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1.0 PURPOSE/SCOPE

This Standing 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 PDX 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
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
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
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

Description	Product No.	Vendor
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.

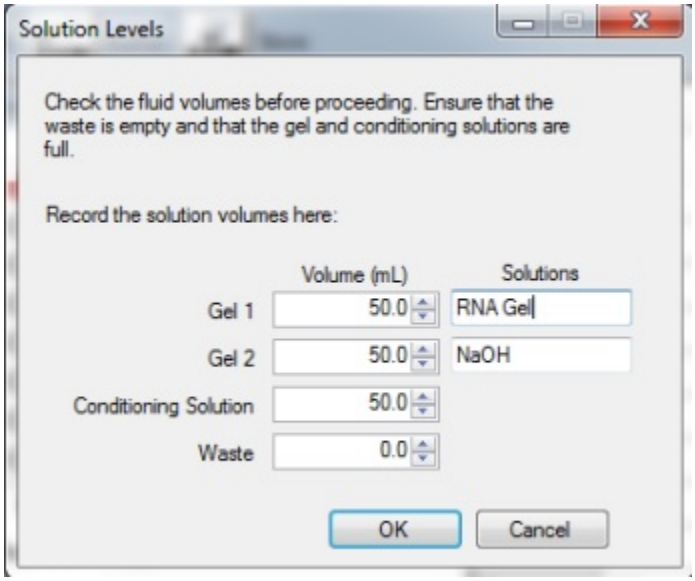
8.1.2 Mix appropriate volumes of Intercalating Dye and RNA Separation Gel necessary for one day of operation. For small volumes use the supplied 50 mL conical centrifuge tube. For larger volumes, use a 250 mL conical centrifuge tube and remove the collar of the tube holder in the instrument reagent compartment.

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

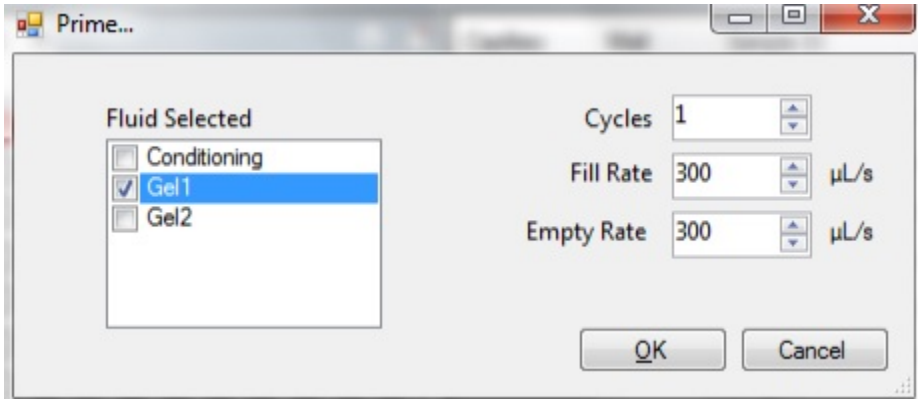
Number of Samples to be Analyzed	Volume of Intercalating Dye	Volume of RNA Separation Gel
95	4.0 uL	40 mL
190	8.0 uL	80 mL
285	12.0 uL	120 mL
380	16.0 uL	160 mL
475	20.0 uL	200 mL

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 pause icon ( ). To Clear the run queue of all loaded runs, press the clear ( ) 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

8.1.25.2 Click Set Global Configuration

8.1.25.3 Click on the Smear Analysis Tab

- Enter the Start size as 200 bp
- Enter the End size as 6000 bp
- Click Apply

8.1.25.4 Click File

- Click Export Data

8.1.25.5 Click Smear Analysis radio button

- Click Export
- 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:**

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

8.2.1.2 Pre-heat to 94°C & hold

8.2.1.3 94°C for 8 minutes

8.2.1.4 4°C hold

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

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

8.2.2.2 Pre-heat to 25°C & hold

8.2.2.3 25°C for 10 minutes

8.2.2.4 42°C for 15 minutes

8.2.2.5 70°C for 15 minutes

8.2.2.6 4°C hold

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

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

8.2.3.2 Pre-heat to 16°C & hold

8.2.3.3 16°C for 60 minutes

8.2.3.4 16°C hold

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

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

8.2.4.2 Hold at 98°C

8.2.4.3 98°C for 30 seconds

8.2.4.4 15 cycles of:

- 98°C for 10 seconds
- 60°C for 30 seconds
- 72°C for 30 seconds

8.2.4.5 72°C for 5 minutes

8.2.4.6 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 PDXRNAAccesstemplate20160815 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 PDXRNAAccesstemplate20160815 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 PostPCRSPRI' of the "Illumina RNA Access Workbook", then save the file.

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

8.4.6 Open "1 RNA Access cDNA Library Preparation" method in the Illumina RNA Access folder.

8.4.7 cDNA Library Preparation

8.4.7.1 Prepare indexing setup

8.4.7.1.1 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

8.4.7.1.2 Click on the 'Indexing' sheet within the "Illumina RNA Access Workbook"

- 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.

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

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

8.4.7.2 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.

8.4.7.2.1 Mix well using a P1000 pipette. **

1st Strand Master Mix	Per well	Total (uL)
1st Strand Master Mix	9	87.1
Superscript II	1	9.7
Total	**10**	**96.8**
2nd Strand Master Mix	Per well	Total (uL)
2nd Strand Master Mix	20	190.1
Resuspension Buffer (RSB)	1	47.5
Total	**25**	**237.6**
A-tailing Mix	Per well	Total (uL)
A-tailing Mix	12.5	132
Resuspension Buffer (RSB)	2.5	26.4
Total	**15**	**158.4**
Ligation Mix	Per well	Total (uL)
Ligase Mix	2.5	57.2
Resuspension Buffer (RSB)	2.5	57.2
Total	**5**	**114.4**
PCR Mix	Per well	Total (uL)
PCR Master Mix (PMM)	25	238

PCR Primer Mix (PPC)	5	47.6
Total	**30**	**285.6**

8.4.7.3 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.

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

8.4.7.5 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 + RSB)	Stop Ligase 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				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

8.4.7.6 Broadcast reagents to match the number of sample columns for Ampure beads, PEG buffer, and Resuspension Buffer.

8.4.7.7 Begin the 'Elution 2 -Frag – Prime' program on the thermocycler.

8.4.8 Prepare the SciClone automation deck with boxes of tips and the reagent plates previously prepared.

8.4.8.1 Press the play button and follow prompts for placement of consumables and reagent plates.

8.4.8.2 Once completed, place RNA samples on the deck.

8.4.8.3 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 uL 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 PostPCRSPRI' 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 μL 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 µl of library sample with 1 µl 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

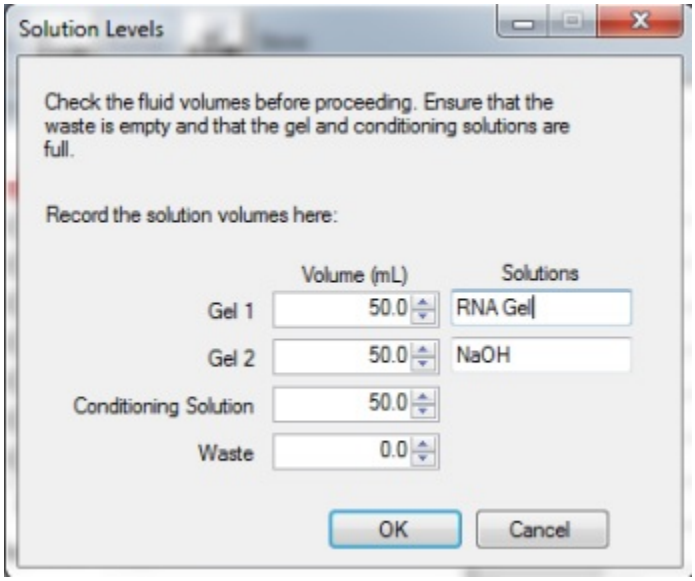
8.14.4 Mix appropriate volumes of Intercalating Dye and Separation Gel necessary for one day of operation. Use the supplied 50 mL conical centrifuge tube to allow a small minimum working volume. For larger volumes, use a 250 mL conical centrifuge tube and remove the collar of the tube holder in the instrument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

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

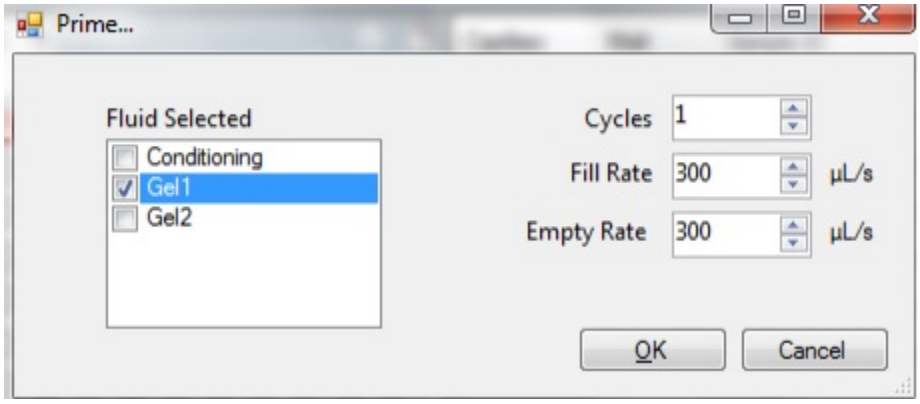
# of samples to be analyzed	Volume of Intercalating Dye	Volume of RNA Separation Gel
95	4.0 uL	40 mL
190	8.0 uL	80 mL
285	12.0 uL	120 mL
380	16.0 uL	160 mL
475	20.0 uL	200 mL

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 µL/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 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.

8.14.16 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.

8.14.17 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 pause icon (). To Clear the run queue of all loaded runs, press the clear () button.

8.14.22.1 Using ProSize 2.0, perform a smear analysis to quantify the amount of amplified library.

- Click Option
- Click Set Global Configuration

8.14.22.2 Click on the Smear Analysis Tab

- Enter the Start size as 150 bp
- Enter the End size as 600 bp
- Click Apply

8.14.22.3 Click File

- Click Export Data
- Click Smear Analysis radio button
- Click Export
- Open the exported smear analysis csv file. The column labeled ng/uL contains the library concentration data.

8.14.22.4 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.

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

8.16.4 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

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

8.16.6 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.

8.16.7 Open Maestro software on the SciClone.

8.16.8 Open "Illumina RNA Access Workbook" worksheet in Excel (shortcut is available on the desktop).

8.16.9 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.

8.16.10 Open "4 RNA Access Hyb Setup" method in the Illumina RNA Access folder.

8.16.11 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.

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

8.16.12.1 Automation will then proceed with the following:

- Addition of 55 uL of Enrichment Hyb Solution to 45 uL of sample
- Pipette mixing

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

8.16.14 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 µl 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

8.17.6.1 Automation will proceed with the following:

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

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

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

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

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

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

8.17.6.8 Add 75 uL of secondary Hyb Solution to each sample.

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

8.17.8 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

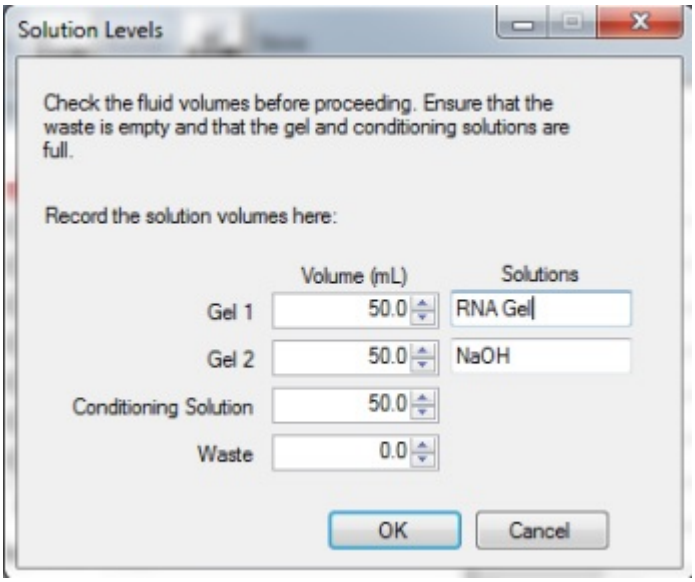
8.20.4 Mix appropriate volumes of Intercalating Dye and Separation Gel necessary for one day of operation. Use the supplied 50 mL conical centrifuge tube to allow a small minimum working volume. For larger volumes, use a 250 mL conical centrifuge tube and remove the collar of the tube holder in the instrument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

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

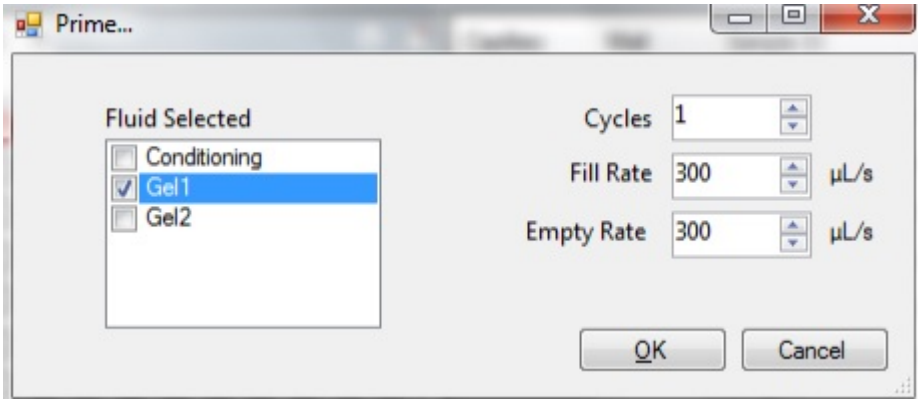
# of samples to be analyzed	Volume of Intercalating Dye	Volume of RNA Separation Gel
95	4.0 uL	40 mL
190	8.0 uL	80 mL
285	12.0 uL	120 mL
380	16.0 uL	160 mL
475	20.0 uL	200 mL

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 pause icon (). To Clear the run queue of all loaded runs, press the clear () 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

- Click Set Global Configuration

8.20.23.2 Click on the Smear Analysis Tab

- Enter the Start size as 150 bp
- Enter the End size as 1000 bp
- Click Apply

8.20.23.3 Click File

- Click Export Data
- Click Smear Analysis radio button
- Click Export
- 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.

8.21 Quantify Library using ddPCR

8.21.1 Refer to SOP titled PDX ddPCR Library Quantification Protocol, SOP document number MCCRD-SOP0004.

8.22 Dilute, Denature and Cluster Pools for Sequencing

8.22.1 Refer to SOP titled PDX Dilute Denature and Cluster Protocol, SOP document number MCCRD-SOP0005.

8.23 Sequencing Pools

8.23.1 Refer to SOP titled Sequencing PDX Library Pools, SOP document number MCCRD-SOP0009.

9.0 REVISION HISTORY

Document Number	Version	Description of Revision	Effective
MCCRD-SOP0006	1.0	Original Release	12/01/2014