Non-Controlled Version **Note: Controlled Versions of this document are subject to change without notice*

96-well Plate-based Strand-specific cDNA Synthesis using Maxima H Minus on Hamilton NIMBUS

I. Purpose

To synthesize cDNA for strand specific RNA-Seq (WTSS) Illumina library construction using Maxima H Minus Reverse Transcriptase from Thermo-Fisher in a 96-well format.

II. Scope

All procedures are applicable to the BCGSC Library Core group and the Library Technical Development group.

III. Policy

This procedure will be controlled under the policies of the Genome Sciences Centre, as outlined in the Genome Sciences Centre High Throughput Production Quality Manual (QM.0001). Do not copy or alter this document. To obtain a copy see a QA associate.

IV. Responsibility

It is the responsibility of all personnel performing this procedure to follow the current protocol. It is the responsibility of the Group Leader to ensure personnel are trained in all aspects of this protocol. It is the responsibility of Quality Assurance Management to audit this procedure for compliance and maintain control of this procedure.

V. References

Reference Title	Reference Number
N/A	N/A

VI. Related Documents

Document Title	Document Number
Operation and Maintenance of the Agilent 2100 Bioanalyzer for DNA samples	LIBPR.0017

VII. Safety

All Laboratory Safety procedures will be complied with during this procedure. The required personal protective equipment includes a laboratory coat and gloves. See the material safety data sheets (MSDS) for additional information.

VIII. Materials and Equipment

Name	Supplier	Cat. No.
Fisherbrand Textured Nitrile gloves	Fisher	
RNAse Zap	Ambion	9780
Ice bucket – Green	Fisher	11-676-36
wet ice	In house	N/A
RNAse free 1.5 ml eppendorf tube	Ambion	12400
Axygen plate PCR 96 fullskt BRC 50/CS	Fisher	14222327
Sterile Filtered Conductive 50µL Tips in Frames	Hamilton	235979
Sterile Filtered Conductive 300µL Tips in Frames	Hamilton	235938
Gilson P20 pipetman	Mandel	GF23600
Gilson P200 pipetman	Mandel	GF-23601
Gilson P1000 pipetman	Mandel	GF-23602
Diamond Filter tips DFL10	Mandel Scientific	GF-F171203
Diamond Filter tips DF30	Mandel Scientific	GF-F171303
Diamond Filter tips DF200	Mandel Scientific	GF-F171503
Diamond Filter tips DF1000	Mandel Scientific	GF-F171703
P2-20 Rainin Lite Manual 12-channel	Rainin	L12-20
P20-200 Rainin Lite Manual 12-channel	Rainin	L12-200
P200 Barrier Rainin tips	Rainin	RT-L200F
P20 Barrier Rainin tips	Rainin	RT-L10F
VX-100 Vortex Mixer	Rose Scientific	S-0100
Large Kimwipes	Fisher	06-666-117
Black ink permanent marker pen	VWR	52877-310
Bench Coat	Fisher	12-007-186
Small Autoclave waste bags 10”X15”	Fisher	01-826-4
70% Ethanol	In house	
Mini-centrifuge	Eppendorf	5417R
Peltier Thermal Cycler	MJ Research	PTC-225
Plastic seal (3M)	Qiagen	19570
Aluminium Foil Seal	VWR	60941-126
Ampure XP beads	Beckman	A63882
Maxima H Minus First Strand cDNA Synthesis Kit	Thermo-Fisher	K165B001
NEBNext Ultra Directional Second Strand Synthesis Module	NEB	E7550L

Actinomycin D 10 mg/mL	In house	
Actinomycin D 5mg	MJS Biolynx	ENZGR3000005
PCR Clean DX (ALINE beads)	ALINE Biosciences	C-1003-450
G127187 DNA Engine Tetrad 2 Peltier Thermal	MJ Research	10177BD
NIMBUS Liquid Handling Workstation	Hamilton	

IX. GENERAL GUIDELINES

1. General guidelines and input material

1.1 The input material for this procedure is polyA+ RNA or Ribo-depleted RNA which has been DNAse I treated. The input volume for this protocol is 34.3µL/well.

1.2 The same positive control (such as Universal Human Reference RNA) and negative controls used in the polyA+ isolation protocol or Ribo-depleted RNA should be carried into this protocol. An additional UHR control, 12ng, should be added at the cDNA stage for samples in the RBD_1.1 pipeline

1.3 Ensure proper personal protective equipment is used when handling sample plates, reagents and equipment. Treat everything and adhere to strict RNA handling techniques.

1.4 Wipe down the assigned workstation, pipetman, tip boxes and small equipment with RNAse Zap (Ambion) followed by DEPC-treated water. Ensure you have a clean working surface before you start.

1.5 Double check the QA release and/or expiry date of each reagent and enzyme.

1.6 The 10µg/µL stock of Actinomycin D should be thawed just before 1st strand synthesis brew set up. All thawed but unused Actinomycin D should be discarded to the appropriate waste container (Sybr-green waste). See Appendix C for making up working stock of Actinomycin D.

1.7 Reactions in plates should never be vortexed and plate covers are never to be re-used. Single/Multi-Channel pipettors should be used for mixing reactions in a circular mixing motion by aspirating volume from the bottom of the well and dispensing higher up at the liquid level.

X. PROCEDURE

Note: ALINE beads can be used as a direct replacement of Ampure XP beads in steps that specify the use of Ampure XP beads

Note: All version numbers for Nimbus protocols have been removed on this document. They are present when running the protocol. If you are unsure which version to use, consult your supervisor.

1. First strand cDNA: Upstream Preparation

1.1. Retrieve and thaw Thermo-Fisher reagents at room temperature: 5X First Strand Buffer, 10mM dNTP mix, Random primers (200ng/µL).Once thawed, pulse-vortex, quick spin and keep reagents on ice. The buffer contains DTT, make sure it is vortexed well and that all precipitate goes to solution. Enzymes should be left in the freezer until ready to use.

1.2. Retrieve and thaw 10µg/µL Actinomycin D solution at room temperature. Once thawed, vortex rigorously and quick spin.

1.3. Retrieve the plate containing polyA+ or Ribo-depleted RNA fraction. If stored in -80°C, thaw it on ice followed by a quick spin at 4°C, 2000g for 1min. Place it on ice.

1.4. Generate the "**SS_cDNA_1st _strand_Maxima H Minus**" brew using LIMS.

LIMS: Prepare Standard Solutions > SS_cDNA_1st_strand_Maxima_H_Minus > follow the prompts > Save Standard Solution

1.5. Retrieve 1D Large label from 5th floor printer outside the RNA room and brew mix check-list label from RNA Room printer.

1.6. If LIMS is down, enter the number of samples to be processed and print the ss-cDNA Worksheet located in:

R:\Lib core\Work Sheets and Calculators\Strand Specific\96-well Plate-based Strand-specific cDNA synthesis with Maxima H Minus

2. Heat Denaturation of mRNA/ Ribo-depleted RNA

2.1. Heat denature the poly(A)+ or Ribo-depleted RNA at 70°C for 5 min using tetrad thermo-cycler.

2.2. After 5 min of denaturation, chill the plate on ice for at least 1min. Spin down the plate at 4°C, 2000g for 1min and place it back on ice.

3. First strand cDNA: Reaction Brew

3.1 Prepare the reaction brew in a non-stick tube and check off reagents as they are added on the worksheet. Mix the brew by repeated pulse-vortexing followed by a quick spin. Label the First Strand brew as FS brew and place on ice until ready to use.

3.2 Make sure to mix each reagent well and quick spin before adding to the brew and add enzyme last.

3.3 The reaction set up for 1 reaction is shown below.

Solution	µL **(per 1rxn)**
mRNA/Ribo-depleted RNA	34.3
5X First Strand Buffer	10
Actinomycin D (10µg/µL)	0.2
10mM dNTP mix	2.5
Random primers (200ng/µL)	2
Maxima H Minus enzyme mix	1
Total Reaction volume	**50**

First Strand Brew Mix (15.7µL)

3.4 Add 15.7µL of the FS brew into each well of a new plate. Check that the appropriate volume is added to all wells.

3.5 Log into Nimbus Program as follows to add 1st strand brew to heat denatured poly(A)+ or ribo-depleted RNA.

Open file: **Production** > *toggle to workflow* > **Transcriptome cDNA > Transcriptome cDNA Scheduler.wfl > 1st strand cDNA Synthesis**

3.6 After Nimbus program completion, seal the plates and quick spin at 4ºC for 1 minute. Inspect the reaction plates for any variations in volume.

3.7 In the tetrad thermo-cycler incubate the 1st strand cDNA synthesis reaction at 25°C for 10 min followed by 50°C for 1h. The program is located in

TETRAD: CDNA >CDNA1MAX

Important: during incubation, continue onto section 4

4. Post-First Strand cDNA: Preparation

4.1 During the 1 hour 1st strand cDNA synthesis reaction, retrieve the following:

4.1.1. Ampure XP beads and 70% Ethanol (prepared using DEPC-treated water) should be at room temperature for at least 30 minutes before usage.

4.1.2. NEBNext Second Strand buffer mix. Thaw them at RT and once thawed immediately transfer to ice. Leave enzymes in the freezer until ready to use.

4.2. After the 1st strand reaction is over, remove the plate from the thermo-cycler, spin it down at 2000g, for 1min.

5. Post–First Strand cDNA: Aline/Ampure XP bead clean up of RNA/DNA hybrid.

5.1. The input volume for this step is 50µL per well. Manually dispense 110µL of Aline/Ampure XP beads to the required wells.

5.2. To clean up the 1st strand cDNA synthesis reaction using Aline/Ampure XP beads on Nimbus as follows:

Open file: **Production** > *toggle to workflow* > **Transcriptome cDNA > Transcriptome cDNA Scheduler.wfl > Bead Clean 1st Strand cDNA Product**

5.2.1. During the bead clean incubation steps, it is recommended to prepare the Second Strand brew (section 6)

5.2.2. After the Magnet Elution time, transfer the RNA/DNA hybrid into a new well. *This is a safe stopping point. If needed, the plate can be stored at* ***-80°C.***

6. Second Strand cDNA: Reaction Brew

6.1. Generate the **"SS_cDNA_2nd_Strand_ultra"** brew using LIMS.

LIMS: Prepare Standard Solutions > SS_cDNA_2nd_Strand_ultra > follow the prompts > Save Standard Solution

6.2. Retrieve 1D Large label from 5th floor printer outside the RNA room and brew mix check-list label from RNA Room printer.

6.3. If LIMS is down, enter the number of samples to be processed and print the ss-cDNA Worksheet located in:

R:\Lib core\Work Sheets and Calculators\Strand Specific\96-well Plate-based Strand-specific cDNA synthesis with Maxima H Minus

6.4. Prepare Strand specific 2nd strand cDNA synthesis brew following the printed LIMS calculator or following the printed Excel spreadsheet. Add enzymes last.

6.5. Mix the brew by repeated pulse-vortexing followed by a quick spin. Label the brew as SS Brew and place on ice until ready to use.

6.6. The reaction setup for 1 reaction is as follows:

Solution	**1 rxn (µL)**	
First Strand sscDNA	35	
DEPC-dH_2O	7.5	Second Strand Brew Mix (15µL)
NEBNext 2nd strand buffer mix	5	
NEBNext 2nd strand Enzyme mix	2.5	
Reaction volume	**50**	

6.7. Add 15µL of the SS brew into each well of a new plate. Check that the appropriate volume is added to all wells.

6.8. Log into Nimbus Program as follows to add 2nd strand brew to first strand cDNA.

Open file: **Production** > *toggle to workflow* > **Transcriptome cDNA > Transcriptome cDNA Scheduler.wfl >2nd Strand cDNA synthesis**

6.9. After Nimbus program completion, seal the plates and quick spin at 4ºC for 1 minute. Inspect the reaction plates for any variations in volume.

6.10. In the tetrad thermocycler incubate the plate at 16°C for 1h. The program is located in

TETRAD: CDNA > cDNA2NEB

6.11. After thermo-cycler program is finished, remove the plate and spin it at 4°C, 2000g for 1min.

Note: ***This is a safe stopping point. If needed, the plate can be stored at -20°C, or on the tetrad overnight if there is insufficient time to take the plate out for cold storage.***

7. Post–Second Strand cDNA: Aline/Ampure XP bead clean-up of DS cDNA

7.1. The input volume for this step is 50µL per well. Manually dispense 110 µL of Aline/Ampure XP beads to the required wells.

7.2. Clean up the double strand cDNA using Aline/Ampure XP beads on Nimbus as follows:

Open file: **Production** > *toggle to workflow*> **Transcriptome cDNA > Transcriptome cDNA Scheduler.wfl > Bead Clean2nd Strand cDNA Synthesis Product**

8. HS Agilent QC of double stranded ss-cDNA for RBD_1.1 samples

8.1. For quality and quantity control check of the cDNA, use 1µL of each sample or representation of samples including the UHR controls and the negative control for High Sensitivity DNA Agilent assay according to protocol: LIBPR.0017. The non-ribodepleted UHR control may be the only sample that has visible cDNA yields. All other samples should have equal or lower yields of cDNA. The ribodepleted UHR should give a cDNA yield of <100% of the cDNA level from non-ribodepleted UHR.

8.2. Check with your supervisor if the yield is >100%. The calculations are based on agilent smear analysis (mass) within 50b-9kb range. The non-ribodepleted control will also serve as a reliable positive control for cDNA synthesis and to ensure that shearing worked later in the library construction protocol. The ribodepleted UHR control and the samples are barely detectable before iPCR.

Appendix A: LIMS

1. Pass barcode to supervisor to add control for cDNA synthesis (for RBD_1.1 samples only)
2. A-Strand Specific cDNA Synthesis: RNA_strategy is 'strand specific' and the pipeline will be SSTRA_3.1, RBD_2.0, or RBD_1.1.
3. Bioanalyzer Run – QC Category: cDNA QC for RBD_1.1 samples.
4. Spike_In_cDNA: supervisor to add appropriate spike-ins to the cDNA.
5. Pass barcode to supervisor to add controls for library construction.

Appendix B - Actinomycin D

1. Actinomycin D is toxic and gloves must be worn when handling it.

2. Actinomycin D powder is hygroscopic and sensitive to light. When stored in original vial, manufacture sealed and protected from light and moisture, at 4°C, it remains unchanged for the amount of time specified by the manufacture (it is shown on the label). When receiving Actinomycin D powder in LIMS enter that date as the expiration date of the powder.

3. Dilute solutions of Actinomycin D are very sensitive to light. This product tends to adsorb to plastic and glass on standing in solution. It is important to take the aliquots out of the freezer only shortly before you are ready to use it, and after thawing using ActD solution quickly. Once taken out from the freezer, an aliquot should either be used up or discarded – it should never be re-frozen for later use.

4. The shelf life of re-suspended Actinomycin D when kept at 4°C and above is only few hours. It is therefore important to perform the re-suspension procedure in an efficient and quick manner.

5. Protected from light, frozen aliquots of 10µg/µL (or 10mg/mL which is 8mM) are expected to be stable up to 3 months at -20°C. After 3 months, all unused tubes of re-suspended, frozen Actinomycin D should be discarded and a new re-suspension batch should be made.

6. Generate the **Actinomycin 10µg/µL** storage stock mix of Actinomycin D in LIMS. Set the expiry date for 3 months.

   6.1. Log into LIMS. Go to "Solutions" page. Under "Mix Standard Solutions" select **Actinomycin 10µg/µL** and 1 x 1 for samples (see image below). Click "Mix Standard Solution" button.

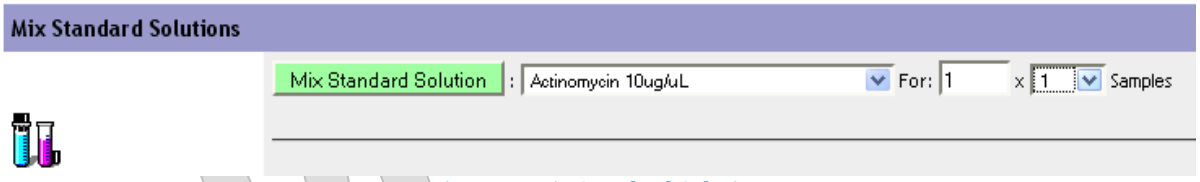

Figure 1: Mix Standard Solution

   6.2. In the Parameters section enter the mg of Actinomycin D in the vial (see image below). Click "Re-calculate Standard Solution".

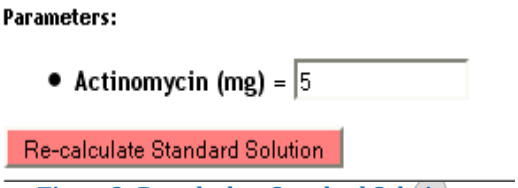

Figure 2: Re-calculate Standard Solution

6.3. In the Other Parameters section enter "Reagent" for Type, expiration date for 3 months ahead, select "Lib Construction" group, 2D solution labels. Click on the "Save Standard Mixture" button (see image below):

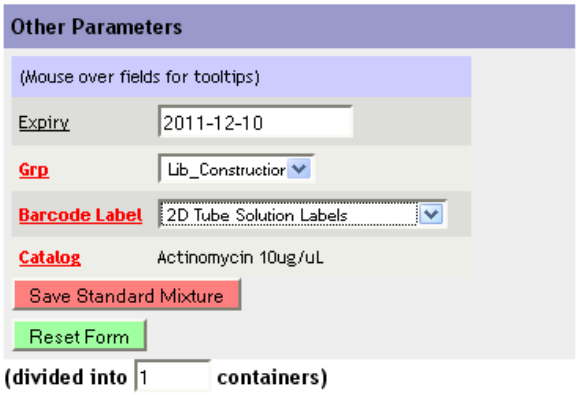

Figure 3: Save Standard Mixture

6.4. Retrieve both the solution barcode and the calculator from the corresponding printers.

6.5. Using the newly generated barcode perform an aliquot step in LIMS dividing the total volume into 15µL per tube. Select 2D solution barcodes.

7. Add the amount of room temperature DMSO listed on the large LIMS calculator to the amber vial containing 5mg of Actinomycin D powder. Close the vial.

8. Mix well few times by gentle pulse-vortexing. Make sure that the powder is fully re-suspended.

9. Open the vial and mix by pipetting using P200.

10. Using 1.5mL amber tubes immediately aliquot Actinomycin D solution 15µL per tube. Place the tubes on ice.

11. Attach 2D solution barcodes generated in step 6.5 on the tubes.

12. Cover each tube with parafilm creating tight seal.

13. As soon as possible place tubes in 9x9 box, protected from light, in the -20°C freezer.