Non Controlled Version

**Note: Controlled Versions of this document are subjected to change without notice*

SLX Transcriptome Library Construction Day 1

I. Purpose

To purify the polyA+ fraction from a sample of DNase-treated Total RNA

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
Product Manual for µMACS mRNA Isolation Kit	Cat. No. 130-075-102
Product Manual for Agilent 6000 RNA Nano Kit	Cat. No. 5067-1511
Operation and Maintenance of the Agilent 2100 Bioanalyzer for RNA samples	LIBPR.0018

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 sheets (MSDS) for additional information.

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		✓

RNAse free 1.5 ml eppendorf tube Non Stick	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	Intersciences	Bt10XL		✓
Neptune barrier tips 20 µl	Intersciences	Bt20		✓
Neptune barrier tips 200 µl	Intersciences	Bt200		✓
Neptune barrier tips 1000 µl	Intersciences	Bt1000		✓
VX-100 Vortex Mixer	Rose Scientific	S-0100		✓
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		✓
DEPC water	Ambion	9922		✓
Mussel Glycogen	Roche Diagnostics	901393		✓
100% Ethanol	Commercial Alcohols	NA	NA	NA
3M Sodium Acetate, pH 5.5	Invitrogen	9740		✓
Mini-centrifuge	Eppendorf	5417R		✓
Multistand	Miltenyi Biotech	130-042-303		✓
µMACS Separation Unit	Miltenyi Biotech	130-042-602		✓
µMACS mRNA Isolation Kit	Miltenyi Biotech	130-075-102		✓
µ Columns	Miltenyi Biotech	130-042-701		✓
Agilent 2100 Bioanalyzer	Agilent Technologies	G2939A		✓
Agilent RNA 6000 Nano Kit	Agilent Technologies	5067-1511		✓
Eppendorf Thermomixer 1.5 mL	Eppendorf	21516-166		✓

VIII. PROCEDURE

A. RNA/Pre-ditag workstation:

1. Retrieval of reagents and equipment preparation

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

1.2. Wipe down with DEPC-water

1.3. Lay down new bench coat

1.4. Preheat a heat block to 70°C.

1.5. Set a thermomixer to 25°C.

1.6. Take out ~1.0mL aliquots, one per library, each of Lysis/Binding buffer and Wash Buffer from the 4°C fridge and warm up to 25°C in a thermomixer for at least 5mins.

1.7. Place one 100µl aliquot of Elution Buffer per library to preheat in the 70°C heat block.

1.8. Double check that the sample has been DNaseI treated, information should be written on the sample tube and in LIMS. If samples have not been DNaseI treated, consult with supervisor.

2. mRNA Isolation

Note: If mRNA is to be isolated from 2 (or more) Total RNA samples, then process them one at a time, leaving the sample to precipitate in Ethanol before continuing with the next sample. After the final sample has been precipitating in Ethanol for a minimum of 20 minutes, all the samples can be spun down for 30 minutes.

Also **if flow through (total RNA minus PolyA+) needs to be kept for future use, follow the mRNA isolation protocol below in Appendix A. After mRNA Isolation, continue with Step 3 and 4 in the protocol.**

2.1. Place a µMACS column in the µMACS magnetic separator, using the metal stand to hold the separator in place. Place a 2mL collection tube below the column to collect the flow through fractions.

2.2. Rinse the column with 100µl Lysis/Binding buffer (warmed up to 25°C). The column is "flow stop" and does not run dry.

2.3. Retrieve the RNA sample, add SuperaseIN in a ratio of 1/20 if DNase treatment was not done at GSC. Heat 10-20µg of the DNase-treated total RNA sample (some libraries might need less than 10µg or more than 20µg of Dnase-treated total RNA, amount to be decided by supervisor according to yield of DNased RNA) in a non-stick 1.5mL tube for 3 minutes at 70°C, then place the tube on ice for 2 minutes to snap cool. Return any remaining DNased RNA to -80°C until required.

2.4. Take the sample tube out of the ice and dilute the total RNA with at least 1 equal volume of Lysis/Binding buffer to a final minimum volume of 250µl. Do not place sample back on ice after diluting.

2.5. Add 25µl oligo(dT) microbeads to the total RNA sample. Invert to mix. Pulse spin quickly after mixing; make sure microbeads do not settle.

2.6. Load the sample onto the column.

2.7. Rinse the column 3 times with 200µl Lysis/Binding buffer, collecting the washes into the same 2mL tube.

2.8. Rinse the column 8 times with 100µl Wash buffer (warmed up to 25^{o}C), changing the collection tube when necessary.

2.9. Remove the collection tube and discard the flow-through fractions.

2.10. Elute the mRNA into a fresh 1.5ml tube with 100µl Elution Buffer preheated to 70°C. It is important to transfer the buffer quickly to the column to maintain the temperature.

2.11. Ethanol precipitate the mRNA (polyA+ fraction) as follows:

Reagent	Volume
polyA+ fraction RNA	100µl
3M Sodium acetate, pH 5.5	10µl
Mussel Glycogen	3µl
Ice cold 100% EtOH	250µl

2.12. Cool the ethanol-precipitated polyA+ RNA at -20°C for a minimum of 20 minutes.

2.13. Spin down the RNA in the Eppendorf mini-centrifuge at 14,000 rpm at 4°C for 30 minutes.

2.14. Wash the RNA pellet with 1 ml ice cold 70% EtOH, and spin at 14,000 rpm at 4^{o}C for 2 minutes.

2.15. After spinning down the tube briefly, discard the supernatant and remove any residual EtOH with a P10 pipette tip.

2.16. Dry the pellet carefully for 5 to 10 minutes on ice or as needed. Resuspend the polyA+ RNA pellet in 9µl DEPC water. Label tube 'poly A+ mRNA'.

2.17. Remove 1.0µl of the purified RNA into a new tube and label for Agilent analysis. Add 0.5µl DEPC water to allow for evaporation when heating the Agilent aliquot.

2.18. Store the remaining polyA+ RNA at -80°C until required.

B. Agilent Workstation:

3. Retrieval of reagents and equipment preparation

3.1. Allow the Agilent RNA Nano kit reagents to warm to room temperature for 30 minutes.

4. Agilent RNA Nano Assay

4.1. Prepare the gel-dye mix as directed by the Agilent Nano Assay protocol.

4.2. Prepare the chip according to the Agilent Nano Assay protocol and LIBPR.0018

4.3. Load the aliquot of polyA+ RNA purified from the oligo(dT) column.

4.4. Choose the "mRNA Nano assay" setting to run the chip.

Note: Samples may also be run using the "Total Eukaryotic RNA assay" setting; however the percentage of rRNA contamination calculation is not a feature of this assay.

4.5. Save the assay results in the Agilent sub folder within the Library Core Folder.

4.6. After the chip has finished running check the results for sample integrity and concentration as follows:

4.6.1. The mRNA (polyA+) sample profile should appear as a curve with a peak height occurring between 1 to 2kb. The yield from10µg total RNA starting material is variable, usually between 50 and 300ng mRNA. The concentration may fall below the quantitative range for the mRNA Nano assay (25-250ng/µl), in which case the Agilent assay serves as a qualitative check of the oligo(dT) column elution products, and the concentration of the purified mRNA is an estimate.

4.6.2. Discuss the profile of the mRNA with the supervisor to determine whether the sample is of sufficient quantity and quality to continue the protocol. Some samples with excessive rRNA contamination may require a second column purification to reduce the background rRNA.

CHANGE HISTORY

Description of Change	Rev. Number	Submitted by	Effective Date
1) Protocol created (March 16 and March 25, 2007) 2) Editing (June 16, 2008)	1	Helen McDonald Anna-liisa Prabhu	April 28, 2009
3) Protocol Edited (CR.672)	2	Angela Tam	October 14, 2009

4) Protocol Edited (CR.744 and CR.755)	3	Angela Tam	April 26, 2010

Appendix A

Note: If flow through (total RNA minus PolyA+) needs to be kept for future use, follow the mRNA isolation protocol below instead of Step 2. After mRNA Isolation, continue with Step 3 and 4 in the above protocol.

1. mRNA Isolation

1.1. Place a MACS µcolumn in the µMACS magnetic separator, using the metal stand to hold the separator in place.

1.2. Label one 2ml tube with library name and "flow-through fraction" on the tube. Label another 2ml as "waste" with library name indicated in brackets.

1.3. Position the waste 2 mL tube below the column.

1.4. Rinse the column with 100 µl Lysis/Binding buffer (at room temperature).

1.5. **Remove** the waste collection tube and set aside.

1.6. Place the 2ml tube labeled as "flow-through fraction" below the column.

1.7. Heat 1-10 µg of the DNase-treated total RNA sample (amount to be decided by APC according to yield of DNased RNA) in a non-stick 1.5 mL tube for 3 minutes at 70°C, then place the tube on ice to snap cool. Return any remaining DNased RNA to -80°C until required.

1.8. Take the sample tube out of the ice and dilute the total RNA with at least 1 equal volume of Lysis/Binding buffer to a final minimum volume of 250 µl.

1.9. Add 25 µl oligo(dT) microbeads to the total RNA sample. Invert to mix.

1.10. Load the sample onto the column. The flow-through should be collecting in"flow-through fraction" labeled tube.

1.11. Rinse the column with 200 µl Lysis/Binding buffer, collect this 1st wash into the same 2 mL "flow-through" tube. Total volume should be 450µl. This fraction contains total

RNA without the polyA+ component. Remove this tube and place on ice until ready to precipitate.

1.12. Place the waste collection tube below the column to collect all subsequent washes.

1.13. Rinse the column 2 more times with 200 µl Lysis/Binding buffer.

1.14. Rinse the column 8 times with 100 µl Wash buffer (at room temperature).

1.15. Remove the waste collection tube and set aside. This tube can be stored in a -20°C freezer. Discard later after verifying that desired fractions have been collected.

1.16. Elute the mRNA into a fresh 1.5 ml tube with 100 µl Elution Buffer preheated to 70°C. It is important to transfer the buffer quickly to the column to maintain the temperature. Label this tube as poly A+ mRNA and with library name.

1.17. Ethanol precipitate the mRNA (polyA+ fraction) and the total RNA(minus polyA+) as described below.

Precipitate the 100µl polyA+ RNA fraction as follows:

Reagent	Volume
polyA+ fraction RNA	100 µl
3M Sodium acetate, pH 5.5	10 µl
Mussel Glycogen	3 µl
100% EtOH	250 µl

Precipitate the 450µl total RNA fraction as follows:

Reagent	Volume
Total RNA fraction	450 µl
3M Sodium acetate, pH 5.5	45µl
Mussel Glycogen	1.5µl
100% EtOH	1200 µl

Gently vortex/flick tubes and then invert tubes several times to mix well.

1.18. Cool the ethanol-precipitated polyA+ mRNA and Total RNA(minus polyA+) tubes at -20°C for a minimum of 20 minutes.

1.19. Spin down the RNA in centrifuge at 14,000 rpm at 4°C for 40 minutes.

1.20. Wash both samples with 1 ml chilled 70% EtOH, and spin at 14,000 rpm for 4 minutes.

1.21. After spinning the tube briefly, discard the supernatant and remove any residual EtOH with a P10 pipette tip.

1.22. Dry pellet carefully at room temperature.

1.23. Resuspend the polyA+ mRNA pellet in 9 µl DEPC water.

1.24. Remove 1.0 µl of the polyA+ purified RNA into a new tube and label for Agilent analysis. Add 0.5 µl DEPC water to allow for evaporation when heating the Agilent aliquot.

1.25. Store the remaining polyA+ mRNA at -80°C until required.

1.26. Resuspend the total RNA pellet in 5.5µl - 12µl DEPC water/ DEPC water with SuperaseIn. Volume amount is based on the original starting amount of total RNA loaded onto the column.
If input amount is 5µg or more: resuspend in 12µl DEPC water and then add 0.5µl SuperaseIn.
If input amount is less than 5µg but more than 1.7µg: resuspend in7.5µl DEPC water and add 0.5µl SuperaseIn.
If starting amount is 1.7µg or less then resuspend in 5.75µl of SuperaseIn treated DEPC water.

1.27. Remove 0.75 µl of the purified flow-through total RNA into a new tube and label for Agilent analysis. Add 0.75 µl DEPC water to allow for evaporation when heating the Agilent aliquot.

1.28. Store the remaining total RNA (minus polyA+) at -80°C until required.

1.29. Proceed to Step 3 and Step 4 for Agilent QC. Also QC the total RNA (minus polyA+) sample in Step 4. If QC is done on both total RNA (minus polyA+) and mRNA samples, select "Total Eukaryotic RNA assay" on Agilent.