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SLX Transcriptome Library Construction Day 2

I. Purpose

To synthesize double-stranded cDNA from the polyA+ fraction of the DnaseI treated RNA sample.

II. Scope

All procedures are applicable to the BCGSC FG library construction core group.

III. Policy

All production procedures shall be documented and controlled by approved systems.

IV. Responsibility

It is the responsibility of all personnel performing this procedure to follow the current protocol. It is the responsibility of the Production Coordinator 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

SOP/Reference Title	SOP/Reference Number
Invitrogen's Superscript™ Double-Stranded cDNA Synthesis kit Manual	Cat. No. 11917-010, Rev. date: 070323
Operation of Covaris E-Series	LIBPR.0041

VI. 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 sheet (MSDS) for additional information. **Steps that involve the use of PCI should be performed in the fume hood.**

VII. Materials and Equipment

Name	Supplier	Number	Model or Catalogue #	
Fisherbrand Textured Nitrile gloves - large	Fisher	270-058-53		✓
RNAse Zap	Ambion	9780		✓
Ice bucket – Green	Fisher	11-676-36		✓
wet ice	In house	N/A	N/A	N/A
RNAse free 1.5 ml eppendorf tube	Ambion	12400		✓

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RNAse free 1.5 ml eppendorf tube Non Sick	Ambion	12450		✓
Gilson P2 pipetman	Mandel	GF-44801		✓
Gilson P10 pipetman	Mandel	GF-44802		✓
Gilson P20 pipetman	Mandel	GF23600		✓
Gilson P200 pipetman	Mandel	GF-23601		✓
Gilson P1000 pipetman	Mandel	GF-23602		✓
Neptune barrier tips 10 µl	CLP	Bt10XL		✓
Neptune barrier tips 20 µl	CLP	Bt20		✓
Neptune barrier tips 200 µl	CLP	Bt200		✓
Neptune barrier tips 1000 µl	CLP	Bt1000		✓
VX-100 Vortex Mixer	Rose Scientific	S-0100	✓	
Eppendorf Thermomixer 1.5 mL	Eppendorf	21516-166		✓
Large Kimwipes	Fisher	06-666-117		✓
Black ink permanent marker pen	VWR	52877-310		✓
Bench Coat (Bench Protection Paper)	Fisher	12-007-186		✓
Small Autoclave waste bags 10"X15"	Fisher	01-826-4		✓
Gibco Ultrapure water	Gibco	10977015		✓
DEPC water	Ambion	AM9922		✓
100% Ethanol	Commercial Alcohols Inc	N/A	N/A	N/A
Mini-centrifuge	Eppendorf	5417 R	✓	
Random hexamer primer, 50 ng/µl (25uM)	Invitrogen	from kit #18090-019		✓
SS Rand Primer DS cDNA Synth Kit	Invitrogen	A10836		✓
RNAse OUT TM (40U/µl)	Invitrogen	10777-019		✓
Phenol/Chloroform/Isoamyl Alcohol	Fisher	HP1752-1000		✓
Mussel Glycogen	Roche Diagnostics	901393		✓
TE buffer pH 8.0	Ambion	AM9849		✓
2 ml Phase Lock Gel tubes	Eppendorf	955154045		✓

VIII. Procedure

RNA/5th floor Pre-PCR Workstation:

1. Retrieval of reagents and equipment preparation

1.1. Wipe down the workbench, small equipment, and ice bucket with RNase Zap (Ambion) and Ethanol.

1.2. Wipe down with DEPC-water.

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1.3. Lay down new bench coat.

1.4. Retrieve all non-enzymatic reagents. Thaw and store on ice.

1.5. Preheat two heat blocks: set to 70°C and 45°C.

2. First strand cDNA synthesis

2.1. Retrieve the purified mRNA sample (8.0 µl) from the -80°C freezer and add 4.0 µl random hexamer primers.

2.2. Heat-denature the mRNA at 70°C for 2 minutes.

2.3. Place the sample on ice to chill briefly.

2.4. Pulse spin the tube and add the reagents in the following table to the mRNA / random primer mix (keep remaining dNTP for 2nd strand synthesis):

Reagent	Volume
5x First strand buffer	4.0 µl
RNaseOUT™ (40U/µl)	0.4 µl
10 mM dNTP mix	1µl
0.1 M DTT	2 µl
Total Volume	**19.4 µl**

2.5. Incubate on the bench for 10 minutes at room temperature to allow the random hexamers to anneal to the mRNA.

2.6. Add 1.0 µl Superscript II Reverse Transcriptase (200 U/µl). Mix the tube gently and pulse spin.

2.7. Incubate the first strand synthesis reaction at 45°C for one hour.

2.8. Remove the tube to ice.

2.9. Chill one block to 16°C.

3. Second strand cDNA synthesis

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3.1. Prepare the 2nd Strand reaction in a siliconized (non–stick) RNase–free 1.5 mL tube on ice:

Reagent	Volume for 1 library	Volume for 2 libraries	Volume for 3 libraries	Volume for 4 libraries
DEPC Water (or Ultrapure water)	36.5 µl	73 µl	109.5 µl	146 µl
5 x Second strand buffer	15 µl	30 µl	45 µl	60 µl
10 mM dNTP mix	1.5 µl	3 µl	4.5 µl	6 µl
E. coli DNA Ligase (10 U/µl)	0.5 µl	1 µl	1.5µl	2 µl
E. coli DNA Polymerase (10U/µl)	1 µl	2 µl	3 µl	4 µl
E. coli RNase H (2 U/µl)	0.5 µl	1 µl	1.5 µl	2 µl
Total Volume	**55 µl**	**110 µl**	**165µl**	**220 µl**

3.2. Add 55µl of 2nd strand reaction mix directly into the tube containing the 20 µL 1st Strand reaction.

3.3. Mix gently, pulse spin, and incubate the reaction at 16°C for 2.0 hours 15 min.

3.4. Move the reaction to ice and add 75 µL of Nuclease-free water to cDNA to bring up volume to 150µl.

3.5. Spin down a 2ml PLG tube at 14000rpm for 1 minute at room temperature.

3.6. Perform a phenol-chloroform extraction of the cDNA: To a pre-spun 2 mL PLG tube add 150 µl PCI, and then add the cDNA synthesis products. Mix by inversion.

3.7. Spin the PLG tube at 14,000 rpm for 5 minutes at room temperature.

3.8. Remove the aqueous (upper) layer, and transfer to a new 1.5 ml tube.

3.9. Ethanol precipitate the cDNA as follows:

Reagent	Volume
Double-stranded cDNA	150 µl
3M Sodium acetate, pH 5.5	15 µl
Mussel Glycogen	1.5 µl
Ice cold 100% EtOH	375µl

3.10. Mix and chill the tube at -20°C for a minimum of 20 minutes.

3.11. Centrifuge the tube at 4°C for 30 minutes at 14,000 rpm.

3.12. Wash the cDNA pellet with 1 ml 70% EtOH and spin at 14,000 rpm for 2 minutes.

3.13. Remove all traces of EtOH and allow the pellet to air dry.

3.14. Resuspend pellet in 40 µl TE.

3.15. Unless supervisor specifies otherwise, the cDNA will require sonication to reduce the larger fragments down to the desired size range for library construction. If the samples are to be sonicated the following day, store tubes in 6th floor library construction room -20°C freezer in the appropriate box.

3.16. If there is sufficient time to sonicate sample(s) on the same day, follow "LIBPR.0041 Operation of Covaris E-Series" for sonication using the Covaris to shear the cDNA.

3.17. Only after sample has been **sonicated**, add 10x loading dye and label tubes as 'sonicated'.

3.18. Next stage is to isolate the appropriate fraction of sonicated fragments on an 8% acrylamide gel as outlined in the protocol (SLX PET). Continue with the protocol (SLX PET) to prepare the cDNA for sequencing.

CHANGE HISTORY

Description of Change	Rev. Number	Submitted by	Effective Date
1) Protocol created (Nov 7, 2007) 2) Editing (June 16, 2008)	1	Helen McDonald Anna-Liisa Prabhu	April 28, 2009
3) Editing (CR.742)	2	Angela Tam	April 26, 2010

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Appendix A – LIMS SLX-Transcriptome

1. Scanner Protocol

1.1. Retrieve the total RNA sample and determine the amount of sample that will be used. Under the Gene Expression tab, **scan the RNA sample. Save the tube set.**

1.2. Select the **SLX-Transcriptome** protocol, continue prep.

1.3. Within the Transcriptome protocol change the pipeline from SLX-GE to TRA:Transcriptome.

1.4. Aliquot the **amount** (volume) of DNased RNA that you are going to use to a new **non-stick tube**.

1.5. Enter the starting amount of RNA (pure number) in ng (for example, if a library is started with 10 µg enter 10,000). Fill any comments. Complete the aliquot. **Record the new tube set number**. Pick up the new tube barcode from the barcode printer, stick to the tube.

1.6. Scan the oligo(dT) Microbeads reagent barcode.

1.7. Save the purified mRNA product to the appropriate rack in the -80°C freezer.

1.8. Enter the Agilent results. Enter the plate attribute for: Agilent Run ID and mRNA concentration.

1.9. Outside the protocol, edit the volume of the mRNA samples.

1.10. Aliquot the **amount** (volume) of mRNA that is going to be used for cDNA synthesis to a new **non-stick tube**.

1.11. Scan in Random Hexamer Primer

1.12. Scan in Superscript II RT

1.13. Scan in E. coli DNA Ligase

1.14. Scan in E. coli DNA Polymerase

1.15. Scan in E. coli RNaseH

1.16. Save the double-stranded cDNAs to the appropriate rack in the -20°C freezer. Edit the volume of the tube by editing the plate field.