Tailing

7.3.1 Prepare the End Repair and A-tailing reaction mix on ice.

Component	Volume
End Repair and A-Tailing Buffer	7 ul
End Repair and A-Tailing Enzyme Mix	3 ul
Total Volume	10 ul

7.3.2 Add 10 ul of the End Repair and A-Tailing reaction mix to each well of the plate containing the fragmented samples.

7.3.3 Mix thoroughly and centrifuge briefly.

7.3.4 Incubate the reaction in a thermal cycler using the following conditions:

Step	Temp	Time
End Repair and A-Tailing	65°C	30 min
HOLD	4°C	∞

Notes: Set the heated lid to 85°C and volume 60 ul.

7.4 Adapter Ligation

7.4.1 Prepare the Ligation reaction mix on ice.

Component	Volume
Nuclease-Free Water	5 ul
Ligation Buffer	30 ul
DNA Ligase	10 ul
Total Volume	45 ul

7.4.2 Add 5 ul of SureSelect XT2 adapters to each well of the end repair/a-tailing reaction plate.

7.4.3 Add 45 ul of ligation mix to each well of the reaction plate.

7.4.4 Mix thoroughly and centrifuge briefly.

7.4.5 Incubate at 20°C for 15 minutes.

7.5 Post Ligation Clean Up

7.5.1 Add 88 ul of Ampure XP beads to each well of the adaptor ligation reaction product.

7.5.2 Mix thoroughly by pipetting up and down several times.

7.5.3 Incubate the plate at room temperature for 15 minutes to bind the DNA to the beads.

7.5.4 Place the plate on a magnet to capture the beads, incubate until the liquid is clear.

- 7.5.5 Carefully remove and discard the supernatant.
- 7.5.6 Keep the plate on the magnet and add 200 ul of 80% ethanol.
- 7.5.7 Incubate the plate on the magnet at room temperature for 30 seconds.
- 7.5.8 Carefully remove and discard the ethanol.
- 7.5.9 Keep the plate on the magnet and add 200 ul of 80% ethanol.
- 7.5.10 Incubate the plate on the magnet at room temperature for 30 seconds.
- 7.5.11 Carefully remove and discard the ethanol.
- 7.5.12 Seal the plate with an adhesive seal and centrifuge briefly.
- 7.5.13 Remove any remaining ethanol.
- 7.5.14 Dry the beads at room temperature for 5 minutes or until all of the ethanol has evaporated.
- 7.5.15 Remove the plate from the magnet.
- 7.5.16 Resuspend the beads in 25 ul of elution buffer, pipette up and down to mix.
- 7.5.17 Incubate the plate at room temperature for 2 minutes to elute the DNA off the beads.
- 7.5.18 Capture the beads by placing the plate on the magnet and incubate until the liquid is clear.
- 7.5.19 Transfer 20 ul of the clear supernatant to a new BioRad Hard Shell plate.

7.6 Pool Samples (pool samples according to the requirements needed for mean depth of sequencing requested)

- 7.6.1 Pool xx samples into 1 pool: yy ul of each sample in 1 Eppendorf tube. (for example: for 0.2X mean of depth of sequencing required, pool 48 samples (xx=48) with 5 ul of each sample (yy=5) in a pool; for 5x mean of depth of sequencing required, pool 3 samples (xx=3) in a pool; and for 20x mean of depth of sequencing required, only 1 sample will represent an entire pool and will be sequenced in a single lane of HiSeq 2500).

7.7 Size Selection Double Cut

- 7.7.1 Perform the first (0.7x) size cut (to exclude library molecules larger than ~450 bp) by taking 50 ul of the pooled samples and adding 35 ul of the Ampure XP beads.
- 7.7.2 Mix thoroughly by pipetting up and down several times.
- 7.7.3 Incubate the plate at room temperature for 15 minutes to bind the library molecules larger than ~450 bp to the beads.
- 7.7.4 Place the plate on a magnet to capture the beads, incubate until the liquid is clear.
- 7.7.5 Carefully transfer 80 ul of the clear supernatant containing the library molecules smaller than ~450 bp to a new plate.
- 7.7.6 Perform the second size cut (0.9x) to retain library molecules >250 bp by adding 10 ul of Ampure XP beads to the 80 ul of supernatant from the first cut.
- 7.7.7 Mix thoroughly by pipetting up and down several times.
- 7.7.8 Incubate the plate at room temperature for 15 minutes to bind the library molecules larger than ~250 bp to the beads.
- 7.7.9 Place the plate on the magnet to capture the beads, incubate until the liquid is clear.

- 7.7.10 Carefully remove and discard the supernatant containing library molecules smaller than ~250 bp.
- 7.7.11 Keep the plate on the magnet and add 200 ul of 80% ethanol.
- 7.7.12 Incubate the plate on the magnet at room temperature for 30 seconds.
- 7.7.13 Carefully removed and discard the ethanol.
- 7.7.14 Keep the plate on the magnet and add 200 ul of 80% ethanol.
- 7.7.15 Incubate the plate on the magnet at room temperature for 30 seconds.
- 7.7.16 Carefully removed and discard the ethanol.
- 7.7.17 Seal the plate with an adhesive seal and centrifuge briefly.
- 7.7.18 Remove any remaining ethanol.
- 7.7.19 Dry the beads for 5 minutes at room temperature or until all of the ethanol has evaporated.
- 7.7.20 Remove the plate from the magnet.
- 7.7.21 Resuspend the beads in 25 ul of elution buffer, pipette up and down to mix well.
- 7.7.22 Incubate the plate at room temperature for 2 minutes to elute DNA off the beads.
- 7.7.23 Capture the beads by placing the plate on the magnet and incubate until the liquid is clear.
- 7.7.24 Transfer 20 ul of the clear supernatant to a new plate/tube.

7.8 Final Library Quality Assessment using the Tape Station

- 7.8.1 Allow High Sensitivity D1000 ScreenTapes, High Sensitivity D1000 sample buffer, and High Sensitivity D1000 ladder to equilibrate to room temperature for 30 minutes prior to use.
- 7.8.2 Launch TapeStation software. Select well positions to be run on the virtual plate set-up. This will calculate the necessary number of tapes and amount of ladder necessary.
- 7.8.3 Flick the required number of D1000 ScreenTapes to eliminate bubbles in the separation channel. Bubbles in the separation chambers can interfere with sample loading.
- 7.8.4 Load a High Sensitivity D1000 ScreenTape in the processing bucket and provide tips. Load the required number of additional High Sensitivity D1000 ScreenTapes in the holding rack.
- 7.8.5 Vortex and spin down the ScreenTape reagents prior to use.
- 7.8.6 If running 15 samples or fewer, prepare 4 µl of ladder by mixing 2 µl D1000 Sample Buffer with 2 µl D1000 ladder and place in a strip tube. If running more than 15 samples, dilute 15 µl of D1000 ladder (yellow cap) with 15 µl of D1000 sample buffer (green cap) and place in a strip tube.
 - 7.8.6.1 Cap strip and vortex mix using the IKA vortexer and adapter at 2000 rpm for 1 minute.
 - 7.8.6.2 Spin the plate down.
- 7.8.7 Dilute 2 µl of each final library pool sample with 2 µl of D1000 sample buffer and load on an Eppendorf skirted PCR plate.
 - 7.8.7.1 Seal the plate with pierceable foil.

7.8.7.2 Mix sample plate on the IKA vortexer with adapter at 2000 rpm for 1 minute.

7.8.7.3 Spin the plate down.

7.8.8 Load the sample plate into the TapeStation, close the lid, and click *Start*.

7.9 **Quantify the pooled libraries using ddPCR**

7.9.1 Refer to ddPCR Library Quantitation SOP (**APPENDIX 5F**).

7.10 **Dilute, Denature, and Cluster Final Pools for Sequencing**

7.10.1 Refer to Illumina Sequencing SOP(**APPENDIX 5H**).

7.10.1.1 Pools should be clustered at a concentration of 13.26 pM with a 1% phiX spike-in for each sample.

APPENDIX 5F: Library Quantitation using ddPCR for Sequencing on Illumina HiSeq2500

1.0 PURPOSE/SCOPE

This Standard Operating Procedure (SOP) describes the steps necessary to use digital droplet PCR (ddPCR) to quantitate libraries and/or pools of libraries for sequencing of samples from ETCTN project on the Illumina HiSeq 2500. This protocol begins with sample dilution, PCR plate set-up and processing, and concludes with data analysis. This SOP is intended for processing up to 24 samples per instrument run. **This SOP is for research purposes only and no clinical samples will be processed using this SOP. Any deviation from this SOP will be noted but will not be formally documented.**

2.0 REFERENCE DOCUMENTATION

Document Number	Title
6407	Droplet Digital PCR Applications guide, BioRad bulletin http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf
10031906	QX200™ Droplet Reader and QuantaSoft™ Software Instruction Manual http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10031906.pdf
10023997	PX1™ PCR Plate Sealer Instruction Manual http://www.bio-rad.com/webroot/web/pdf/lsr/literature/bulletin_10023997.pdf
10021377	C1000 Touch™ Thermal Cycler Instruction Manual http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10021377.pdf
6402	ddPCR™ Library Quantification Kit for Illumina TruSeq Flier http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6402.pdf
10043138	Automated Droplet Generator Instruction Manual http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10043138.pdf

Document Number	Title
10031986	ddPCR™ Library Quantification Kit for Illumina TruSeq Product Insert http://www.biorad.com/webroot/web/pdf/lsr/literature/10031986.pdf

3.0 BACKGROUND

ddPCR is used to reproducibly determine the concentrations of library samples and library pools to achieve accurate cluster density for sequencing on the Illumina HiSeq 2500. The ddPCR Library Quantification Kit for Illumina TruSeq is used on the Bio-Rad QX200 AutoDG ddPCR systems and allows absolute quantification of both the P5 and P7 adapter sequences. The multiplexed, probe-based method also provides library quality information.

4.0 DEFINITIONS

Abbreviation/Term	Definition
ddPCR	Droplet Digital PCR

5.0 EQUIPMENT

Description	Model #	Vendor
Single Channel Pipettes (p2, 20, 200, 1000)	Variable	Rainin
Multi-Channel Pipettes (LTS 2, 20,200)	Variable	Rainin
Vortex	58816-121	VWR
MiniFuge	93000-196	VWR
PX1 PCR Plate Sealer	181-4000	Bio-Rad
QX200 AutoDG ddPCR system including laptop and droplet reader	186-4100	Bio-Rad
Plate Centrifuge	022628203	Eppendorf
C1000 Touch thermal cycler	1851197	Bio-Rad

Description	Model #	Vendor
PCR Workstation	AC648LFUVC	AirClean Systems

6.0 REAGENTS/MATERIALS

Description	Product No.	Vendor
Ion AmpliSeq Library Kit v2.0*	4475345	Life Technologies
MPACT Custom Primer Panel	IAD27536	Life Technologies
ddPCR Library Quantification Kit for Illumina TruSeq	1863040	Bio-Rad
PCR Plate Heat Seal, foil, pieceable	1814040	Bio-Rad
DG32™ Automated Droplet Generator Cartridges	1864108	Bio-Rad
Nuclease-free Molecular Biology Grade Water (not DEPC-Treated)	AM9937	Life Technologies
MicroAmp Optical 96-well PCR Plates	N8010560	Life Technologies
MicroAmp 96-well Optical Adhesive Film	4311971	Life Technologies
1.5mL Lo-Bind Tubes	022431021	Eppendorf
200-1000 µL Aerosol Barrier Pipette Tips	RT-L1000FLR	Rainin
20-200 µL Aerosol Barrier Pipette Tips	RT-L-200FLR	Rainin
0.2-20 µL Aerosol Barrier Pipette Tips	RT-L10FLR	Rainin
ddPCR™ Droplet Reader Oil	1863004	Bio-Rad

Description	Product No.	Vendor
Automated Droplet Generation Oil for Probes	1864110	Bio-Rad
Pipet Tip Waste Bins for the AutoDG™ System	1864125	Bio-Rad
Pipet Tips for the AutoDG™ System	1864121	Bio-Rad
Eppendorf™ 96-Well twin.tec™ PCR Plates	E951020346	Fisher Scientific
Buffer EB	19086	Qiagen
Disposable Pipetting Reservoirs	89094-662	VWR
96 well 0.8mL Storage Plates (MIDIPlates)	AB-0859	Fisher Scientific

7.0 AUTOMATION METHODS

The AutoDG droplet generator is pre-programmed. The user must load the instrument with the necessary consumables, select the columns to be processed, and the type of probes that are to be used.

8.0 SAFETY

8.1 Lab coats, safety glasses, gloves must be worn at all times when handling hazardous or sensitive equipment, samples, reagents, and materials. These safety measures must also be followed when in close proximity to those who are working with these items.

9.0 ASSAY GUIDELINES

- 9.1 Once thawed, the library quantification kit can be stored at 4°C for 2 weeks. Repeated freeze thawing is not recommended.
- 9.2 Thaw reagents at room temperature and then store on ice during use. After thawing, vortex and centrifuge reagents prior to pipetting.
- 9.3 For accurate quantitation, the ddPCR concentrations should be between 100-5000 copies/ul.

- 9.4 Assaying 2 dilution points in duplicate ensures that a reliable concentration measurement is obtained. In most cases 10^{-6} and 10^{-7} dilutions of libraries that were originally diluted to a target concentration of 5nM will yield data within the recommended range.

10.0 PROCEDURE

10.1 Program the C1000 Thermal Cycler

- 10.1.1 Program the C1000 thermal cycler with the cycler protocol outlined below.

Temperature	Time (mm:ss)	# Cycles
95°C	HOLD	1
95°C	10:00	1
94°C	00:30	40
61°C	02:00	2°C/sec ramp rate
98	10:00	1
12°C	HOLD	1

*Use a heated lid set to 105°C and set the sample volume to 40 uL.

Note: Use the above thermocycler protocol instead of the protocol outlined in the assay documentation. This protocol has been optimized to maximize the fluorescent intensity between positive and negative droplets.

10.2 Preparation of ddPCR master mix

- 10.2.1 In a clean room or PCR box, prepare the ddPCR master mix as outlined in the table below.

Reagent	Volume/reaction, uL (including 5% overage)	Volume/96 well plate (including 5% overage)	Final concentration
2x ddPCR Supermix for Probes (no dUTP)	11.55	1108.8	1x
20x ddPCR library quantification assay	1.155	110.88	1x
Nuclease-free water	5.775	554.4	
Total volume to add per well	17.6 uL (+5% overage)	17.6 uL (+5% overage)	1x

Note: The AutoDG requires complete columns of samples. If necessary, fill remaining wells in the column with NTC samples (17.6 uL master mix + 4.4 uL nuclease-free water).

10.2.2 Add 17.6 uL master mix into each reaction well of a 96 well twin.tec plate. Seal the plate with adhesive film. Store the plate on ice in the dark until samples are added.

10.3 Create sample dilutions

10.3.1 Using the concentration information from the quality control Fragment Analyzer run, dilute the libraries or library pools with nuclease-free molecular biology grade water. Dilute samples to a final target concentration of about 5nM. Total dilution volumes should be at least 14uL. This ensures enough volume to use as a working stock for the remainder of the workflow.

10.4 Perform serial dilutions of samples

10.4.1 Create serial dilutions to be used for the ddPCR setup. Use EB buffer to dilute samples in a 96 well MIDI plate. Thoroughly mix samples between each step of the dilution series. Dilute each sample row-wise as outlined in the table below.

Dilution step	Column 1	Column 2	Column 3	Column 4
Sample volume, uL	2 uL working stock	2 uL from column 1	2 uL from column 2	10 uL from column 3
EB buffer volume, uL	198 uL	198 uL	198 uL	90 uL
Dilution	100x	100x	100x	10x
Final dilution	10^{-2}	10^{-4}	10^{-6}	10^{-7}

10.5 Combine diluted samples and PCR master mix to create reaction plate

10.5.1 Add 4.4 uL diluted sample to each reaction well containing master mix according to the plate layout below. The total volume in each well should be 22 uL.

Sample #1 10^6	Sample #1 10^6	Sample #1 10^7	Sample #1 10^7	Sample #9 10^6	Sample #9 10^6	Sample #9 10^7	Sample #9 10^7	Sample #171 10^7	Sample #171 10^7	Sample #171 10^7	Sample #171 10^7
Sample #2 10^6	Sample #2 10^6	Sample #2 10^7	Sample #2 10^7	Sample #10 10^6	Sample #10 10^6	Sample #10 10^7	Sample #10 10^7	Sample #18 10^7	Sample #18 10^7	Sample #18 10^7	Sample #18 10^7

Sample #3 10 ⁶	Sample #3 10 ⁶	Sample #3 10 ⁷	Sample #3 10 ⁷	Sample #11 10 ⁶	Sample #11 10 ⁶	Sample #11 10 ⁷	Sample #11 10 ⁷	Sample #19 10 ⁷	Sample #19 10 ⁷	Sample #19 10 ⁷	Sample #19 10 ⁷
Sample #4 10 ⁶	Sample #4 10 ⁶	Sample #4 10 ⁷	Sample #4 10 ⁷	Sample #12 10 ⁶	Sample #12 10 ⁶	Sample #12 10 ⁷	Sample #12 10 ⁷	Sample #20 10 ⁷	Sample #20 10 ⁷	Sample #20 10 ⁷	Sample #20 10 ⁷
Sample #5 10 ⁶	Sample #5 10 ⁶	Sample #5 10 ⁷	Sample #5 10 ⁷	Sample #13 10 ⁶	Sample #13 10 ⁶	Sample #13 10 ⁷	Sample #13 10 ⁷	Sample #21 10 ⁷	Sample #21 10 ⁷	Sample #21 10 ⁷	Sample #21 10 ⁷
Sample #6 10 ⁶	Sample #6 10 ⁶	Sample #6 10 ⁷	Sample #6 10 ⁷	Sample #14 10 ⁶	Sample #14 10 ⁶	Sample #14 10 ⁷	Sample #14 10 ⁷	Sample #22 10 ⁷	Sample #22 10 ⁷	Sample #22 10 ⁷	Sample #22 10 ⁷
Sample #7 10 ⁶	Sample #7 10 ⁶	Sample #7 10 ⁷	Sample #7 10 ⁷	Sample #15 10 ⁶	Sample #15 10 ⁶	Sample #15 10 ⁷	Sample #15 10 ⁷	Sample #23 10 ⁷	Sample #23 10 ⁷	Sample #23 10 ⁷	Sample #23 10 ⁷
Sample #8 10 ⁶	Sample #8 10 ⁶	Sample #8 10 ⁷	Sample #8 10 ⁷	Sample #16 10 ⁶	Sample #16 10 ⁶	Sample #16 10 ⁷	Sample #16 10 ⁷	Sample #24 10 ⁷	Sample #24 10 ⁶	Sample #24 10 ⁷	Sample #24 10 ⁷

10.5.2 Seal plate with an optical seal and vortex thoroughly. Quick spin the plate. Inspect the plate to ensure there are no bubbles in the wells.

Note: Plate should be stored on ice away from light if it is not being immediately loaded onto the AutoDG for droplet generation.

10.6 Generate droplets

10.6.1 Load consumables on AutoDG instrument. For a run with 96 reactions:

10.6.1.1 Place 3 unused droplet generation cartridges on the back row

10.6.1.2 Remove lids and place 2 full boxes of droplet generation tips on the middle row

10.6.1.3 Place an empty waste bin on the middle row to the left

10.6.1.4 Place the 96 well cold chill block to the front right. Place an unused twin-tec 96 well plate in the chill block. Droplets will be deposited into this plate after they are formed.

7.10.1.2 Ensure that there is enough droplet generation oil to complete the run

10.6.2 Configure the AutoDG using the touchscreen on the instrument

10.6.2.1 'Probes' should be selected as oil type

10.6.2.2 Configure the sample plate by selecting the columns containing samples

10.6.2.3 Place the reaction plate on the deck to the front left. Carefully remove the adhesive film.

10.6.2.4 Lower the lid of the AutoDG. Push start to generate droplets.

Note: Droplet generation will take approximately 40 minutes for a full 96 well plate of samples. Final volume in each reaction well is approximately 40 uL.

10.7 Seal the reaction plate

10.7.1 After droplet generation is complete, place a pierceable foil seal on the 96 well plate containing the droplets.

10.7.2 Remove the reaction plate from the AutoDG and seal the plate on the plate sealer.

10.7.2.1 Seal at 180 °C for 5 seconds

10.8 Amplification

10.8.1 Place the reaction plate on the thermal cycler and start the program outlined in step 10.1.1.

Note: The amplification protocol takes about 2.5 hours.

Note: If droplets are not immediately read plate can be stored for up to 2 days at 4-12 °C away from light.

10.9 Configure the plate template

10.9.1 Open the Quantasoft software and load the plate template file located on the S drive. File location is: S:\Experiments\PDX\Library preps\templates. File name is: ddPCR quant template.qlt. If desired, update the sample names on the template to match the reaction plate setup.

10.9.1.1 ABS should be selected as Experiment Type in the well editor

10.9.1.2 ddPCR Supermix for Probes (no dUTP) should be selected as Supermix Type in the well editor

10.9.1.3 Ch1 Unknown in Target 1 and Ch2 Unknown in Target 2 should be selected in the well editor

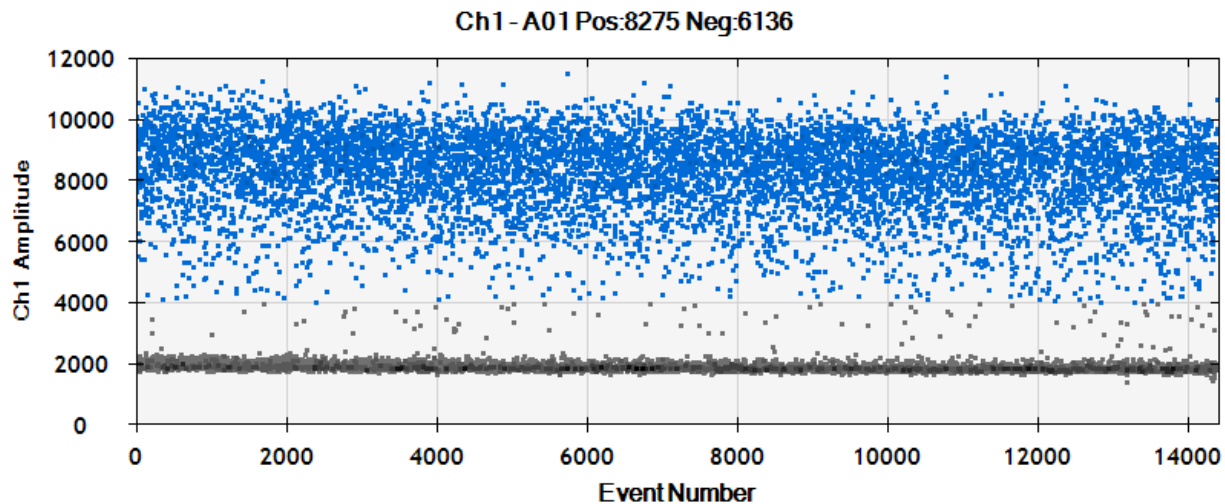
10.9.1.4 Load the reaction plate onto the droplet reader.

10.9.1.5 In the software, click Run and select the FAM/HEX dye set.

Note: A complete plate of 96 reactions will take approximately 2 hours to read.

10.10 Data analysis

10.10.1 Click Analyze in the Quantasoft software. Select the dilutions corresponding to a sample in the well selector under analyze. Examine the automatic thresholding applied to the amplitude data. It may be necessary to manually set the threshold between the positive and negative droplets using the amplitude and/or histogram plots. See below for an example of thresholding. Repeat for each dilution of each sample.



10.10.2 Assess data quality

10.10.2.1 Concentration data for 10^6 dilutions should be about 10 times concentration data for 10^7 dilutions.

10.10.2.2 Replicate data should be similar.

10.10.2.3 Total number of droplets per reaction should be 12000-20000.

10.10.2.4 Concentration data for Ch1 and Ch2 should be similar. If these numbers are not similar it may indicate an issue with library quality. Properly formed library molecules will be positive for both the Ch1 and Ch2 targets.

10.10.2.5 When looking at the 2D plot, ddPCR data can also be used to assess library quality.

10.10.2.5.1 Inserts of varying sizes can be observed along a diagonal path. Larger fragments are nearer the negative droplets.

10.10.2.5.2 Adaptor/adaptor ligations will appear as a population of droplets in the upper right-hand corner.

See below for an illustration of library quality information that can be determined from a 2D plot.

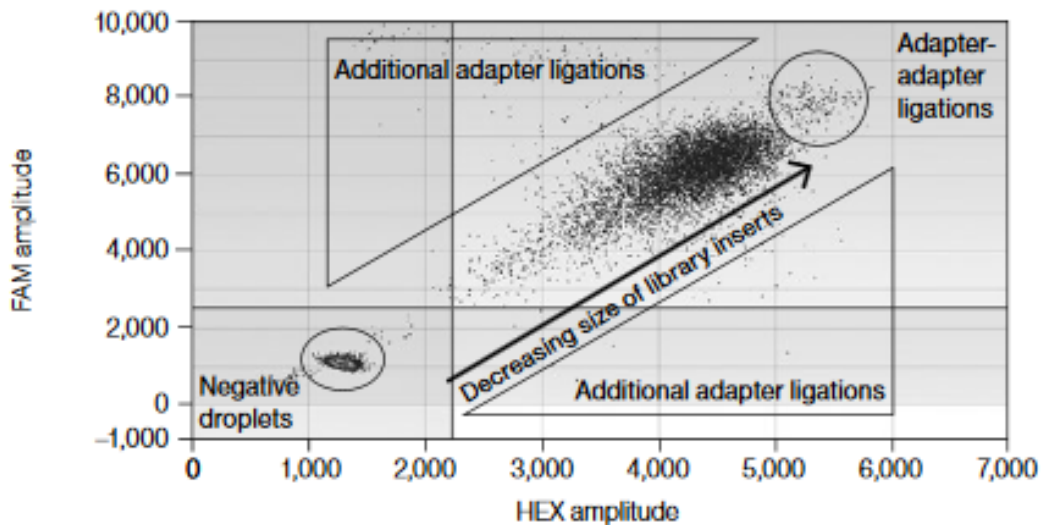


Fig. 1. Illumina TruSeq library quantified using the ddPCR Library Quantification Kit for Illumina TruSeq.

10.10.3 Save the post-analysis plate information in Setup, Plate, Save As.

10.10.4 Export both the merged and single well information and save as a csv file.

10.11 Concentration calculations

10.11.1 Using the merged information that was exported and saved in step 10.10.4, transfer sample name and concentration (in copies/uL) data to the ddPCR calculations template file located on the S drive.

File location: S:\Experiments\PDX\Library preps\templates

10.11.2 For each dilution for each sample, compare the Ch1 and Ch2 data and copy and paste the lower concentration into the lowest conc, copies/uL column. Once this concentration information is entered, the nM concentration of the library will be automatically calculated.

10.11.2.1 Calculation example: A library at the 10^6 dilution yielded 2000 copies/uL in the ddPCR mixture. Multiply 2000 by 10^6 and by 5 to account for the dilution in the reaction mixture ($2000 \times 10^6 \times 5 = 10^{10}$ copies/uL of original stock library). To obtain nM: 10^{10} copies/uL $\times 10^6$ uL/L / 6.023×10^{23} copies/mole = 1.66×10^{-8} M or 16.6 nM.

Use the ddPCR data from the least diluted reactions that yielded concentration information between 100 and 5000 copies per reaction. For most samples this means the 10^6 data will be used. Use these concentrations to further dilute the samples for pooling or clustering.

APPENDIX 5G: Denaturing and Clustering of Libraries for Sequencing on HiSeq 2500 at MoCha Laboratory

1.0 PURPOSE/SCOPE

This Standard Operating Procedure (SOP) describes the steps necessary to denature, dilute and cluster library samples on the cBot for sequencing of ETCTN samples on the Illumina HiSeq 2500. This protocol begins with sample denaturation and dilution and concludes with loading the cBot for cluster generation. This SOP is intended for processing up to 8 lanes of samples per instrument run. **This SOP is for research purposes only and no clinical samples will be processed using this SOP. Any deviation from this SOP will be noted but will not be formally documented.**

2.0 REFERENCE DOCUMENTATION

Document Number	Title
15050107 v02	HiSeq and GAllx Systems: Denature and Dilute Libraries Guide http://support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/hiseqkits/hiseq-ga-denaturing-diluting-libraries-reference-guide-15050107-02.pdf
15006165 v02	cBot System Guide http://support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/cbot/cbot-system-guide-15006165-02.pdf
15050104	HiSeq Cluster Kit v4 Reference Guide

3.0 BACKGROUND

The cBot uses bridge amplification to create hundreds of millions of single-molecule DNA templates simultaneously on a sequencing flowcell. Clonal clusters are created from single DNA templates on a flowcell, preparing them for sequencing on the HiSeq 2500.

4.0 DEFINITIONS

Abbreviation/Term	Definition
EBT	Buffer EB + 0.1% Tween 20

5.0 EQUIPMENT

Description	Model #	Vendor
Single Channel Pipettes (p2, 20, 200, 1000)	Variable	Rainin
Vortex	58816-121	VWR
MiniFuge	93000-196	VWR
cBot	SY-312-2001	Illumina
Tabletop Centrifuge	5424	Eppendorf

6.0 REAGENTS/MATERIALS

Description	Product No.	Vendor
Nuclease-free Molecular Biology Grade Water (not DEPC-Treated)	AM9937	Life Technologies
1.5mL Lo-Bind Tubes	022431021	Eppendorf
200-1000 µL Aerosol Barrier Pipette Tips	RT-L1000FLR	Rainin
20-200 µL Aerosol Barrier Pipette Tips	RT-L-200FLR	Rainin
0.2-20 µL Aerosol Barrier Pipette Tips	RT-L10FLR	Rainin
Buffer EB	19086	Qiagen
0.2 mL PCR tube strips	14230210	Fisher Scientific
Sodium hydroxide, 10N volumetric solution	02004123	Fisher Scientific
HiSeq PE Cluster kit v4	401-4001	Illumina
Kimwipes™ Delicate Task Wipers, 1-Ply	06-666	Fisher Scientific
Phix Control kit v3	FC-110-3001	Illumina
Buffer EB	19086	Qiagen
Tween 20	BP337-100	Fisher Scientific
Lens paper 6x8 inches	52845-009	VWR

Description	Product No.	Vendor
Sterile isopropanol wipes prep pads, 70%	22-363-750	Fisher Scientific
Forceps	609T	Lerloy

7.0 AUTOMATION METHODS

The cBot is pre-programmed. The user must load the instrument with the necessary consumables and libraries and select the appropriate recipe to be used.

8.0 SAFETY

8.1 Lab coats, safety glasses, gloves must be worn at all times when handling hazardous or sensitive equipment, samples, reagents, and materials. These safety measures must also be followed when in close proximity to those who are working with these items.

9.0 ASSAY GUIDELINES

- 9.1 Thaw reagents in a water bath at room temperature and then store on ice during preparation.
- 9.2 Unless other noted, store library pool samples on ice during preparation.
- 9.3 Prior to starting, prepare EBT buffer by adding Tween 20 to buffer EB to yield for a final solution of 0.1% Tween 20 in EB.
- 9.4 Freshly prepare 0.1 N NaOH. Prepare at least 1mL of diluted NaOH to prevent small pipetting errors from affecting the final NaOH concentration.
- 9.5 Library pool samples are loaded for clustering at a concentration of 13.26pM with a 1% PhiX spike-in. This results in a cluster density of approximately 1050 K/mm² when sequenced on a HiSeq 2500.

10.0 PROCEDURE

10.1 Dilute pools

- 10.1.1 Following ddPCR pool quantitation, enter the calculated pool concentration values into the sheet called 'pool quant summary' in the library prep excel file.

10.1.2 Dilute each pool to a target concentration of 1.326 nM using the information in the table in the 'pool quant summary' sheet. Use EBT to dilute the pools. Total volume for each diluted pool is 10 uL.

Note: RNA Access pools are sequenced 3 pools to a lane, with lanes run in duplicate. Dilute individual RNA Access pools to 1.326 nM as described above. After diluting individual pools, combine 5 uL of each of the 3 diluted pools together. Use 10 uL of this combined-pool mixture to continue with denaturing and loading for clustering.

10.2 Dilute PhiX

10.2.1 Combine 2 uL of 10nM stock PhiX with 9 uL EBT to yield 2nM PhiX.

10.3 Denature pools and PhiX

10.3.1 Combine 10 uL of each diluted pool or PhiX with 10 uL 0.1N NaOH.

10.3.2 Vortex to mix.

10.3.3 Centrifuge at 280 x g for 1 minute.

10.3.4 Incubate for 5 minutes at room temperature to denature the library into single strands.

10.4 Dilute denatured pools

10.4.1 Add 980 uL pre-chilled HT1 to the denatured pools and PhiX to result in 13.26pM pools and 20pM PhiX.

10.4.2 Invert the tube several times to mix and pulse centrifuge.

10.4.3 Place the denatured samples on ice until ready to proceed.

10.5 Spike in PhiX

10.5.1 Remove and discard 10 uL from each denatured 13.26pM pool sample.

10.5.2 Add 10 uL of denatured 20pM PhiX sample to each pool sample for a 1% PhiX spike-in.

10.5.3 Place samples on ice until ready to prepare the 8 tube strip.

10.6 Prepare an 8 tube strip

10.6.1 Label the tubes of an 8 tube strip 1-8.

10.6.2 Add 75uL diluted/denatured pool sample into the specified tubes.

Note: Tube 1 of the strip corresponds to lane 1 of the flowcell, tube 2 corresponds to lane 2, etc.

10.6.3 Set the tube strip aside on ice until ready to load onto the cBot.

10.6.4 When prompted by the cBot software, load the 8 tube strip onto the cBot in the TEMPLATES tube strip holder.

10.7 Prepare the cBot

10.7.1 Turn on the cBot.

10.7.2 Ensure that the waste bottle is empty and there is sufficient coolant for the run.

10.7.3 Select User Name. Enter your user name and select Enter.

10.7.4 Select start to proceed to the pre-run wash.

10.7.5 Perform a pre-run wash.

10.7.5.1 Raise the cBot lid and fill the wash reservoir, located behind the thermal stage with approximately 12 mL deionized water.

10.7.5.2 Select the checkbox on the screen to indicate that water is present.

10.7.5.3 Select Wash to start the wash.

10.7.5.4 Repeat the Wash 1x for a total of 2 washes.

10.7.5.5 Blot out excess water remaining in the wash reservoir with a Kimwipe

10.7.5.6 Select the checkbox on the screen to indicate that the wash reservoir is dry.

10.7.5.7 Select Next.

10.8 **Setting up and starting the cBot run**

10.8.1 Select Experiment Name. Type the experiment name and select Enter.

10.8.2 Select protocol PE_HiSeq_Cluster_Kit_v4_cBot_recipe_v9.0 and select Next.

10.8.3 Load reagents

10.8.3.1 Remove the clear plastic lid from the thawed cBot reagent plate.

10.8.3.2 Gently press down on the tubes in the plate to make sure they are securely seated.

10.8.3.3 Select Scan Reagent ID to activate the barcode scanner.

10.8.3.4 With the barcode label facing the instrument, hold the reagent plate level and move the reagent plate into the light path of the barcode scanner.

10.8.3.4.1 You will hear a beep when the scanner has successfully read the barcode. The reagent ID appears on the screen.

10.8.3.5 Pull the spring-loaded reagent plate lever towards you to release the clamp.

10.8.3.6 Place the reagent plate onto the reagent stage, positioned with row 1 facing towards the front of the stage. Make sure the beveled corner of the plate is positioned in the front right corner.

10.8.3.7 Release the reagent plate lever to secure the reagent plate.

10.8.3.8 Select the checkbox to indicate the reagent plate is loaded

10.8.3.9 Select Next.

10.8.4 Load the flow cell

10.8.4.1 Lift the flow cell clamp.

10.8.4.2 Wipe the adapter plate on the thermal stage with an isopropanol wipe and allow to dry.

10.8.4.3 Select Scan Flow Cell ID to activate barcode scanner.

10.8.4.4 Hold the flow cell container close to the scanner tray with the barcode positioned toward the instrument.

10.8.4.5 Slowly slide the flow cell container into the light path of the barcode scanner so the entire barcode crosses the scan light at the same time.

10.8.4.5.1 You will hear a beep when the scanner has successfully read the barcode. The flow cell ID appears on the screen.

10.8.4.6 Remove the flow cell from the storage tube using forceps.

10.8.4.7 Holding the flow cell by the edges, rinse the flow cell gently with deionized water and dry thoroughly using a lens cleaning tissue.

10.8.4.8 Place the flow cell on the thermal stage with holes facing upward, lane 1 on the right side, and lane 8 and the barcode on the left side.

Note: HiSeq flow cell lane orientation for loading onto the cBot is lane 8-1, left to right.

Note: HiSeq flow cells have a mechanically keyed corner, which provides visual orientation for loading the flow cell. Flow cells should be loaded so that the notched corner is in the far right corner of the thermal stage.

10.8.4.9 Select the checkbox to indicate that the flow cell has been loaded.

10.8.4.10 Select Next

10.8.5 Load the manifold

10.8.5.1 Remove the manifold from the packaging and inspect the sippers. Sippers should be straight. Ensure that the black rubber gaskets are evenly seated.

10.8.5.2 Position the manifold over the flow cell with the sipper comb pointing toward the front of the cBot.

10.8.5.3 Align the manifold with the guide pins on the thermal stage and set the manifold into place on top of the flow cell.

10.8.5.4 Wiggle the manifold to make sure it is evenly seated over the flow cell. The manifold must be evenly seated to form a tight seal.

10.8.5.5 Select the checkbox to indicate the manifold is seated.

- 10.8.5.6 Close the flow cell clamp to lock the manifold in position. Make sure the bracket snaps securely under the white clip.
- 10.8.5.7 Select the checkbox to indicate the flow cell clamp is closed
- 10.8.5.8 Connect the outlet end of the manifold to the outlet port in the wash reservoir and make sure it is evenly seated.
- 10.8.5.9 Snap the outlet clamp closed to secure the outlet end of the manifold.
- 10.8.5.10 Select the checkbox to indicate that you have connected the manifold to the outlet port and the rear clamp is secured.
- 10.8.5.11 Align the sipper comb with the two metal guide pins on the front edge of the thermal stage.
- 10.8.5.12 Snap the sipper comb into place using the plastic tabs on either side of the sipper comb. Make sure the sippers are straight and perpendicular to the reagent plate.
- 10.8.5.13 Select Next.

10.8.6 Load templates

- 10.8.6.1 Select Enter Template Name.
- 10.8.6.2 Type the template ID and select Enter.
- 10.8.6.3 Load the 8 tube strip containing the template into the TEMPLATE row of the tube strip holder such that the tube labeled **#8 is on the left** side and the tube labeled **#1 is on the right** side.
- 10.8.6.4 Select the checkbox to indicate that templates have been loaded.

10.8.7 Perform a Pre-Run check and start the run

- 10.8.7.1 Select Next. The system will automatically perform a pre-run check.
- 10.8.7.2 After successful completion of the pre-run check, close the cBot lid and select Start. The Run Status screen opens and the run begins.

10.9 cBot post run procedures

- 10.9.1 When the run is complete, select Unload.
- 10.9.2 Raise the cBot lid.
- 10.9.3 Release the outlet clamp securing the outlet end of the manifold.
- 10.9.4 Disconnect the outlet end of the manifold from the outlet port in the wash reservoir.
- 10.9.5 Remove the sipper comb from the metal guide pins using the plastic tabs on either side of the sipper comb.

10.9.5.1 When the sipper comb is removed, the system selects the checkbox indicating that the manifold is removed.

10.9.6 Release the flow cell clamp.

10.9.7 Carefully remove the manifold from the cBot, making sure that the flow cell is still on the thermal stage.

10.9.8 Lift the flow cell from the thermal stage and place in the flow cell conical. Store on ice or at 4°C until loaded onto the HiSeq.

Note: The clustered flow cell is stable for 10 days if properly stored in storage buffer at 4°C.

10.9.9 Pull the reagent lever toward you to release the reagent plate.

10.9.10 Remove the reagent plate. Ensure that remaining reagent levels are equal across the rows.

10.9.11 Remove the 8 tube strip containing templates. Ensure that remaining template levels are equal.

10.9.12 Select the checkbox to indicate that reagents and templates have been unloaded. The Wash button becomes active when all components have been removed.

10.9.13 Perform a post-run wash

10.9.13.1 Fill the wash reservoir with approximately 12 mL deionized water. You must have sufficient volume to prevent air from entering the lines.

10.9.13.2 Select the checkbox on the screen to indicate water is present. The Wash button becomes active.

10.9.13.3 Select Wash.

10.9.13.4 Repeat the wash 1x for a total of 2 washes.

10.9.13.5 When the wash is complete use a Kimwipe to blot out any excess water remaining in the wash reservoir.

10.9.13.6 Select the checkbox on the screen to indicate the wash reservoir is dry. The Exit button becomes active.

10.9.13.7 Select Exit. The Start screen opens.

APPENDIX 5H: Sequencing of Libraries on HiSeq2500 at MoCha Laboratory

1. PURPOSE/SCOPE

This Standard Operating Procedure (SOP) describes the steps necessary to configure a run, prepare reagents, and load a flow cell clustered with library pools for sequencing on the Illumina HiSeq 2500. This protocol includes procedures for HiSeq maintenance and water washes, sample sheet generation, reagents preparation and loading, software run configuration, and loading a flow cell to begin sequencing. This SOP is intended for processing 8 lanes of samples per flow cell. Each flow cell is capable of sequencing 24 whole exome or 48 RNA Access samples. Each HiSeq is capable of sequencing 2 flow cells simultaneously. **This SOP is for research purposes only and no clinical samples will be processed using this SOP. Any deviation from this SOP will be noted but will not be formally documented.**

2. REFERENCE DOCUMENTATION

Document Number/Name	Title
Sample Sheet template	Sample Sheet.csv S:\Experiments\PDX\Library preps\templates\sample sheet template
Flow Cell Master loading list	2015_Flowcell_Loading_MasterList_PMM_031302015.xlsx S:\Bioinformatics\Illumina\2015_Flowcell_Loading_MasterList_PMM_031302015.xlsx
15035786 v01	HiSeq 2500 System Guide http://support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/hiseq2500/hiseq-2500-system-guide-15035786-01.pdf

3. BACKGROUND

The HiSeq 2500 is an ultra-high-throughput sequencing system that allows RNA and whole exome sequencing of samples using Illumina's SBS (sequencing by synthesis) technology. Samples are sequenced using the HiSeq v4 high output run parameters.

4. DEFINITIONS

Abbreviation/Term	Definition
HCS	HiSeq Control Software

5. EQUIPMENT

Description	Model #	Vendor
Single Channel Pipettes (p200, 1000)	Variable	Rainin
Advanced High Volume Plate Stirrer	12621-056	VWR Scientific
SCIENCEWARE® Carboys with Spigot, Bel-Art, 8L	118470020	VWR Scientific
VWR® Circulus™ Magnetic Stir Bar, Red	58947-907	VWR Scientific
Argos Technologies Omega Single-Channel Pipet Controller	03-391-253	Fisher Scientific
HiSeq 2500	HiSeq 2500	Illumina

6. REAGENTS/MATERIALS

Description	Product No.	Vendor
HiSeq® SBS Kit v4 (250 cycles)	FC-401-4003	Illumina
200-1000 µL Aerosol Barrier Pipette Tips	RT-L1000FLR	Rainin
20-200 µL Aerosol Barrier Pipette Tips	RT-L-200FLR	Rainin
ProClin 300	48912-U	Sigma-Aldrich
Kimwipes™ Delicate Task Wipers, 1-Ply	06-666	Fisher Scientific
Centrifuge tubes, 250 mL	430776	Corning
Conical tubes, 15 mL	430052	Corning
Tween 20	BP337-100	Fisher Scientific
Lens paper 6x8 inches	52845-009	VWR Scientific
Sterile isopropanol wipes prep pads, 70%	22-363-750	Fisher Scientific
Disposable Standard Serological Pipets, 25 mL capacity	13-678-14B	Fisher Scientific
Disposable Standard Serological Pipets, 10 mL capacity	13-678-12E	Fisher Scientific
Forceps	609T	Lerloy
Laboratory grade water 18 M Ohm	n/a	n/a

7. AUTOMATION METHODS

The cBot is pre-programmed. The user must load the instrument with the necessary reagents and clustered flow cell(s) and select the appropriate settings to be used.

8. SAFETY

- 8.1. Lab coats, safety glasses, gloves must be worn at all times when handling hazardous or sensitive equipment, samples, reagents, and materials. These safety measures must also be followed when in close proximity to those who are working with these items.
- 8.2. The paired-end reagents contain formamide, a probable reproductive toxin. Wear protective equipment when handling. Handle used reagents as chemical waste and discard in accordance with governmental safety standards.

9. ASSAY GUIDELINES

- 9.1. Thaw frozen reagents in a water bath at room temperature and then store on ice during preparation. Alternatively, sequencing reagents can be thawed for about 16 hours at 4°C.
 - 9.1.1. *Note:* If thawing reagents using a water bath, thaw CRM in a separate water bath. Always replace gloves after handling CRM. Once thawed, store CRM separately on ice.
 - 9.1.2. *Note:* Protect IRM from light during and after thawing.
- 9.2. Library pool samples are loaded for clustering at a concentration of 13.26pM with a 1% PhiX spike-in. This results in a cluster density of approximately 1050 K/mm² when sequenced on a HiSeq 2500.
- 9.3. A maintenance wash and water wash must be performed within 24 hours of starting a sequencing run. The HCS will not allow you to start a sequencing run if the wash has not been completed within the past 24 hours.
- 9.4. 2 flow cells may be loaded and sequenced at the same time on a single HiSeq 2500. Separate reagents must be prepared for each flow cell.

10. PROCEDURE

10.1. Start the HiSeq 2500

- 10.1.1. Start the instrument control computer.
- 10.1.2. If necessary, log onto the operating system.
- 10.1.3. Turn on the main power switch to the On position. If you are facing the front of the instrument, the power switch is on the left side.
- 10.1.4. Wait for the instrument drive called DoNotEject to initialize.

Note: Never eject this drive—it contains hardware configuration files.
- 10.1.5. Copy the Illumina Maintenance folder onto the computer's Desktop from drive D:

10.1.6. Right click on drives D: and E: and Quick Format them

10.1.7. Copy the Illumina Maintenance Folder from the Desktop back onto drive D:

10.1.8. If necessary, reconnect network drive where data files will be directed.

10.1.9. Start the HiSeq Control Software.

Note: The HCS Welcome Screen is split into 2 panels, one for flow cell A and 1 for flow cell B. You can run a set up for both flow cells in parallel using the software interface.

Note: The HCS provides commands to begin a sequencing run, wash the instrument, perform a system check, and change modes. The current mode appears at the top of the screen. When a run is complete, the software returns to the Welcome Screen.

10.2. Perform a maintenance wash

10.2.1. Prepare maintenance wash solution.

Note: The prepared maintenance wash solution can be stored for up to 30 days at room temperature.

10.2.1.1. Prepare 250 mL of 10% Tween 20 by combining 225 mL of laboratory-grade water with 25 mL Tween 20.

10.2.1.2. Place a stir bar in an empty carboy.

10.2.1.3. Combine 750 mL laboratory-grade water, 250 mL 10% Tween 20, and 1.5 mL ProClin 300 in the carboy.

10.2.1.3.1. These volumes result in a 2.5% Tween 20, 0.15% ProClin 300 solution

10.2.1.4. Place the carboy on the stir plate and stir until thoroughly mixed.

10.2.1.5. Add 4 L laboratory-grade water to the solution.

10.2.1.5.1. These volumes result in a 0.5% Tween 20, 0.03% ProClin 300 wash solution.

10.2.1.6. Stir until solution is thoroughly mixed.

10.2.1.7. Store maintenance wash solution at room temperature until ready to use.

10.2.2. Prepare the HiSeq 2500 for a maintenance wash and start the wash.

10.2.2.1. From the Welcome Screen select Wash → Maintenance

10.2.2.2. Select Yes to wash PE reagent positions.

10.2.2.3. Select Next.

10.2.2.4. Fill a 250 mL centrifuge bottle with 250 mL maintenance wash solution for each SBS reagent position in the HiSeq. Screw a funnel cap onto each bottle. 8 bottles will be needed for each side of the HiSeq.

- 10.2.2.5. Fill a 15 mL conical tube with 12 mL maintenance wash solution for each paired end position in the HiSeq. Do not place lids on the tubes. 10 tubes will be needed for each side of the HiSeq.
- 10.2.2.6. Load the bottles and tubes onto the instrument in the assigned reagent rack positions. Lower the sippers into the tubes and close the door to the reagent compartment on the HiSeq.
- 10.2.2.7. Empty the waste bottle.
- 10.2.2.8. Select the Wash solution loaded and template loading station closed checkbox, then select Next.
- 10.2.2.9. Remove the front and back gaskets from both flow cell positions on the HiSeq.
- 10.2.2.10. Wipe down the flow cell stage with an isopropanol wipe and allow to dry completely.
- 10.2.2.11. Place new gaskets on the front and back slots of the flow cell holders.
- 10.2.2.12. Load a used flow cell into both flow cell holders and engage the vacuum.
- 10.2.2.13. Make sure the Vacuum engaged checkbox is selected and select Next.
- 10.2.2.14. Select Next to start the wash.
- 10.2.2.15. When the wash is complete, select Return to Start.

10.3. Perform a Water Wash.

- 10.3.1. From the welcome screen, select Wash→Water
- 10.3.2. Select Yes to wash paired end reagent positions, then select Next.
- 10.3.3. Load the instrument with laboratory-grade water
 - 10.3.3.1. Fill a 15 mL conical tube with 12 mL water for each paired end position in the HiSeq. Do not place lids on the tubes. 10 tubes will be needed for each side of the HiSeq.
 - 10.3.3.2. Fill a 15 mL conical tube with 12 mL water for each paired end position in the HiSeq. Do not place lids on the tubes. 10 tubes will be needed for each side of the HiSeq.
 - 10.3.3.3. Load the bottles and tubes onto the instrument in the assigned reagent rack positions. Lower the sippers into the tubes and close the door to the reagent compartment on the HiSeq.
- 10.3.4. Empty the waste bottle.
- 10.3.5. Make sure a used flow cell is loaded.
- 10.3.6. Select Next
- 10.3.7. Perform a fluidics check:

10.3.7.1. Select solution 2 from the drop-down list. Accept the default pump values.

10.3.7.2. Inspect the flow cell for bubbles passing through the lanes and leaks near the gaskets.

10.3.7.3. Separate the fluidics waste lines into 15 mL conical tubes.

10.3.7.4. Select Next to start the water wash. Approximate run time is 60 minutes.

10.3.7.5. When the wash is complete check void volume consistency across all tubes.

10.3.7.6. Return the tubing to the waste bottle.

10.4. Create a Sample Sheet

10.4.1. Using the Sample Sheet template csv file on the S drive as a guide, enter sample information for the flow cell.

10.4.2. The ETCTN sample IDs should be used in the Sample ID column on the Sample Sheet file.

10.4.3. Do not use dashes (-). Underscores (_) should be used instead.

10.4.4. Ensure that there are not hidden empty characters on the sheet.

10.4.5. Save the file in the desired location.

10.4.6. File name should be flow cell ID.

10.5. Enter flow cell information on the Flow Cell master file

10.5.1. Enter sample and flow cell information on the Flow Cell Master file on the S drive.

10.6. Enter run parameters

10.6.1. From the HCS Welcome Screen, select Sequence → New Run.

10.6.2. Follow the on-screen prompts to configure the run

10.6.2.1. Integration Screen

10.6.2.1.1. Select None to proceed without using BaseSpace.

10.6.2.1.2. Select Next

10.6.2.2. Storage Screen

10.6.2.2.1. Select the Save to an output folder checkbox and select Browse to navigate to the desired network location.

10.6.2.2.2. Select Zip to BCL files? YES

10.6.2.2.3. Select Bin Q Scores? YES

10.6.2.2.4. In Save Auxiliary Files options select Save All Thumbnails

10.6.2.2.5. Select Next

10.6.2.3. Flow Cell Setup Screen

10.6.2.3.1. Scan the flow cell barcode of the flow cell to be sequenced.

10.6.2.3.2. Confirm that the flow cell type is HiSeq Flow Cell v4. Flow cell type should be selected automatically based on the flow cell ID.

10.6.2.3.3. Enter an experiment name.

10.6.2.3.4. Enter a user name.

10.6.2.3.5. Select Next.

10.6.2.4. Advanced Screen

10.6.2.4.1. Do not check the box next to Confirm the First Base.

10.6.2.4.2. Check the box next to each lane for Align to PhiX.

10.6.2.4.3. Select Next.

10.6.2.5. Recipe Screen

10.6.2.5.1. For Index Type select Custom

10.6.2.5.2. Select Paired End for Flow Cell Format

10.6.2.5.3. Enter the number of cycles for Read 1, Read 2 and the Index Reads

10.6.2.5.3.1. Read 1: 126

10.6.2.5.3.2. Index 1: 9

10.6.2.5.3.3. Index 2: 0

10.6.2.5.3.4. Read 2: 126

10.6.3. *Note*; The number of cycles performed in a read is 1 more cycle than the number of cycles analyzed. For example, to perform 125 cycles for cycle 1, enter 126.

10.6.3.1. Confirm default chemistry settings. Fields will be auto-populated depending on the selected index option type:

10.6.3.1.1. SBS: HiSeq SBS Kit v4

10.6.3.1.2. Index: HiSeq v4 Single Index

10.6.3.1.3. PE turnaround: HiSeq PE Cluster kit v4

10.6.3.2. Do not check the box next to Use Existing Recipe. Allow the software to create the recipe using the run parameters.

10.6.3.3. Sample Sheet Screen

10.6.3.3.1. Select Browse to navigate to the location where the sample sheet was stored.

10.6.3.3.2. Select Next.

10.6.3.4. Reagents Screen

- 10.6.3.4.1. Scan the SBS reagent kit ID. The kit ID is used to determine reagent kit type and run mode compatibility.
- 10.6.3.4.2. Scan the Paired-End Cluster reagent kit ID.
- 10.6.3.4.3. Select 250 cycles as the SBS reagent kit for the run
- 10.6.3.4.4. Select Prime SBS Reagents to prime reagents before starting a run. Always prime reagents before loading a new flow cell.
- 10.6.3.4.5. Select Next
- 10.6.3.5. Review Screen
 - 10.6.3.5.1. Review the run parameters on the Review Screen to ensure no entry errors were made.
 - 10.6.3.5.2. Select Next to proceed or Back to change parameters.

10.7. Load and prime reagents

10.7.1. Load SBS reagents

- 10.7.1.1. Invert each reagent bottle to mix.

Note: After handling the bottle of CRM, discard gloves and replace them.

- 10.7.1.2. Place each reagent bottle onto the rack in the associated number position.

- 10.7.1.3. Place a reagent bottle containing PW1 into position 2. This bottle is not included with the SBS reagents.

Table 1 SBS Reagent Positions

Position	Reagent	Description
1	IRM	Incorporation Reagent Master Mix
2	PW1	25 ml of PW1 or laboratory-grade water
3	USM	Universal Scan Mix
4	SBS Buffer 1 (SB1)	High Salt Buffer
5	SBS Buffer 2 (SB2)	Incorporation Wash Buffer
6	SBS Buffer 2 (SB2)	Incorporation Wash Buffer
7	CRM	Cleavage Reagent Mix
8	SBS Buffer 3 (SB3)	Cleavage Buffer

- 10.7.1.4. Remove the cap from each reagent bottle and replace it with a funnel cap.

Note: Handle the bottle of CRM last, after you have loaded all other reagents, to prevent cross-contamination. Always replace gloves after handling CRM.

- 10.7.1.5. Open the reagent compartment door.

10.7.1.6. Raise the sippers, slide the reagent rack into the reagent compartment, and lower the sippers.

10.7.1.7. Select the PW1 (25 mL) loaded checkbox.

10.7.2. Load Indexing Reagents

10.7.2.1. Invert each reagent tube to mix.

10.7.2.2. Remove caps and place each reagent tube onto the rack in the associated number position.

Table 3 Paired-End Flow Cells

Position	Reagent	Description
10	FRM*	Fast Resynthesis Mix
15	FDR	Fast Denaturation Reagent (contains formamide)
17	HP12	Index Sequencing Primer i7

10.7.3. Load Paired End Reagents

10.7.3.1. Invert each reagent tube to mix

10.7.3.2. Remove caps and place each reagent tube onto the rack in the associated number position.

Table 4 Paired-End Flow Cell

Position	Reagent	Description
10	FRM*	Fast Resynthesis Mix
11	FLM2	Fast Linearization Mix 2
13	AMS	Fast Amplification Mix
14	FPM	Fast Amplification Premix
15	FDR*	Fast Denaturation Reagent (contains formamide)
16	HP11	Read 2 Sequencing Primer

* If you loaded indexing reagents for a single-index run, FRM is already loaded in position 10.

10.7.3.3. Place 15 mL conicals filled with PW1 in unused positions 12, 18 and 19.

10.7.3.4. Slide the reagent rack into the reagent compartment.

10.7.3.5. Lower the sippers into the paired-end reagent tubes.

10.7.3.6. Select Next.

10.7.4. Prime Reagents

Note: Always use a used flow cell to prime reagents.

10.7.4.1. Load a priming flow cell

10.7.4.1.1.1. *Note:* Do not remove or replace the flow cell gaskets during this step.

- 10.7.4.1.2. Rinse the used priming flow cell with laboratory-grade water. Dry with a lens wipe.
- 10.7.4.1.3. Using an alcohol wipe, wipe the surface of the flow cell holder. Do not allow alcohol to drip into the vacuum holes or around the manifolds.
- 10.7.4.1.4. Place the flow cell on the flow cell holder with inlet and outlet ports facing down. Make sure that the arrow on the left edge of the flow cell, which indicates flow direction, points towards the instrument.
- 10.7.4.1.5. Gently slide the flow cell towards the top and right guide pins until it stops.
- 10.7.4.1.6. Slowly move the flow cell level to position 1 to engage the vacuum. When the flow cell level is blinking green, the vacuum is engaged.
- 10.7.4.1.7. Wait for 5 seconds, and slowly move the flow cell level to position 2. When the flow cell level is solid green, the manifolds are in position and the flow cell is ready.
- 10.7.4.1.8. Make sure the Vacuum Engaged checkbox is selected and select Next.
- 10.7.4.2. Confirm proper flow
 - 10.7.4.2.1. Select solution 2 (laboratory-grade water, PW1) from the drop-down list.

Note: Use water to confirm proper flow on a used flow cell only. Never use water to confirm proper flow on a clustered flow cell.
 - 10.7.4.2.2. Confirm the default values:
 - 10.7.4.2.2.1. Volume: 125
 - 10.7.4.2.2.2. Aspirate rate: 250
 - 10.7.4.2.2.3. Dispense rate: 2000
 - 10.7.4.2.3. Select Pump
 - 10.7.4.2.4. Visually inspect the flow cell for bubbles passing through the lanes and leaks near the manifold. If you see excessive bubbles, check the gaskets for obstructions. If problems persist, remove the flow cell, repeat the cleaning steps and reload the flow cell.
- 10.7.4.3. Position tubing and start prime
 - 10.7.4.3.1. Remove the 8 lines of waste tubing for each flow cell from the waste container.
 - 10.7.4.3.2. Place each waste tubing into an empty 15 mL conical, 1 conical per line. Priming waste is collected and measured after the priming step.

10.7.4.3.3. Select Start Prime

10.7.4.3.4. When priming step is complete, measure the waste in each conical. Volume in each tube should be 1.75 mL.

10.7.4.3.5. Return the waste tubing to the waste container.

10.7.4.3.6. Select Next

10.8. Load a flow cell

10.8.1. Remove the used flow cell

10.8.1.1. Slowly move the flow cell lever to position 1 to disengage the manifolds.

10.8.1.2. Slowly move the flow cell lever to position 0 to disengage the vacuum seal and release the flow cell.

10.8.1.3. Remove the used flow cell.

10.8.2. Clean the flow cell holder using an isopropanol wipe.

10.8.3. Clean the flow cell

10.8.3.1. Remove the clustered flow cell from the container using forceps

10.8.3.2. Rinse the flow cell with laboratory-grade water and dry with lens paper.

10.8.3.3. Protect the flow cell from dust until you are ready to load it onto the instrument.

10.8.4. Load the sequencing flow cell

Note: Do not replace the manifold gaskets. Replace the manifold gaskets before the maintenance wash.

10.8.4.1. Place the flow cell on the flow cell holder with the inlet and outlet ports facing down and the barcode on the right. Make sure that the arrow on the left edge of the flow cell points towards the instrument.

10.8.4.2. Gently slide the flow cell towards the top and right guide pins until it stops.

10.8.4.3. Slowly move the flow cell level to position 1 to engage the vacuum. When the flow cell level is blinking green, the vacuum is engaged.

10.8.4.4. Wait for 5 seconds, and slowly move the flow cell level to position 2. When the flow cell level is solid green, the manifolds are in position and the flow cell is ready.

10.8.4.5. Make sure the Vacuum Engaged checkbox is selected and select Next.

10.8.5. Confirm proper flow

10.8.5.1. Select solution 5 from the drop-down list

10.8.5.2. Enter the following default values:

- 10.8.5.2.1. Volume: 250
- 10.8.5.2.2. Aspirate Rate: 250
- 10.8.5.2.3. Dispense Rate: 2000
- 10.8.5.3. Select Pump
- 10.8.5.4. Visually inspect the flow cell for bubbles passing through the lanes or leaks near the manifolds. If bubbles are present, check the manifold gaskets for obstructions and repeat the pump process using solution 6 to avoid depleting solution 5. Reduce the aspirate rate to 100, and pump another 250 uL to the flow cell.
- 10.8.5.5. Select Next. Ensure that the flow cell lever is green and close the flow cell compartment door.
- 10.8.5.6. Confirm that the checkboxes Vacuum Engaged and Door Closed are selected, and select Next
- 10.8.5.7. Select Start to start the sequencing run.

APPENDIX 31: RNASeq Analysis Pipeline SOP at MoCha Laboratory

1.0 PURPOSE/SCOPE

This Standard Operating Procedure (SOP) describes the pipeline and data analysis specifications for HiSeq RNASeq Pipeline for ETCTN project used/performed by the Molecular Characterization and Clinical Assay Development Laboratory (MoCha) at the Frederick National Laboratory for Cancer Research. **This SOP is for research purposes only and no clinical samples will be processed using this SOP. Any deviation from this SOP will be noted but will not be formally documented.**

2.0 PROCEDURE

2.1 FASTQ files are generated with bcl2fastq (version: 2.17.1.14, Illumina). Adaptors are trimmed within this process using the default cutoff of the adapter-stringency option.

2.2 The fastq files are mapped to human transcriptome based on exon models from hg19 using Bowtie2 (version 2.2.6) [1]. The resulting SAM files are converted to BAM format using samtools [2] and the coordinations in BAM are converted to the genomic (hg19) coordinations using RSEM (version 1.2.31). Gene and transcript quantifications are also done using RSEM.

- `rsem-calculate-expression --output-genome-bam --bowtie2 --forward-prob 0 --paired-end Sample_R1.fastq.gz Sample_R2.fastq.gz hg19_UCSC_RefGene_rsem`

3.0 REFERENCES

- 1. Langmead B, Salzberg S. Fast gapped-read alignment with Bowtie 2. *Nature Methods*. 2012, 9:357-359.
- 2. Li H., Handsaker B., Wysoker A., Fennell T., Ruan J., Homer N., Marth G., Abecasis G., Durbin R. and 1000 Genome Project Data Processing Subgroup (2009) The Sequence alignment/map (SAM) format and SAMtools. *Bioinformatics*, 25, 2078-9.
- 3. Li, B. and Dewey, C. N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics*. 12:323.

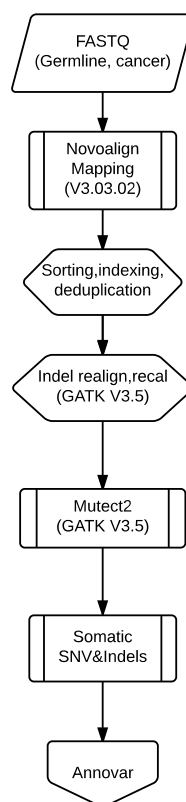
APPENDIX 5I: WES Data Analysis Pipeline

This Standard Operating Procedure (SOP) describes the pipeline and data analysis specifications for whole exome sequencing data from samples available as tumor-normal pairs. This procedure is used/performed by the Molecular Characterization and Clinical Assay Development Laboratory (MoCha) at the Frederick National Laboratory for Cancer Research for the specified tumor samples. **This SOP is for research purposes only and no clinical samples will be processed using this SOP. Any deviation from this SOP will be noted but will not be formally documented.**

BACKGROUND

Mutect is a somatic variant developed by Broad Institute (1) and is based on the Bayesian approach using a hidden Markov Model. Mutect2 is not only a somatic SNV caller but indel caller which combines Mutect with the assembly-based machinery of HaplotypeCaller from GATK (2).

Somatic variant calling pipeline for Whole Exome from Tumor samples from tumor-normal pairs



PROCEDURE

1. Compressed FASTQ files (fastq.gz) are generated with bcl2fastq (version: 2.17.1.14, Illumina). Adaptors are trimmed within this process using the default cutoff of the adapter-stringency option.
2. The fastq files are mapped to human genome reference hg19 using the Novoalign (version 3.03.02) from Novocraft. The resulting SAM files are converted to BAM format using samtools (3). The resulting BAM files are sorted, indexed and duplicates removed all by novosort (Novocraft).
 - 2.1. Align fastq.gz files
 - 2.1.1.1. `novoalign -f Sample_R1.fastq.gz Sample_R2.fastq.gz -d hg19.nix -a -H 20 -l 60 -t 99 -r Exhaustive 10 -k 2>Sample_stats.txt | samtools view -bS ->Sample.bam`
 - 2.1.1.2. `novosort -m 14G -c 6 --removeduplicates --keep-tags Sample.bam -i -o Sample_sorted_dedup.bam`
3. Qualimap v2.1.3 is used to assess mapping quality, mean coverage of the samples [2].
 - 3.1. Qualimap QC metrics generation:
 - 3.1.1.1. `qualimap bamqc -bam Sample_sorted_dedup.bam --java-mem-size=3G -gd HUMAN -gff SS_V5.bed -nt 2 -outdir Sample -outformat PDF`
4. Somatic mutations will be called using GATK 3.5 (Mutect2)
5. `java -Xmx30g -jar GATK.jar -T RealignerTargetCreator -R hg19.genome.fa -I Sample_sorted_dedup.bam -L SS_V5.bed -known Mills1kg_gold_standard.indels.b37.vcf -known 1kg_phase1_indels.vcf -nt 8 -o Sample_realignment_targets.list`
 - 5.1. `java -Xmx30g -jar GATK.jar -T IndelRealigner -R hg19.genome.fa -dcov 50000 -I Sample_sorted_dedup.bam -targetIntervals Sample_realignment_targets.list -known 1kg_phase1_indels.vcf -known Mills1kg_gold_standard.indels.b37.vcf -o Sample_realigned.bam`
 - 5.2. `java -Xmx30g -jar GATK.jar -T BaseRecalibrator -R hg19.genome.fa -I Sample_realigned.bam -L SS_V5.bed -knownSites dbSNP_138.b37.vcf -knownSites 1kg_phase1_indels.vcf -knownSites Mills1kg_gold_standard.indels.b37.vcf -o Sample_recal_data.table`
 - 5.3. `java -Xmx30g -jar GATK.jar -T PrintReads -R hg19.genome.fa -L SS_V5.bed -I Sample_realigned.bam -BQSR Sample_recal_data.table -o Sample_recal.bam`
 - 5.4. `java -Xmx30g -jar GATK.jar -T MuTect2 -R hg19.genome.fa -I:tumor tumor_recal.bam -I:norm1 norm_recal.bam -L SS_V5.bed -dbSNP dbSNP_138.b37.vcf -cosmic COSMIC.vcf -o Sample_somatic.vcf`
6. Annotation of the variants using ANNOVAR (4)

REFERENCES

1. Cibulskis K, Lawrence MS, Carter SL, Sivachenko A, Jaffe D, Sougnez C, et al. Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. *Nat Biotechnol*. Marts 2013;31(1546-1696):213-9.
2. Aaron McKenna et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Research* 2010, 20:1297-1303.

3. Li H., Handsaker B., Wysoker A., Fennell T., Ruan J., Homer N., Marth G., Abecasis G., Durbin R. and 1000 Genome Project Data Processing Subgroup (2009) The Sequence alignment/map (SAM) format and SAMtools. *Bioinformatics*, 25, 2078-9. [PMID: [19505943](#)]
4. Wang K, Li M, Hakonarson H. ANNOVAR: Functional annotation of genetic variants from next-generation sequencing data *Nucleic Acids Research*, 38:e164, 2010.

APPENDIX 5J: RNASeq Transcriptome Data Analysis Pipeline and Specifications

PURPOSE/SCOPE

This Standard Operating Procedure (SOP) describes the pipeline and data analysis specifications for HiSeq RNASeq Pipeline for ETCTN samples used/performed by the Molecular Characterization and Clinical Assay Development Laboratory (MoCha) at the Frederick National Laboratory for Cancer Research. **This SOP is for research purposes only, and no clinical samples will be processed using this SOP. Any deviation from this SOP will be noted but will not be formally documented.**

PROCEDURE

- FASTQ files are generated with bcl2fastq (version: 2.17.1.14, Illumina). Adaptors are trimmed within this process using the default cutoff of the adapter-stringency option.
- The fastq files are mapped to human transcriptome based on exon models from hg19 using Bowtie2 (version 2.2.6) [1]. The resulting SAM files are converted to BAM format using samtools [2] and the coordinations in BAM are converted to the genomic (hg19) coordinations using RSEM (version 1.2.31). Gene and transcript quantifications are also done using RSEM.
- `rsem-calculate-expression --output-genome-bam --bowtie2 --forward-prob 0 --paired-end Sample_R1.fastq.gz Sample_R2.fastq.gz hg19_UCSC_RefGene_rsem`

REFERENCES

1. Langmead B, Salzberg S. Fast gapped-read alignment with Bowtie 2. *Nature Methods*. 2012, 9:357-359.
2. Li H., Handsaker B., Wysoker A., Fennell T., Ruan J., Homer N., Marth G., Abecasis G., Durbin R. and 1000 Genome Project Data Processing Subgroup (2009) The Sequence alignment/map (SAM) format and SAMtools. *Bioinformatics*, 25, 2078-9.
3. Li, B. and Dewey, C. N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics*. 12:323.