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SLX- miRNA2

I. Purpose

To prepare total RNA samples for small RNA sequencing.

II. Scope

All procedures are applicable to the BCGSC FG-Library production team.

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
Preparing samples for Small RNA sequencing using the alternative v1.5 protocol	Illumina © 2009
Operation and maintenance of the Agilent 2100 Bioanalyzer for DNA samples.	LIBPR.0017
Illumina Concentration Checked	LIBPR.0030

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

VII. Materials and Equipment

Name	Supplier	Number: #	Model or Catalogue #	
Illumina Small RNA Sample prep kit	Illumina	FC-15002615		✓
SRA 5' miRNA Adapter (5µM)	Illumina	1000595		✓

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10X v1.5 sRNA 3' Adapter (100µM)	Illumina	15000263		✓
T4 RNA Ligase 2, truncated (200U/ul)	NEB	M0242L		✓
10X T4 RNL2 truncated reaction buffer	NEB	B02425		✓
T4 RNA Ligase (5U/µL)	Ambion	AM2140		✓
10X T4 RNA Ligase Buffer	Ambion	AM2140		✓
RNase Out (40U/ul)	Invitrogen	10777-019		✓
DEPC water	Ambion	9922		✓
10 mM ATP, molecular grade	NEB	9804		✓
100 mM MgCl2	Ambion	AM9530G		✓
5X Novex TBE buffer	Invitrogen	LC6675		✓
dNTP Mix (10 mM each)	Invitrogen	46-0519		✓
0.1M DTT	Invitrogen	Y00147		✓
Superscript II Reverse Transcriptase (200U/ul)	Invitrogen	18064 014		✓
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
Nuclease Free 2.0 ml eppendorf tube	Ambion	12400		✓
RNAse free 1.5 ml eppendorf tube	Ambion	12400		✓
RNAse free 1.5 ml eppendorf tube Non Sick	Ambion	12450		✓
RNase free 0.5 mL Non-stick Microfuge tubes	Ambion	12350		✓
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		✓
Galaxy mini-centrifuge	VWR	37000-700		✓
VX-100 Vortex Mixer	Rose Scientific	S-0100		✓
Large Kimwipes	Fisher	06-666-117		✓
Black ink permanent marker pen	VWR	52877-310		✓
Eppendorf BenchTop Refrigerated Centrifuge 5810R	Eppendorf	5810 R		✓
Eppendorf Thermomixer 1.5 mL	Eppendorf	21516-166		✓
Bench Coat (Bench Protection Paper)	Fisher	12-007-186		✓
Small Autoclave waste bags 10"X15"	Fisher	01-826-4		✓
DEPC water	Ambion	9922		✓
DNA*AWAY*	MBS	7010		✓
Mussel Glycogen (20mg)	Roche	10 901 393 001		✓
3 M Sodium Acetate	Sigma	EC 211-162-9		✓
Anhydrous Ethyl Alcohol (100% Ethanol)	Commercia	People Soft ID:		✓
70% Ethanol	In house			
Agilent DNA 1000 Series II Kit	Agilent	5067-1504		✓

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DNA 1000 Gel Matrix	Agilent	5067-1504		✓
DNA 1000 Dye Concentrate	Agilent	5067-1504		✓
DNA 1000 Marker	Agilent	5067-1504		✓
DNA 1000 Ladder	Agilent	5067-1504		✓
Agilent DNA 1000 Chips	Agilent	6064-8230		✓
Agilent Chip Priming Station	Agilent	Chip Priming	✓	✓
IKA Works Vortexer	Agilent	MS2S9-Agilent-	✓	
22R Microfuge Centrifuge	Bechman	22R Centrifuge	✓	
GeneAmp PCR System 9700	ABI	PCR System 9700	✓	
Agilent Electrode Cleaner	Agilent	6064-8230		✓
Peltier Thermal Cycler	MJ	PTC-225	✓	
Power Supply, LVC2kW, 48VDCV	Tyco	RM200HA100	✓	
Razor Blades	VWR	55411-050		✓
Small RNA RT Primer, 100uM	Illumina	FC-102-1010		✓
5x Phusion HF buffer	Finnzymes	F-518		✓
Small RNA PCR Primer 1 (GX1- Illumina)	IDT			
Small RNA PCR Primer 2 (GX2- Illumina)	IDT			
Phusion Hot Start High Fidelity DNAPolymerase (2U/ul)	New England Biolabs	F-540L		✓
8%TBE Gels, 1.0mm, 10 well	Invitrogen	EC6215BOX		✓
XCell *SureLock* Mini-Cell	Invitrogen	EI0001		✓
10x BPB/XC loading buffer	In house	N/A		N/A
Saran Wrap	BCCA	SW183		✓
25 bp DNA ladder	Invitrogen	10597-011		✓
SybrGreenI	CAMBEX	50513		✓
Sybr Green Imager		In House		
Dark Reader (Transilluminator)	InterSicenc	DR-190M	✓	
Gel Elution buffer	In house			
Spin-X Filter Columns	Costar	8160	✓	

Small RNA adapters:

5'miRNA Adapter (5 µM)
5' GUU CAG AGU UCU ACA GUC CGA CGA UC 3'

3'miRNA2 Adapter (from Illumina) (preadenylated, 10 µM working stock)
5' ATC TCG TAT GCC GTC TTC TGC TTG T3'

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Oligonucleotide Sequences (DGE–Small RNA Sample Prep Kit)

Small RNA RT primer (100 µM):
5' CAA GCA GAA GAC GGC ATA CGA 3'

miRNA PCR Primer P1 (25 µM):
5' CAA GCA GAA GAC GGC ATA CGA 3'

miRNA PCR Primer P2 (25 µM):
5' AAT GAT ACG GCG ACC ACC GAC AGG TTC AGA GTT CTA CAG TCC GA 3'

VIII. Procedure

RNA/Pre-PCR workstation

Note: If total RNA quality / quantity needs to be checked, proceed to Step 1; otherwise, proceed directly to Step 2.

1. QC prior to beginning procedure

1.1. Evaluate total RNA by running the extracted RNA sample on Agilent Bioanalyzer using the RNA Nano total eukaryotic RNA assay.

1.2. RNA must be of high quality (RIN > 7.0) for this procedure.

1.3. Do NOT treat total RNA sample with DNase I prior to starting the miRNA2 protocol.

2. Retrieval of reagents and equipment preparation

2.1. Print off the worksheet as shown in Appendix B to record the solution and plate numbers of the reagents and samples used in the assay.

2.2. Put on a disposable lab coat and clean pair of gloves.

2.3. Wipe down the assigned specific workstation, pipetors, and small equipment with RNaseZap (Ambion) and then DEPC- treated water.

2.4. Lay down a new benchcoat.

2.5. Change gloves.

2.6. Set one heat block to 70°C and another to 22°C.

2.7. Retrieve ice and all required reagents.

2.8. Thaw all reagents on ice; vortex and pulse spin.

3. Ligation of 3'miRNA2 Adapter

3.1. Set up the ligation reaction in a nuclease-free 1.5 mL microcentrifuge tube on ice.

Reagent	**Volume**
Total RNA **(1µg)**	5.0 µL
10uM 3'miRNA2 Adapter	1.0 µL

3.2. Incubate the reaction at 70°C for 2 minutes, then immediately return it to ice.

3.3. Quick spin, and then add the following reagents to the RNA adapter mixture on ice.

Reagent	**Volume**
Total RNA + 3'miRNA2 Adapter	6.0 µL
10X T4 RNL2 truncated buffer	1.0 µL
100 mM MgCl2	0.8 µL
T4 RNA Ligase 2, truncated (200U/ul)	1.5 µL
RNase Out (40U/ul)	0.5 µL
Total volume	9.8 µL

3.4. Incubate at 22°C for one hour.

4. Ligation of the 5' miRNA adapter

4.1. With 5 minutes remaining on the 3' adapter ligation, **prepare the 5' adapter for ligation by heating it (2 µL for 4 libraries) at 70^{o}C for 2 minutes**, then immediately transferring it to ice.

4.2. After the 1 hour 3' adapter ligation reaction, add the following reagents to the 3' adapter-ligated total RNA reaction.

Reagent	**Volume**
3' Adapter ligation reaction	9.8 µL
10 mM ATP	1.0 µL
5' miRNA Adapter***- **linearized and chilled**	0.5 µL
T4 RNA ligase (5U/ul)	1.0 µL
Total volume	12.3 µL

***Note: indexed adapters may be used in place of the standard 5' miRNA Adapter

4.3. Incubate the reaction at 20°C for one hour and store at 4°C or proceed to reverse transcription.

5. Reverse transcribe small RNA ligated with adapters

5.1. Set one heat block to 65°C and one heat block to 48°C.

5.2. Dilute SRA RT primer (100uM) 1 in 5 with DEPC water to 20uM and store on ice.

5.3. Combine the following reagents in a sterile, nuclease-free 1.5 mL microcentrifuge tube on ice. Store the remaining 4.3 µL ligation reaction in the -80°C freezer in the current rac labeled as 'Illumina Micro RNA ligation intermediate products'.

Reagent	**Volume**
Ligated total RNA	8.0 µL
SRA RT primer, 20uM	2.0 µL

5.4. Heat the mixture at 65°C for 10 minutes, then immediately return to ice.

5.5. Prepare the following reagents in the order listed, mix well, and then add 8.0 µL to the ligated RNA & RT primer. The total volume should now be 18 µL.

Reagent	**Volume (per tube)**	**Master mix (9 libraries)**

5X first strand buffer	4.00 µL	39.6 µL
10 mM dNTP	1.25 µL	12.4 µL
100 mM DTT	2.0 µL	19.8 µL
RNase Out (40U/ul)	0.75 µL	7.43 µL
Total	**8.0 µL**	**79.1 µL**
Ligated RNA & RT primer	10.0 µL	
Total	**18.0 µL**	

5.6. Heat the mixture at 48°C for 3 minutes. Remove the tube and set the heat block to 44°C.

5.7. Add 2.0 µL of SuperScript II Reverse Transcriptase (200U/ul). The total volume should now be 20 µL.

5.8. Incubate the mixture at 44°C for 1 hour. Store the first strand cDNA at -20^{o}C or proceed to PCR amplification.

5.9. Prepare a 10 µL aliquot of the reverse transcribed template for PCR.

5.10. Store the back up fraction in the current rac labeled as 'Illumina Micro RNA RT intermediate products'.

RNA/Pre-PCR Biological Safety Cabinet (BSC)

6. PCR amplify small RNA with ligated adapters

6.1. Put on a clean pair of gloves.

6.2. Wipe down the BSC, pipettors, small equipment and ice bucket with RNase Zap (Ambion).

6.3. Thaw all required reagents, vortex and then pulse spin.

6.4. Prepare the PCR brew mix as listed below in a nuclease free 1.5ml tube on ice.

Reagent	**Volume (per tube)**	**Master mix (9 libraries + 1 control)**

Ultra pure water	26.25 µL	263 µL
5X Phusion HF buffer	10.0 µL	100 µL
Primer GX1 or miRNA P1	1.0 µL	10 µL
Primer GX2 or miRNA P2	1.0 µL	10 µL
10 mM dNTP	1.25 µL	12.5 µL
Phusion DNA polymerase (2U/ul)	0.5 µL	5.0 µL
Total	**40.0 µL**	**263 µL**

6.5. Label individual 0.2 mL thin walled PCR tubes with sample or control name and the number of PCR cycles.

6.6. Add 40 µL of the master mix brew into four labeled tubes; the remaining master mix will be used for a no template control.

6.7. Add 10 µL of nuclease-free water to the no template control tube.

6.8. In the 6th floor biosafety cabinet add 10 µL of the reverse transcribed template to the PCR brew mix.

Library Production Room BSC

6.9. Put on a clean pair of gloves and a disposable lab coat.

6.10. Wipe down the 6th floor BSC, pipettors, and Tetrad with DNA Away (Thermo Fisher Scientific – Molecular BioProducts, USA).

6.11. Turn on the Tetrad.

6.12. Carefully aliquot 10 µL of the reverse transcribed template into the appropriate 0.2 mL PCR tubes containing 40 µL of PCR brew mix.

6.13. Put the sample and control PCR tubes in the alpha unit, ensuring the lid is properly in place. Start the "MIR15" program.

PCR Conditions for 'MIR 15":

98°C 30 sec
98°C 10 sec

60°C 30 sec 15 Cycles of PCR
72°C 15 sec
72°C 5 min

6.14. While the PCR reaction is running set up the gel running apparatus as described below.

Library Production Room Gel Preparation/Gel Running Workstations

7. PCR product purification

7.1. Put on a clean pair of gloves.

7.2. Cut open an 8% TBE Novex gel cassette pouch to remove the gel cassette, and drain away the gel packaging buffer. Handle the gel cassette by the edges only. Rinse the gel cassette with deionized water.

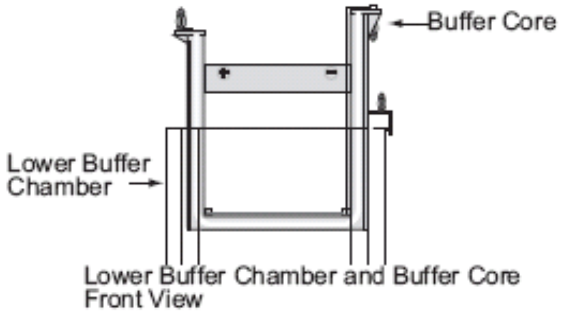
7.3. Peel off the tape covering the slot on the back of the gel cassette.

7.4. In one fluid motion, pull the comb out of the cassette.

7.5. Use a 1 mL pipette to gently wash the cassette wells with 1X TBE running buffer. Repeat twice, and then fill the sample wells with running buffer.

7.6. Assemble the gel apparatus as follows:

7.6.1. Lower the Buffer Core into the Lower Buffer Chamber so that the negative electrode fits into the opening in the gold plate on the Lower Buffer Chamber as shown in the figure.



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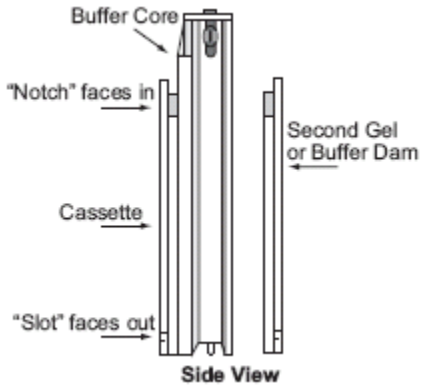
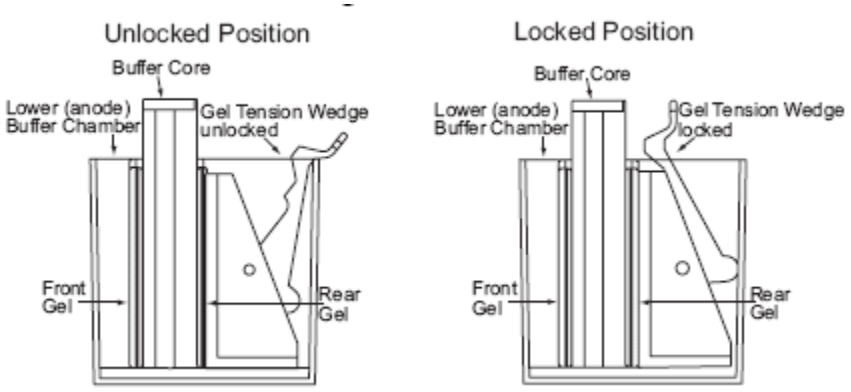
7.6.2. Insert the Gel Tension Wedge into the XCell *SureLock* cell behind the buffer core. Make sure the Gel Tension Wedge is in its unlocked position, allowing the wedge to slip easily into the XCell *SureLock* unit.

7.6.3. Insert the gel cassette into the lower buffer chamber in front of the core, with the well side of the cassette facing in towards the buffer core. The slot on the back of the cassette must face out towards the lower buffer chamber. Place the Buffer Dam behind the core.



7.6.4. Pull forward on the Gel Tension Lever in a direction towards the front of the XCell *SureLock* unit until lever comes to a firm stop and the gel/buffer dam appear snug against the buffer core.



7.7. Fill the Upper Buffer Chamber with 200 mL of the 1X TBE running buffer. Ensure that the Upper Buffer Chamber is not leaking. If the level of the running buffer drops, the apparatus will need to be reassembled.

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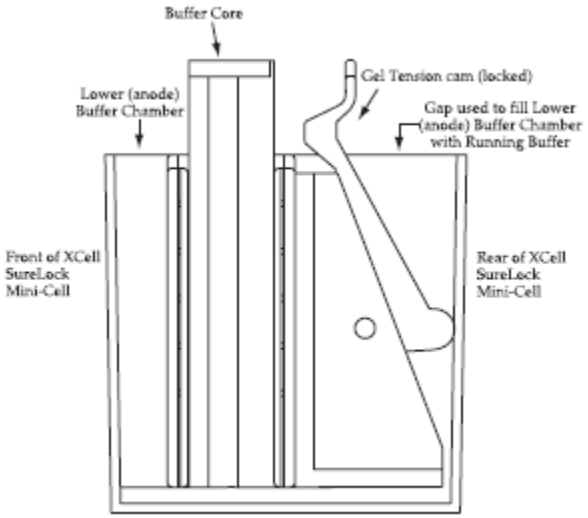
7.8. Fill the Lower Buffer Chamber with approximately 200 mL of running buffer through the gap between the Gel Tension Wedge and the back of the Lower Buffer Chamber as shown below:



7.9. Align the lid on the Buffer Core. The lid can only be firmly seated if the (-) electrode is aligned over the banana plug on the right.

Caution: Power must be off before connecting the XCell *SureLock* Mini Cell to the power supply.

7.10. After the PCR reaction is finished give the tubes a quick spin and then add 6 µL of 10X Bromophenol Blue/Xylene Cyanol loading dye to each sample and the control tube.

7.11. Label the gel apparatus with library name, start time, finish time, date, and initials.

7.12. Load ~20 µL of the no template control into a well on the left side of the gel.

7.13. In the next well load 10 µL of 25 bp DNA ladder (20 ng/µL).

7.14. Leave a gap of approximately 5 wells, and carefully load the sample into as many wells as required, keeping in mind that one well holds a maximum volume of 25 µL.

7.15. Replace the lid of the apparatus, and start the gel run.

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7.16. Run the 8% TBE gel at 200V for 40 minutes. The xylene cyanol corresponds to the 200 bp DNA marker, and it will have migrated approximately 1/2 of the length of the gel.

7.17. Prepare 2 tubes of 0.5 mL and 2 mL tubes for shearing the gel slices for each sample. Make a hole through the bottom of 0.5 mL tubes with an 18 gauge needle. Place each 0.5 mL tube into a 2 mL tube. Label each 2 mL tube on the side of the tube with the library name, "# cycles PCR", date, and initials.

7.18. Prepare fresh SybrGreenI DNA stain; 10 µL stock in 100 mL 1x TBE. Minimize exposure to light.

7.19. Stop the gel run after 40 minutes and dismantle the PAGE apparatus.

7.20. Using a clean tray, stain the gel for 5 minutes.

7.21. Scan the gel; save image in the appropriate network directory and print hardcopies. For example: Library Name_15cycles_Date_Initials.

7.22. Lay the gel down on the Dark Reader. Using a brand new razor blade carefully cut out the band(s) corresponding to 92-110 bp. See the gel below as reference

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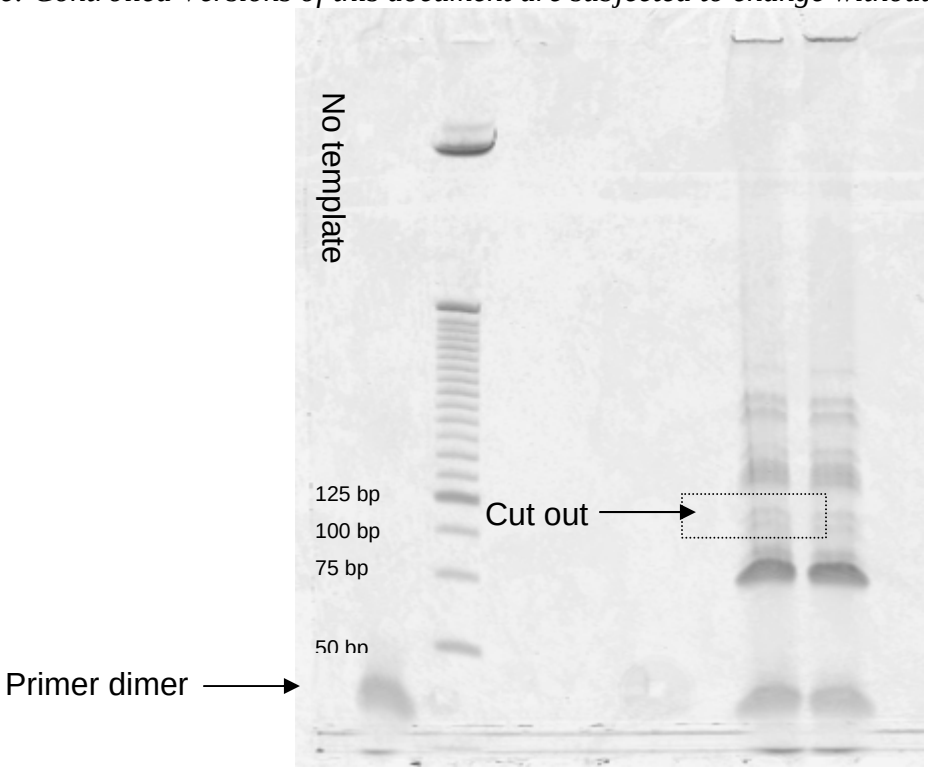

7.23. Cut the gel slice corresponding to the 14-30 nt miRNA with 5' and 3' adapters (92-110 bp). It is better to err towards higher rather than lower for downstream sequencing, i.e. cut at, and just above 100 bp.

7.24. Clean scanner surface with dH_2O and remove any pieces of gel material.

7.25. Clean Scanner area: discard dye and rinse trays, and discard SybrGreen waste and sharps material appropriately.

7.26. With the lids tailing (left of tube position), spin at 12,000 rpm / 4°C for 3 minutes. The gel slices should shear through the holes and collect into the bottom of the 2 mL tubes.

7.27. For each gel slice that was sheared into a 2 mL tube, add ~200 µL of elution buffer (5:1, LoTE:7.5M Ammonium Acetate). Ensure that all sheared gel pieces are covered with elution buffer. Add more buffer if needed.

7.28. Mix well by vortexing. Pulse spin.

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7.29. If time permits, place gel slurries at 65°C for one hour and then go to step 7.5. If time does not permit, place gel slurries at 4°C overnight in the appropriate shelf and rack and then go to step 7.1.

7.30. Dispose of all waste and clean up workstations.

7.31. Place used racks in bleach decontamination soak.

8. Precipitate and Purify the 100bp PCR products

8.1. Put on a clean pair of gloves.

8.2. Retrieve the gel slurries from the previous day's PAGE gel from 4°C.

8.3. Vortex and pulse spin.

8.4. Heat the gel slurries at 65°C for 15 minutes in the preheated heat block.

8.5. Vortex the tubes, pulse-spin and transfer the gel slurry from each tube onto the top of a Spin-X filter column. Spin the sample through the spin column into the collection tube at 12,000 rpm for 3 minutes at 4°C.

8.6. Check each Spin Column tube and ensure that all buffer has spun through the filter. Re-spin the tubes if there is still liquid trapped in the gel material.

8.7. Transfer the eluate from one sample into a single sterile 1.5 mL tube, up to a maximum total volume of 400 µL. The total volume of eluate will depend on the amount of excised gel and the volume of added elution buffer; adjust the amount of ethanol used to precipitate the PCR products accordingly, and add the reagents to the eluate:

REAGENT	VOLUME
Eluate	400 µL
3 M Sodium Acetate	40 µL
Mussel Glycogen (20 mg/mL)	3 µL
100 % Ethanol	1000 µL
TOTAL VOLUME	**1443µL**

8.8. Vortex and pulse spin. Chill the tubes at minus 20°C for a minimum of 20 minutes.

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8.9. Spin at 14,000 rpm / 4°C for 30 minutes.

8.10. Wash the pellet with 1 mL of 70% EtOH by adding the EtOH solution and inverting the tube. Spin at 14,000 rpm / 4°C for 2 minutes. Discard the supernatant as previously.

8.11. Repeat the 70% EtOH wash.

8.12. Pulse spin the sample tube and carefully remove any residual ethanol by using a P200 pipette tip first to remove the majority of the supernatant, then finally using a P10 pipette tip to remove the last trace of solution. Mark the outside bottom of the tube to better locate the pellet when resuspending in buffer.

8.13. Allow the tube to air-dry for approximately 5 to 10 minutes at room temperature, until the white precipitate is no longer visible.

8.14. Resuspend each sample in a total volume of 12 µL Qiagen EB buffer. Note: this volume may be decreased if the PCR product yield is low as determined by the intensity of staining on the previous day's gel. Supervisor will advise if changes to the volume are necessary.

8.15. If the purified PCR product was EtOH precipitated in more than one 1.5 mL tube, then pool the aliquots belonging to a single sample into one tube containing 12µl (caution: take care to pool tubes belonging to same sample).

Agilent / Qubit area:

9. Agilent DNA 1000 chip analysis and Qubit quant

9.1. Run the Agilent DNA 1000 assay according to the manufacturer's protocol and described in LIBPR.0017. Load a 1 µL aliquot of the purified PCR product sample onto the Agilent DNA 1000 chip.

9.2. Measure the concentration of the purified PCR product sample using Qubit by following the manufacturer's protocol. Use only 1 µL of the purified sample for this measurement.

9.3. Determine the molarity of the purified sample based on the base pairs measured by Agilent and concentration in ng/µL measured by Qubit.

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9.4. Remove an aliquot of the sample to make a 8 nM dilution of the library for Illumina sequencing. Dilute using Qiagen EB buffer supplemented with 0.1% Tween-20. In the case of a very dilute sample, no dilution of the stock may be necessary. Keep in mind that a minimum of 4 µL of 8 nM material is required for cluster generation, but if possible at least 20 µL of 8 nM diluted sample should be prepared for sequencing.

9.5. Place the 8 nM diluted DNA sample in the "To Double Check Box" (rac41513) and the undiluted sample in the completed Illumina Micro RNA libraries box. Complete the Illumina Concentration checked protocol as described in LIBPR.0030.

9.6. Dispose all waste and clean the workstation area.

9.7. Place used racks in bleach decontamination soak.

9.8. Complete LIMS protocol as described in Appendix A.

CHANGE HISTORY

Description of Change	Rev. Number	Submitted by	Effective Date
1) Protocol created	1	Angela Tam	February 03, 2010

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Appendix A – LIMS SLX miRNA2

1. Scanner Protocol

1.1. Retrieve your total RNA samples and determine the amount of sample that will be used. Make sure you are under the Gene Expression tab; **scan the RNA sample plate numbers**. **Save the tube set**.

1.2. Select the **SLX miRNA2** protocol and select 'Continue Prep'.

1.3. Change the pipeline from SLX-GE to **MR2: SLX-miRNA2**.

1.4. Aliquot (transfer) the appropriate total RNA volume to a new 1.5 mL non-stick tube. Use commas between volumes when inputting information for multiple libraries. Select 'Completed Aliquot RNA to 1.5 mL tube'. **This barcode represents the ligation product.**

1.5. Scan the 3' miRNA2 adapter solution number and select 'Completed Add 3' miRNA2 adapter'.

1.6. Scan the T4 RNA ligase 2 solution number and select 'Completed Add T4 RNA Ligase 2'.

1.7. Scan the 5' miRNA adapter solution number and select 'Completed Add 5'miRNA adapter'.

1.8. Scan the T4 RNA ligase solution number and select 'Completed Add T4 RNA Ligase'.

1.9. Click on 'Completed decant' to set the ligation volume to 0.

1.10. Resupend and store the ligated RNA. Scan EB barcode and enter the volume of the ligation product (12.3 uL). Enter the rac number and store the ligation product in the -80C freezer. Select 'Completed Store ligated RNA'.

1.11. Transfer 8 uL of the ligated RNA into a new 1.5 mL tube and select 'Completed Aliquot to 1.5 mL tube'. **This barcode represents the template barcode.**

1.12. Scan the Superscript II solution number and select 'Completed Add Superscript II'.

1.13. Scan the sRNA RT primer solution number and select 'Completed Add sRNA RT primer'.

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1.14. Click on 'Completed Decant#2' to set the template volume to 0.

1.15. Resuspend and store PCR template. Scan the EB solution number and enter the volume of the RT template (20 uL). Enter the appropriate rac labeled as 'Illumna Micro RNA RT intermediate products' and store the template in the -20C freezer on the 5th floor. Select 'Completed Resuspend and Store Template'.

1.16. Transfer 10 uL of the RT template (1st strand cDNA) into a new 1.5 mL non-stick tube and select 'Completed Aliquot 1st strand cDNA to 1.5 mL tube'. **This barcode represents the PCR product barcode.**

1.17. Scan the P1 primer solution number and select 'Completed Add primer P1'.

1.18. Scan the P2 primer solution number and select 'Completed Add Primer P2'.

1.19. Scan the Phusion Polymerase solution number and select 'Completed Add Phusion Polymerase'.

1.20. Enter the number of PCR cycles used for library enrichment and select 'Completed Enter in PCR cycles'.

1.21. Click on 'Decant#3'to set the PCR product volume to 0.

1.22. Resuspend and store purified PCR product. After size selection of the 92-110 bp bands, enter the volume of the PCR product after running on Agilent bioanalyzer and quantifying by Qubit (10 uL). Scan the EB solution number. Enter the appropriate rac labeled 'Illumina miRNA PCR products' and select 'Completed store purified PCR product'.

1.23. Enter the Agilent and Qubit results. Enter the Agilent run ID and final product size in base pairs. Enter the DNA concentration based on the Qubit quant. Select 'Completed Agilent and Qubit Results'.

1.24. Aliquot the sample to a new 1.5 mL tube in order to make a dilution for sequencing and select 'Completed aliquot DNA to 1.5 mL tube'. **This barcode represents the barcode for Illumina sequencing.**

1.25. Click on 'Decant#4' to set the sample for Illumina sequencing to 0.

1.26. Scan EB to resuspend submission sample. Scan the EB solution number and enter the volume of the diluted sample after Qubit quant. Click on 'Completed Scan EB to Resuspend Submission Sample'.

Non Controlled Version

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

1.27. Proceed to the Illumina concentration checked protocol.

Non Controlled Version

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Appendix B –SLX miRNA2 Worksheet

Illumina miRNA2 Library Worksheet (rev. 090618TT)

Library Name: **Date:** **Performed By:**

3' miRNA2 adapter ligation

	Lot/Sol #	Volume Per Tube		Added
Total RNA (1 µg)		5.0	µL	
3'miRNA2 Adapter		1.0	µL	
10X T4 truncated buffer		1.0	µL	
100 mM MgCl2		0.8	µL	
T4 RNA Ligase 2, truncated		1.5	µL	
RNase Out		0.5	µL	
Total		**9.8**	**µL**	

5' miRNA adapter ligation

	Lot/Sol #	Volume Per Tube		Added
3' Adapter ligation reaction		9.8	µL	
10 mM ATP		1	µL	
5' miRNA Adapter-linearized		0.5	µL	
T4 RNA ligase		1	µL	
Total		**12.3**	**µL**	

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RT reaction

	Lot/Sol #	Volume Per Tube			# of rxns*		Added
Purified ligated RNA		8	µL				
Small RNA RT-Primer		2	µL				
5x first strand buffer		4	µL	x	9.9	39.6	
10 mM dNTPs		1.25	µL	x	9.9	12.4	
100 mM DTT		2	µL	x	9.9	19.8	
RNaseOut (40 U/µL)		0.75	µL	x	9.9	7.43	
Superscript II RT (200U/µL)		2.0	µL				
Total						**79.2**	

* Prepare master mix for 9.9 reactions to account for pipetting error

PCR Cycle Used: _____

Amount of Template Used: _____

PCR Brew Preparation	SOL Number	Volume/rxn			# of rxns		Added
Ultra pure water or DEPC water		26.25	µL	x	10	263	
5X Pusion HF buffer		10	µL	x	10	100	
Primer GX1		1	µL	x	10	10	
Primer GX2		1	µL	x	10	10	
10 mM dNTPs		1.25	µL	x	10	12.5	
Phusion Hotstart DNA polymerase		0.5	µL	x	10	5.0	
Total		**40**	**µL**			**400**	

Purification of 92 bp bands

Number of Spin Columns used:

Number of 1.5 mL tubes used:

	Lot Number	Volume per Tube		Added
Eluate		400	µL	
3 M NaOAc		40	µL	
Mussel Glycogen (20 mg/mL)		3	µL	
100% Ethanol		1000	µL	
70% Ethanol		2 x 1000	µL	
Elution Buffer (Qiagen)		12 (final)	µL	