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miRNA3 - Plate Format miRNA Library Construction

I. Purpose

To prepare total RNA samples for small RNA sequencing in a 96 well plate format.

II. Scope

All procedures are applicable to the BCGSC Library Core and TechD Groups.

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 QS 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 Systems to audit this procedure for compliance and maintain control of this procedure.

V. References

Document Title	Document Number
Preparing samples for Small RNA sequencing using the alternative v1.5 protocol	Illumina © 2009

VI. Related Documents

Document Title	Document Number
Operation and Maintenance of the Agilent 2100 Bioanalyzer for DNA samples	LIBPR.0017
Operation and Maintenance of the Agilent 2100 Bioanalyzer for RNA samples	LIBPR.0018
Operation and Maintenance of the LabChip GX for HT RNA Samples using the HT RNA Assay	LIBPR.0052
Operation and Maintenance of the Caliper LabChip GX for DNA Samples using the High Sensitivity Assay	LIBPR.0051

miRNA3 – Plate Format miRNA Library Construction	
Document #: LIBPR.0054	Supersedes: Version 14
Version: 15	Page 2 of 26

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

Document Title	Document Number
Quantifying DNA Samples using the Qubit Fluorometer	LIBPR.0030
Manually pour agarose gels for Barracuda DNA size selection	LIBPR.0055
DNA Size Selection on the Barracuda Robot II	LIBPR.0090

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 sheet (MSDS) for additional information.

VIII. Materials and Equipment

Name	Supplier	Number	Model or Catalogue #	
5' miRNA Adapter (previous Index4) (1µM)	IDT	N/A	N/A	N/A
3' Adenylated Adapter (2.5µM)	IDT	N/A	N/A	N/A
T4 RNA Ligase 2, truncated (200U/µL)	NEB	M0242L		✓
10X T4 RNL2 truncated reaction buffer	NEB	B02425		✓
T4 RNA Ligase (5U/µL)	Ambion	AM2141		✓
RT primer (20µM)	IDT	N/A	N/A	N/A
RNase Out	Invitrogen	10777-109		✓
DEPC water	Ambion	9922		✓
10 mM ATP, molecular grade	NEB	9804		✓
100 mM MgCl ₂	Ambion	AM9530G		✓
Indexed PCR primers (25µM)	IDT	N/A		N/A
PCR primer 2 (25µM)	IDT	N/A		N/A
dNTPs Mix (10 mM each)	Invitrogen	46-0519		✓
0.1M DTT	Invitrogen	Y00147		✓
Superscript II Reverse Transcriptase (200U/µL)	Invitrogen	18064 014		✓
DMSO (dimethyl sulfoxide)	Fisher Scientific	BP231-100		✓
5X First Strand Buffer	Invitrogen	18064-014		✓
Fisherbrand Textured Nitrile gloves – various sizes (XS, S, M)	Fisher Scientific	296359682,296359683, 270-058-52		✓
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		✓
AB1000 Thermo-fast 96 skirted plates	Abgene	SKU AB-1000		✓
Gilson P2 pipetman	Mandel	GF-44801		✓

miRNA3 – Plate Format miRNA Library Construction	
Document #: LIBPR.0054	Supersedes: Version 14
Version: 15	Page 3 of 26

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

Gilson P10 pipetman	Mandel	GF-44802		✓
Gilson P20 pipetman	Mandel	GF23600		✓
Gilson P200 pipetman	Mandel	GF-23601		✓
Gilson P1000 pipetman	Mandel	GF-23602		✓
Diamond Filter tips (DFL10)	Mandel	GF-F171203		✓
Diamond Filter tips (DF30)	Mandel	GF-F171303		✓
Diamond Filter tips (DF200)	Mandel	GF-F171503		✓
Diamond Filter tips (DF1000)	Mandel	GF-F171703		✓
Rainin 200 µL Tips	Rainin	RT-L200F		✓
Rainin 20 µL Tips	Rainin	RT-L10F		✓
Galaxy mini-centrifuge	VWR	37000-700		✓
VX-100 Vortex Mixer	Rose Scientific	S-0100		✓
Black ink permanent marker pen	VWR	52877-310		✓
Sorval LegendRT - Benchtop	Thermo Electron Corp	2701		✓
Bench Coat (Bench Protection Paper)	Fisher	12-007-186		✓
Small Autoclave waste bags 10”X15”	Fisher	01-826-4		✓
DNase AWAY	MBS	7010		✓
Rainin L-12-20 Pipet-Lite LTSmanual 12-CH	Rainin	L-12-20		✓
100% Ethanol	CW stores	23878		✓
70% Ethanol	In house	N/A	N/A	N/A
22R Microfuge Centrifuge	Bechman	22R Centrifuge	✓	
GeneAmp PCR System 9700	ABI	PCR System 9700	✓	
Power Supply, LVC2kW, 48VDCV	Tycos Electronics	RM200HA100	✓	
Razor Blades	VWR	55411-050		✓
NEBNext Ultra II Q5 Master Mix	NEB	M0544L		✓
Thermocycler Thetrad	MJ Researach			✓
10x BPB/XC loading buffer	In house	N/A	N/A	N/A □
100bp ladder	Invitrogen	Cat. No. 15628-19		✓
Dark Reader (Transilluminator)	InterSicence	DR-190M	✓	
Gel Elution buffer	In house	N/A	N/A	N/A
Ultrapure water (GIBCO)	Invitrogen	10977-023		✓
80% EtOH	In house	N/A	N/A	N/A
75% EtOH	In house	N/A	N/A	N/A
Coverall disposable gowns - large	CW stores	00026401		✓
Coverall disposable gowns - medium	CW stores	00026402		✓
VWR® Aluminum Foils (packs of 100)	VWR	60941-126		✓
Foil Tape	General Fasteners	34000740		✓
Elution Buffer (from MinElute PCR Purification Kit)	Qiagen	28004		✓
Hamilton Nimbus P50 tips	Hamilton Co.	235831		✓
Hamilton Nimbus P300 tips	Hamilton Co.	235832		✓
Spin-X Filter Columns	Costar	8160	✓	
Universal P50 Tips	Beckman	CABKA21586		✓

miRNA3 – Plate Format miRNA Library Construction	
Document #: LIBPR.0054	Supersedes: Version 14
Version: 15	Page 4 of 26

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

Large Kimwipes	Fisher Scientific	06-666-1A		✓
2mL safe-lock tubes	VWR International	CA20901-540		✓
Forma Laminar AirFlow Workstation	Fisher Scientific	15 465 267	✓	
Mini Vortex	VWR international	58816-121		✓
Tape pads: Adhesive plate sealer	Qiagen	19570		✓
ALPS 50V Microplate Heat Sealer	Thermo Scientific	AB-1443	✓	
EZPierce 20uM Thermal foil	ThermoFisher	AB1720		✓

Ligation adapters:

miRNA3 3' Adapter (modified at the 3' end - 2.5 µM working stock)
5' /5rApp/ ATCTCGTATGCCGTCTTCTGCTTGT /3ddC/

miRNA3 5' Adapter (1 µM working stock)
5' GUUCAGAGUUCUACAGUCCGACGAUCUGGUCAA3'

cDNA synthesis primer:

miRNA3 RT primer (20 µM working stock):
5' CAAGCAGAAGACGGCATAACGAGAT3' (Tm=58.9C)

PCR primers:

miRNA3 PCR Primer P2 (25 µM working stock):
5' CAA GCA GAA GAC GGC ATA CGA GAT 3'

96 different miRNA3 Indexed PCR Primers P1 (25 µM working stock – NNNNNN is the indexed sequence):

5' AATGATACGGCGACCACCGACAGNNNNNNGTTTCAGAGTTCTACAGTCCGA 3'

IX. Procedure

1. Initial Guidelines and Template QC

- 1.1. Work flow: All of the steps in this protocol up until the PCR set up are to be carried out in the designated RNA-work-only area. PCR setup is to be done in the PCR clean room.

miRNA3 – Plate Format miRNA Library Construction	
Document #: LIBPR.0054	Supersedes: Version 14
Version: 15	Page 5 of 26

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

To add the cDNA template to the PCR brew use the pre-PCR Nimbus located on the 5th floor (FX-5). Post PCR steps are to be carried out on the 6th floor.

- 1.2. All solutions should be aliquoted for single use only.
- 1.3. The starting material for this procedure is total RNA or alternatively the flow-through collected after passing total RNA through mRNA-retaining column.
- 1.4. Evaluate total RNA and flow-through fraction on the Caliper using *LIBPR.0052 – Operation and Maintenance of the LabChipGX for Total RNA Samples using the RNA Assay*. Alternatively, you can use the RNA Nano total eukaryotic RNA assay, refer to *LIBPR.0018 – Operation and Maintenance of the Agilent 2100 Bioanalyzer for RNA samples*. Please confirm with your supervisor which method to use.
- 1.5. For this procedure the RNA should be RIN ≥ 7.0 or RQS of ≥ 6.0 with detectable miRNA in the small RNA assay. The recommended starting amount is 1 μ g of total RNA in a 4 μ L volume. Samples may need to be topped up to 4 μ L with DEPC water. If samples are not above RIN7.0 or RQS 6.0 or there is no detectable miRNA in the small RNA assay, discuss with supervisor to see if samples are good enough for miRNA3 library construction.
- 1.6. Do not treat total RNA sample with DNase I prior to starting the miRNA3 protocol.
- 1.7. Please use VWR aluminum foil seals for incubations and PCR rxns and the foil tape for long term storage. Use the Qiagen clear seals for quick spins.

2. Retrieval of Reagents and Equipment Preparation

- 2.1. Retrieve ice and bring it to the 5th floor RNA area.
- 2.2. Put on clean pair of gloves and new disposable lab coat.
- 2.3. Wipe down the workbench, small equipment, and ice bucket with RNase Zap.
- 2.4. Lay down new bench coat.
- 2.5. Turn on the plate-based centrifuge and pre-chill to 4°C.
- 2.6. Retrieve metal blocks from the 4°C. Wipe them down with RNase Zap and place on ice.
- 2.7. Turn on the Tetrad. Using the ‘Instant’ Function, set alpha unit #1 to 70°C.

miRNA3 – Plate Format miRNA Library Construction	
Document #: LIBPR.0054	Supersedes: Version 14
Version: 15	Page 6 of 26

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

3. Template Transfer

- 3.1. Retrieve the plate containing total RNA or precipitated flow through from the -80°C storage location.
- 3.2. Without removing the foil cover, thaw it on ice.
- 3.3. Spin the plate down for 1 minute at 2000g at 4°C. Keep it on ice.
- 3.4. From each well, aliquot the starting amount and top up to 4uL if needed, into a new 96well AB1000 plate using the Rainin manual 12-channel P2 -20 pipette. Use new tips for each row and mark the side of the plate to keep track of the rows used.
- 3.5. Label the plate with the project, tray number, what is in the plate, rac ID (for TCGA samples), date, and your initials.

E.g. TCGA_tra12345_cDNA_rac65374_111108ND
Or Collaborator's name_tra34567_cDNA_111108ND
- 3.6. Seal both plates and spin down for 1 minute at 2000g at 4°C.
- 3.7. Return the stock plate to -80°C storage location and place the daughter plate on ice.
- 3.8. Add positive and negative controls to the plate. Please consult with the APC which controls should be used and their location on the plate. Use DEPC treated water to top up the volume of controls to 4µL when needed.
- 3.9. Your Supervisor will generate a barcode for the daughter plate. Place the barcode on the plate. **This will be the cDNA Template plate.** Please run this tra# through the “Start of Plate Library Construction” LIMS protocol (See Appendix A).

4. Ligation of 3' Adenylated Adapter

- 4.1. Retrieve one **300 µL aliquot of 2.5 µM 3' Adapter** per 96 well plate processed. Thaw at Room Temperature then immediately place it on ice. Discard leftover adapter. Once thawed, do not re-freeze the adapter again.

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

4.2. Vortex the adapter for few seconds then pulse spin. **Aliquot 25 µL of 3’Adapter into one row of a new 96-well plate which is going to be used as a reservoir.** Remove any air bubbles. Spin down the plate for 1 minute at 2000g at 4C. Keep it on ice.

4.3. Using Rainin 12-channel P2-20 pipette, **add 2 µL of 3’ Adapter into each well containing 4 µL of template.** After each addition, pipette up and down 15 times to mix. To depress full at the end, lift the tips above the liquid level and touch the side of the well. Use new tips for each row. Make sure that there is no liquid left in the tips. Seal the plate using PCR cover and spin down at 2000g for 1 minute at 4°C. Place the plate on ice.

4.4. Current content in each well:

Table 1: Current content in each well

Reagent	Volume
Total RNA or flow through (1µg)	4.0 µL
2.5 µM 3’ miRNA3 adapter	2.0 µL
Total volume	6.0 µL

4.5. Incubate the spun down RNA template + 3’Adapter plate at 70°C for 2 minutes in a Tetrad thermocycler.

4.6. Snap chill on ice for 1 minute

4.7. To prepare Truncated Ligase Brew, retrieve DEPC treated water and 10x T4 RNL2 **truncated** buffer (contains MgCl₂) and thaw at RT. Immediately place them on ice once thawed. Keep enzymes in the freezer until you are ready to add them to the brew.

4.8. Vortex and pulse spin 10x T4 RNL2 **truncated** Ligase buffer.

4.9. Gently flick and pulse spin T4 RNL2 **truncated** Ligase and RNaseOUT enzymes.

4.10. Generate barcode for 3’ Adapter Ligation Brew Mix in LIMS. Under Mix Standard Solution, select “[miRNA3_96_3p_AdapterLig](#)” to set up 3’Adapter Ligation brew.

4.11. Retrieve large barcode and reagent check list from 6th floor printers. Place both in your lab book.

4.12. If LIMS is not working, please use the EXCEL spreadsheet calculator located in directory path: [Library Core\Work Sheets and Calculators\miRNA\Manual](#).

miRNA3 – Plate Format miRNA Library Construction	
Document #: LIBPR.0054	Supersedes: Version 14
Version: 15	Page 8 of 26

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

- 4.13. Add reagents in a 1.5mL tube as listed from top to bottom on the generated checklist. Check off each reagent as they are added to the brew mix.
- 4.14. **Aliquot 38 μ L of the brew into one row (12 wells) of a 96 well AB-1000 brew source plate.** This plate is going to be used as a reservoir.
- 4.15. Spin down both sample plate and brew plate at 2000g for 1 minute at 4°C.
- 4.16. On the tetrad, open the ‘MIRNA’ file. Choose program **3LIG**. Enter in ‘10 μ L’ as the volume and choose alpha unit #4 to run the program.
- 4.17. Once the ‘Sample Temperature’ reaches 22°C, click on ‘Pause’.
- 4.18. Using a Rainin 12-channel P2-20 **add 4 μ L of the brew to each reaction well containing denatured RNA template+adapter.**
- 4.19. Use new tips for each row. After each addition pipette up and down 15 times to mix.
- 4.20. To depress full at the end, lift the tips above the liquid level and touch the side of the well. Make sure there is no liquid left in tips.
- 4.21. Quick spin plate at 2000g for 1 minute at 4°C.
- 4.22. Check the volumes on the plate.
- 4.23. Place the plate in alpha unit #4 and tighten the head.
- 4.24. Click on ‘Resume’. **Incubate at 22°C for 1h using tetrad on program 3LIG.**

5. Ligation of the 5’ miRNA adapter

- 5.1. While the plate is incubating, retrieve one **300 μ L aliquot of 5’miRNA3 Adapter 1uM** per each 96-well plate processed.
- 5.2. Thaw at RT then immediately place it on ice. Vortex the adapter for few seconds then pulse spin.

miRNA3 – Plate Format miRNA Library Construction	
Document #: LIBPR.0054	Supersedes: Version 14
Version: 15	Page 9 of 26

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

- 5.3. **Aliquot 25 μ L/well of 5' Adapter into one row (12 wells) of a 96-well AB-1000 plate to be used as a reservoir.** Quick spin the plate for 1 minute at 4°C at 2000g. Keep plate on ice.
- 5.4. Retrieve and thaw **10 mM ATP**. Once thawed, vortex, pulse spin then place it on ice.
- 5.5. With 5 minutes remaining of the previous 3' Adapter ligation, heat the plate containing 5' miRNA Adapter at 70°C for 2 minutes in a tetrad cyclor (using Instant temp option: 70°C with heated lid) then immediately transfer to ice. Keep on ice for 1 minute then spin at 2000g at 4°C for 1 minute.
- 5.6. Using a Rainin 12 channel P2-20 or robot, **add 2 μ L of 5' miRNA Adapter into each reaction well.**
- 5.7. Use fresh tips for each row. After each addition, pipette up and down 15 times to mix. Make sure no liquid is left in each tip. To depress full at the end lift the tips above the liquid level and touch the side of the well.
- 5.8. Seal with plastic cover and spin down at 2000g for 1 minute at 4°C.
- 5.9. On the tetrad, open the 'MIRNA' file. Choose program **37LIG**. Enter in '14 μ L' as the volume and choose alpha unit #4 to run the program.
- 5.10. Once the 'Sample Temperature' reaches 37°C, click on 'Pause'.
- 5.11. To make the Ligase-ATP Brew Mix: Keep the RNA Ligase enzyme in the freezer until you are ready to add it to the brew (ATP). Gently flick and pulse spin the enzyme before adding to the brew.
- 5.12. Use LIMS to make the brew mix. Under "Mix Standard Solution" use the calculator called "[miRNA3_96_5p_LigaseMix](#)" to make brew.
- 5.13. Retrieve barcode and Reagent Check List from printers. Place in lab book.
- 5.14. If LIMS is not working, please use the EXCEL spreadsheet calculator located in directory path: [Library Core\Work Sheets and Calculators\miRNA\Manual](#).
- 5.15. **Aliquot 20 μ L brew/well to one row (12 wells) of a 96-well AB-1000 reservoir plate for Ligase-ATP brew addition.** Spin down and keep on ice.

miRNA3 – Plate Format miRNA Library Construction	
Document #: LIBPR.0054	Supersedes: Version 14
Version: 15	Page 10 of 26

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

- 5.16. Using Rainin 12 channel P2-20 **add 2 μ L of the RNA Ligase-ATP brew into each reaction well.**
- 5.17. After each addition, pipette up and down 15 times to mix. Make sure no liquid is left in each tip.
- 5.18. Seal with Applied Biosystems PCR cover and spin down at 2000g for 1 minute at 4°C.
- 5.19. Click ‘Resume’. **Incubate the reaction at 37°C in a tetrad cyclor for 1 hour on the 37LIG program.**
- 5.20. When the 5’ adapter ligation is complete, remove reaction plate from the tetrad and spin down at 2000g at 4°C for 1 minute. Transfer double adaptered RNA plate to ice.

6. cDNA Synthesis of Double Adaptered miRNA

- 6.1. Retrieve and thaw 1 tube of **300 μ L of small RT primer 20 μ M working stock.** Vortex RT primer for few seconds then pulse spin. **Aliquot 25 μ L/well into one row (12 wells) of a 96-well AB-1000 brew source plate.** Keep it on ice.
- 6.2. Using Rainin 12 channel P2-20 Barrier **add 2 μ L of “miR3 RT primer” into each reaction well of double-adaptered miRNA.**
- 6.3. After each addition, pipette up and down 15 times to mix. Make sure no liquid is left in each tip.
- 6.4. Seal the plate with Applied Biosystems PCR cover. Spin down 2000g at 4°C for 1 minute.
- 6.5. Change the temperature of α -unit#1 to 65°C using the ‘Instant’ Function.
- 6.6. Incubate the plate at 65°C for 10 minutes.
- 6.7. While the plate is incubating make and barcode First Strand Brew Mix.
- 6.8. Retrieve 1 tube of each per 96-well plate: **5x first strand buffer, 10mM dNTPs, 100mM DTT** at room temp then transfer to ice.
- 6.9. Vortex buffer, dNTPs and DTT then pulse spin.

miRNA3 – Plate Format miRNA Library Construction	
Document #: LIBPR.0054	Supersedes: Version 14
Version: 15	Page 11 of 26

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

- 6.10. Gently flick and pulse spin Reverse Transcriptase and RNaseOUT before adding to the brew.
- 6.11. Make the First Strand Brew Mix, “miRNA3_96_RT_1st_Strand”, in LIMS.
- 6.12. Retrieve barcode and Reagent Check List from the printer and place in your book.
- 6.13. If LIMS is not working, please use the EXCEL spreadsheet calculator located in directory path: [\Library Core\Work Sheets and Calculators\miRNA\Manual](#).
- 6.14. Combine reagents from top to bottom as shown in Reagent Check List in a 1.5mL tube. **Keep on ice.**
- 6.15. **Aliquot 126 µL of the First Strand brew mix into one row (12 wells) of a 96-well AB-1000 brew source plate.** Keep it on ice.
- 6.16. Once the 65°C 10 minute incubation is complete, remove reaction plate from the Tetrad cyclor and immediately quench plate on ice for 1 minute.
- 6.17. Spin the plate down at 2000g at 4°C at for 1 minute.
- 6.18. On the tetrad, open the ‘MIRNA’ file. Choose program **SSIIRT**. Enter in ‘30 uL’ as the volume and choose alpha unit #4 to run the program.
- 6.19. Once the ‘Sample Temperature’ reaches 44°C, click on ‘Pause’.
- 6.20. **Using Raining 12 channel P2-20 Barrier add 14 µL of First Strand brew mix into each reaction well.**
- 6.21. After each addition pipette up and down 15 times to mix. Make sure no liquid is left in the tips.
- 6.22. Seal the plate well with Applied Biosystems PCR cover. Spin at 2000g at 4°C at for 1 minute.
- 6.23. Click ‘Resume’ on the tetrad. **Incubate at 44°C for 1 hour (program SSIIRT).**

miRNA3 – Plate Format miRNA Library Construction	
Document #: LIBPR.0054	Supersedes: Version 14
Version: 15	Page 12 of 26

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

6.24. *Please complete the LIMS portion of the protocol, “Start of Plate Library Construction” and “miRNA3_cDNA”. Refer to Appendix A.*

NOTE 1: This is a safe stopping point.

NOTE 2: This is the last step in the RNA work only area.

7. PCR Amplification with Indexed Primers Added Separately

- 7.1. This step must be carried out in the clean room/PCR set up area.
- 7.2. Put on a clean pair of gloves and wipe down pipettes, small equipment, and ice bucket with DNase away.
- 7.3. **iPCR Brew is made without primers in 510 PCR Clean Room – Laminar Hood.**
- 7.4. **PCR Primer P2 is added to brew in 511 Tissue Culture B Room - Laminar Hood.**
- 7.5. **Indexed P1 Primers (25 μ M) are then added (2.5 μ L/well) on the Nimbus robot on the 5th floor.**
- 7.6. **cDNA Template (15 μ L/well) is added last using the Nimbus robot on the 5th floor.**
- 7.7. **510 PCR Clean Room:** Use 80% EtOH to wipe down the hood and pipettes.
- 7.8. Retrieve ice and place in the ice bucket. Put on a new disposable lab coat and new gloves. Turn on the laminar hood.
- 7.9. Thaw 5x HF buffer and 10 mM dNTP's on ice.
- 7.10. Keep dH₂O and DMSO at Room Temperature.
- 7.11. **511 Tissue Culture Room:** Retrieve ice and place in the ice bucket. Put on a new disposable lab coat and gloves. Turn on the laminar hood.
- 7.12. Decontaminate Room 510 Forma Laminar Airflow Workstation by wiping down the hood and pipettes with DNaseAway.
- 7.13. Retrieve a tube of PCR Primer P2 from -20°C. Thaw on ice.
- 7.14. Thaw indexed P1 primer plate on ice next to FX-5.

miRNA3 – Plate Format miRNA Library Construction	
Document #: LIBPR.0054	Supersedes: Version 14
Version: 15	Page 13 of 26

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

- 7.15. In the 5th floor 510 PCR Clean Room, vortex and spin down all of the tube solutions.
- 7.16. Don't retrieve the Q5 Master Mix until you are ready to add it to the brew.
- 7.17. Gently flick and pulse spin Q5 Master Mix just before adding it to the brew.
- 7.18. In LIMS calculate iPCR Brew Mix. Select “miRNA3_Q5 iPCR brew” calculator, select # of plates and wells. Click “Mix Standard Solution” green button.
- 7.19. Retrieve barcode and Reagent Check List from the printer and place in your book.
- 7.20. If LIMS is not working, please use the EXCEL spreadsheet calculator located in directory path: [Library Core\Work Sheets and Calculators\miRNA\Manual](#).
- 7.21. Mix the brew well and leave on ice.
- 7.22. Put reagents away to its appropriate location. Discard any waste. Clean the hood by wiping it down with 80% EtOH. Turn off the laminar hood.
- 7.23. Transfer the brew tube on ice to the 511 Tissue Culture Room.
- 7.24. Vortex and spin down the P2 PCR Primer. Add the appropriate amount of P2 PCR Primer to iPCR brew in the Room 511 Tissue Culture Room laminar flow hood. Mix the brew well by vortexing.
- 7.25. Dispense 32.5 µL per well of the brew into 96-well AB-1000 PCR plate using a distri-man. Cover with plastic seal and store it on ice.
- 7.26. **Use a PCR-free Nimbus on the 5th floor to add 2.5 µL/well of miRNA3 P1 Indexed Primers (25 µM) and 15µL/well of the synthesized cDNA to the iPCR reaction brew plate.**
- 7.27. Run the Nimbus liquid transfer program twice (first for Index primer addition, and a second time for cDNA template addition, to PCR brew:

<i>Open file: Production > stay in methods > Transfer to AB1000</i>
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Non-Controlled Version *Note: Controlled Versions of this document are subject to change without notice

Follow the prompts using the table below as guideline:				
P1 Indexed Primers (25µM) addition to PCR Brew				
Liquid Type	Destination Plate Type	Source Plate Type	Destination Plate Volume	Transfer Volume
Aqueous (water, Primer)	AB1000	AB1000	32.5 µL	2.5 µL
cDNA Template Addition to PCR Brew				
Viscous (Brews)	AB1000	AB1000	35 µL	15 µL

- 7.27.1. Review the final entered Nimbus parameters carefully before proceeding to the display deck for set up.
- 7.28. After the program is completed, cover iPCR brew plate with plate cover. Cover the stock primer plate with new foil tape.
- 7.29. Spin down the PCR brew plate after index primer addition at 2000g for 1 minute at 4°C and dispose of the tips.
- 7.30. Return primer plate to its -20°C storage location.

NOTE: Primer plate can only be thawed up to 4 times so mark the plate after each use and discard if it has 4 markings. If you thaw the plate but decide not to use it that day – you need to mark it as if used.

*Heat seal the reaction plate using Adhesive foil EZPierce 20uM Thermal foil (Cat. No. AB1720, Thermo Fisher). The equipment used for this is ALPS 50 V Microplate Heat Sealer (Cat. No. AB-1443, Thermo Scientific). Please see Appendix C for instructions.

- 7.31. Seal leftover cDNA plate with new piece of 3M foil tape. Mark top with a marker so it is known it has been used once. Store template in -20°C in the appropriate rac.
- 7.32. Place the plate in the Tetrad (in the 6th floor PPGP area) and close the lid. Ensure good but not overly tight seal. Select and start **“MIR3_15” PCR program.**

miRNA3 – Plate Format miRNA Library Construction	
Document #: LIBPR.0054	Supersedes: Version 14
Version: 15	Page 15 of 26

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

PCR Conditions for ‘MIR3 15’:

98°C	30 sec	} total of 15 Cycles of PCR
98°C	15 sec	
62°C	30 sec	
72°C	15 sec	
72°C	5 min	
4°C forever		

7.33. When PCR reaction is complete, remove the plate from Tetrad cycler and place it on ice for 1 minute then spin it down at 2000g at 4°C for 1 minute.

7.34. If necessary, store at 4°C when overnight or at -20°C if for a longer period.

8. QC PCR Product and Pooling

8.1. Retrieve ice. Put on clean pair of gloves and lab coat.

8.2. Wipe down the workbench, small equipment, and ice bucket with DNaseAway.

8.3. Lay down new bench coat.

8.4. You will use 5 µL of PCR product in 25µL of EB for a 1/6 dilution to do the Caliper QC. Refer to SOP *LIBPR.0051 ‘Operation and Maintenance of the Caliper LabChip GX for DNA Samples using the High Sensitivity Assay’* to QC each well of your PCR plate. The dilution can be done on a 6th floor Nimbus using the "Transfer to AB1000" program.

8.5. Check with the APC if you need to spot check some wells on the Agilent. If you need to spot check some wells on the Agilent, remove 1 uL of PCR product from the appropriate wells for QC and dilute with 1uL of Ultrapure water for a 1/2 dilution. Perform the DNA1000 Series II assay. Refer to *LIBPR.0017 – Operation and Maintenance of the Agilent 2100 Bioanalyzer for DNA Samples*. Attributes do not need to be filled in for the DNA Agilent run generated.

8.6. Discuss the Caliper and Agilent results of your PCR with the APC.

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

NOTE: The samples will be size selected using the Barracuda. Typically 16 wells of a plate make up 1 pool by using 14uL from each well. However, there may be times when the Collaborator will want a different pooling strategy. Your Supervisor will inform you of changes to the pooling strategy and the pooling amounts.

See example below for pooling and rearray:

8.7. Pool each set of 16 wells into a separately, labeled 1.5 mL tube taking 14 μ L from each well (See diagram below). Use a fresh P200 tip each time. You should be left with 6 pools (~224 μ L each). When physically pooling the samples, mark or dot the well as you pool each set of 16 wells to prevent duplication or contamination. Use a new box of tips to do the pooling as a way of gauging which wells have been pooled. Seal the plate with 3M foil tape. Label with the library name, iPCR, date and initials.

16 wells will be pooled to make 6 pooled indexed miRNA3 libraries

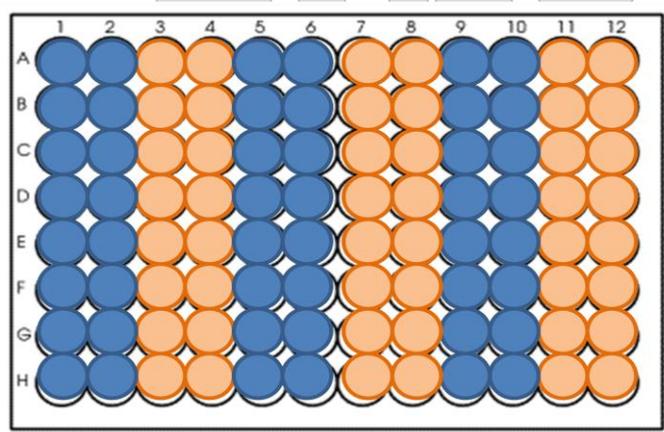


Figure 1: 16 wells to be Pooled

8.8. Manually re-array 26 μ L of your pooled libraries back to the appropriate wells in a new 96-well Abgene 1000 plate. Each of the 8 wells now has a mix of all 16 samples. Seal the plate with 3M foil tape. Label with the library name, iPCR, date and initials.

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

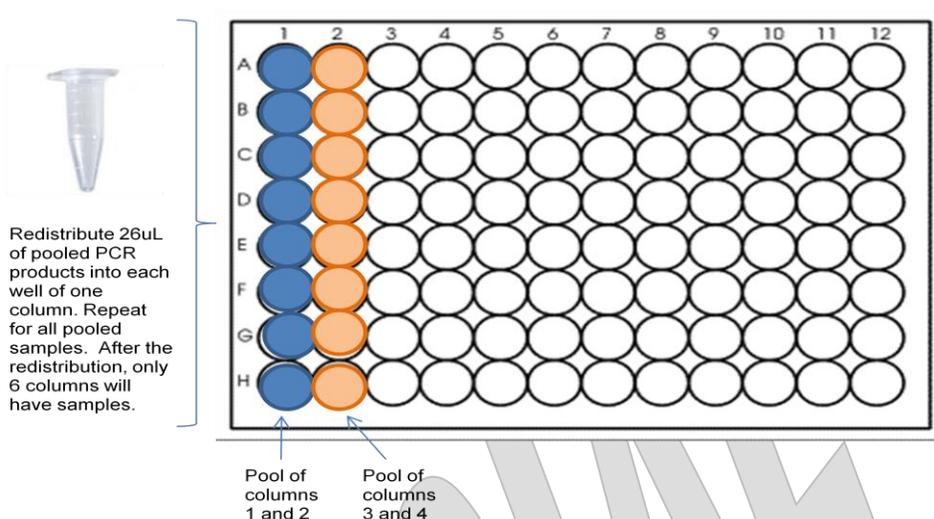


Figure 2: Re-Array 26µL of your pooled libraries back to the appropriate wells in a new 96-well Abgene 1000 plate

- 8.9. Quick-Spin the plate for 1 minute at 2000g at 4°C.
- 8.10. Store the plate in -20°C in the appropriate rac.
- 8.11. *Please complete the LIMS portion of the protocol, “miRNA3_PCR” (Appendix A).*

Note: Please proceed to pouring gels for Barracuda Size selection. Refer to LIBPR.0055 Manually pour agarose gels for Barracuda DNA size selection. In rare cases manual size selection may be needed (see Appendix B). Your supervisor will let you know if this is the case.

Note: For preparing your PCR plate for Barracuda size selection, please refer to LIBPR.0090 DNA Size Selection on the Barracuda Robot II . After preparing your plate, store it at -20C in the appropriate rac until you are ready to load the plate onto the Barracuda. Please complete LIMS, “Plate_Barracuda_Size_Selection” (Appendix A).

Note: Please pool your samples in LIMS (Appendix A).

Note: Please refer to LIBPR.0090 DNA Size Selection on the Barracuda Robot II for precipitation of the Barracuda size selected product. Please complete LIMS, “Plate_Barracuda_PPGP” (Appendix A). Attach the MX barcodes and label with PPGP-15X and store in the appropriate rack

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

Table 2: PPT Brew Mix for samples post-Barracuda

Reagent	volume per tube (uL)	volume per 13 tubes (uL)	volume per 25 tubes (uL)
Eluate	400		
3M NaOAc (1/10)	40	520	1000
mussel glycogen	3	39	75
100% EtOH (2.5 x eluate)	1000	13000	25000
total brew	1043	13559	26075
brew aliquot per tube	1043	1043	1043

9. Agilent / Qubit area:

- 9.1. Run the Agilent DNA 1000 Series II assay of size selected product. Refer to *LIBPR.0017 – Operation and Maintenance of the Agilent 2100 Bioanalyzer for DNA samples* to generate the Bioanalyzer runs for your set of samples.
- 9.2. Check the Agilent profile for correct peak size. The expected miRNA product of 105 bp runs on Agilent Bioanalyzer at about 108-118bp. If there are 2 prominent peaks then the average must be taken (confirm with supervisor if unsure). The DNA concentration will be based on the Qubit quant. Discuss the results with APC. Fill in the following 2 attributes when you are “analyzing” your Agilent runs:

DNA_concentration_ng_uL = the concentration of your miRNA from the Qubit quant

Avg DNA bp size = the size of the miRNA peak from the Agilent profile

- 9.3. Please make sure the volumes for the stock PPGP samples are entered so LIMS can calculate the molarity for you. This molarity is the stock nM concentration.
- 9.4. *Please complete LIMS, “Plate_PPGP_QC” (Appendix A).*
- 9.5. Use the calculator worksheet located in the folder to calculate the dilution factor necessary to reach 20nM. R:\Library Core\Work Sheets and Calculators\Plate Based\miRNA\miRNA_calculate molarity for submitting samples_120717ND.

miRNA3 – Plate Format miRNA Library Construction	
Document #: LIBPR.0054	Supersedes: Version 14
Version: 15	Page 19 of 26

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

9.6. Enter the amount of sample you want to dilute in the ‘amount to be used for dilution (µL)’. Once the amount is entered, dilute the DNA according to the dilution factor generated by the spreadsheet calculator using Qiagen EB buffer. Dilution of the stock is unnecessary if the concentration is 37nM or less.

NOTE: If possible, submit at least 20 µL of the diluted sample.

9.7. Based on the dilution factor, dilute accordingly. If you are unsure of what final volume is needed, consult with APC.

9.8. Quantify 1 µL of the diluted product with the Qubit as per the SOP ***LIBPR.0030 Quantifying DNA Samples on Qubit Fluorometer.***

9.9. Enter the Qubit data into the appropriate sample fields in the calculator worksheet located in the folder to calculate the ‘actual’ nM of your samples. [\Library Core\Work Sheets and Calculators\miRNA\Library Submission Forms](#). The calculation for the molarity is

$$\text{nM} = \text{concentration (ng/}\mu\text{L)} * 1000000 / (\text{bp} * 660)$$

9.10. Please save this worksheet in folder [R:\Library Core\Work Sheets and Calculators\miRNA\Library Submission Forms](#). It should be saved as MX#+date+initials (eg MX0893-MX0904_130117ND).

9.11. A concentration range of 2-37 nM is acceptable. Concentration outside of this range is sometimes acceptable too, discuss with Supervisors in those cases.

9.12. Record the ‘actual’ concentration (nM) on each tube label both on the lid and again on the side of the tube. Place the tubes in the appropriate ‘Next Solexa Run’ box located in the -20C freezer.

9.13. Clean up your work areas. Remove the benchcote. Dispose of waste. Wipe down the workbench, small equipment, and ice bucket with DNaseAway. Place any used racks in the bucket of diluted bleach.

9.14. Return unused stock reagents back into the appropriate location.

9.15. Ensure that all samples are put away in the appropriate location.

9.16. ***Please complete LIMS, “Final_Submission”. Ensure that the final pipeline is ISE: ISE. (Appendix A).***

miRNA3 – Plate Format miRNA Library Construction	
Document #: LIBPR.0054	Supersedes: Version 14
Version: 15	Page 20 of 26

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

Appendix A- LIMS Protocols

1. Starting Plate Library Construction: MIR_3.0: miRNA 3.0
2. miRNA3_cDNA: MIR_3.0: miRNA 3.0
3. miRNA3_PCR: MIR_3.0: miRNA 3.0
4. Caliper run: no attributes to upload
5. Plate_Barracuda_Size_Selection: MIR_3.0: miRNA 3.0
6. Manually Pooling in LIMS
7. Plate_Barracuda_PPGP: MIR_3.0: miRNA 3.0
8. Bioanalyzer run: enter values for **DNA_concentration_ng_uL** and **Avg DNA bp size**
9. Final_Submission: ISE:ISE

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

Appendix B- Manual Size Selection

1. Manual Size Selection - PAGE Gel Electrophoresis

Note: If we presume miRNA size is 22nt, the constructed actual miRNA library size would be 108bp. But it runs at 105bp in gels according to DNA ladder, and runs around 115bp in Agilent DNA chip, so both 105bp and 115bp appeared in the following text.

- 1.1. Prepare one 12% polyacrylamide gel per each pool you want to size select. Follow the recipe below for gel preparation. Make sure to mix the mixture well before addition of the last two components and then again after APS and TEMED addition.

Table 3: 12% POLYACRYLAMIDE GEL:

REAGENT	Per GEL
Ultrapure dH ₂ O	23.5 mL
40% Polyacrylamide (19:1 acrylamide:bis)	10.5 mL
50x TAE	700 µL
10% Ammonium Persulfate (APS)	350 µL
TEMED	30 µL

- 1.2. Pour gel onto gel casting apparatus. Let the gel polymerize for at least 40 minutes.
- 1.3. Set up the PAGE apparatus with cold water circulation and attach a label of colored tape to the gel plates, indicating the library name, date, and your initials.
- 1.4. Load 20 µl of the 10 bp DNA Ladder (20ng/µL) into one well and 20 µL of 25 bp DNA Ladder (20ng/µL) into an adjacent well near the center of the gel. Make sure there is no residual ladder on the outside of the tip when loading ladder into the well.
- 1.5. Add 20 µL of the loading dye to each of the pools.
- 1.6. Split each pool over many wells – one pool per gel. On each gel load 20 µL/well into wells on both sides of the ladders. Avoid the very edge wells if possible.
- 1.7. Immediately after loading, run the gel @ **200 V** for 6 hours. Change the running buffer half way through the run.
- 1.8. Using colored tape, attach a label to the gel apparatus which states the Pool name, start time, finish time, date, and your initials).

miRNA3 – Plate Format miRNA Library Construction	
Document #: LIBPR.0054	Supersedes: Version 14
Version: 15	Page 22 of 26

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

1.9. Dispose of all waste.

2. Gel Scan and Cutting DNA Fraction from PAGE

- 2.1. Put on a clean pair of gloves.
- 2.2. Set up sets of 0.5 mL and 2 mL tubes for shearing the gel slices: 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. You will need one set of tubes per pool.
- 2.3. Label each 2 mL tube on the side with the pool, name, size fraction, date, and initials.
- 2.4. Cover the Dark Reader screen with a fresh sheet of plastic wrap.
- 2.5. Prepare fresh stain: 10 μ L stock in 100 mL 1x TAE. Minimize exposure to light.
- 2.6. Stop the gel run after 6 hours and dismantle the PAGE apparatus.
- 2.7. Using a clean post PCR dedicated tray, stain the gel for at least 3 minutes.
- 2.8. Check the staining on the dark reader (ladders should be clearly visible). If it is stained well, retrieve the gel from the staining solution and place onto the Mylar sheet.
- 2.9. Log onto the computer and scan image on high sensitivity setting. Store the image in the appropriate network directory and file folder.

**Name the file with Library Name_size selection__DateInitials,
i.e.MX050_ size selection gel_070315mm.gel**
- 2.10. Print the image and paste it into your lab notebook.
- 2.11. Lay the Mylar sheet with the gel on top onto the Dark Reader and cut out the region containing miRNA which is around 105 bp. Make the first cut with the blade at around 100bp. The second cut should be above 105 bp mark.

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

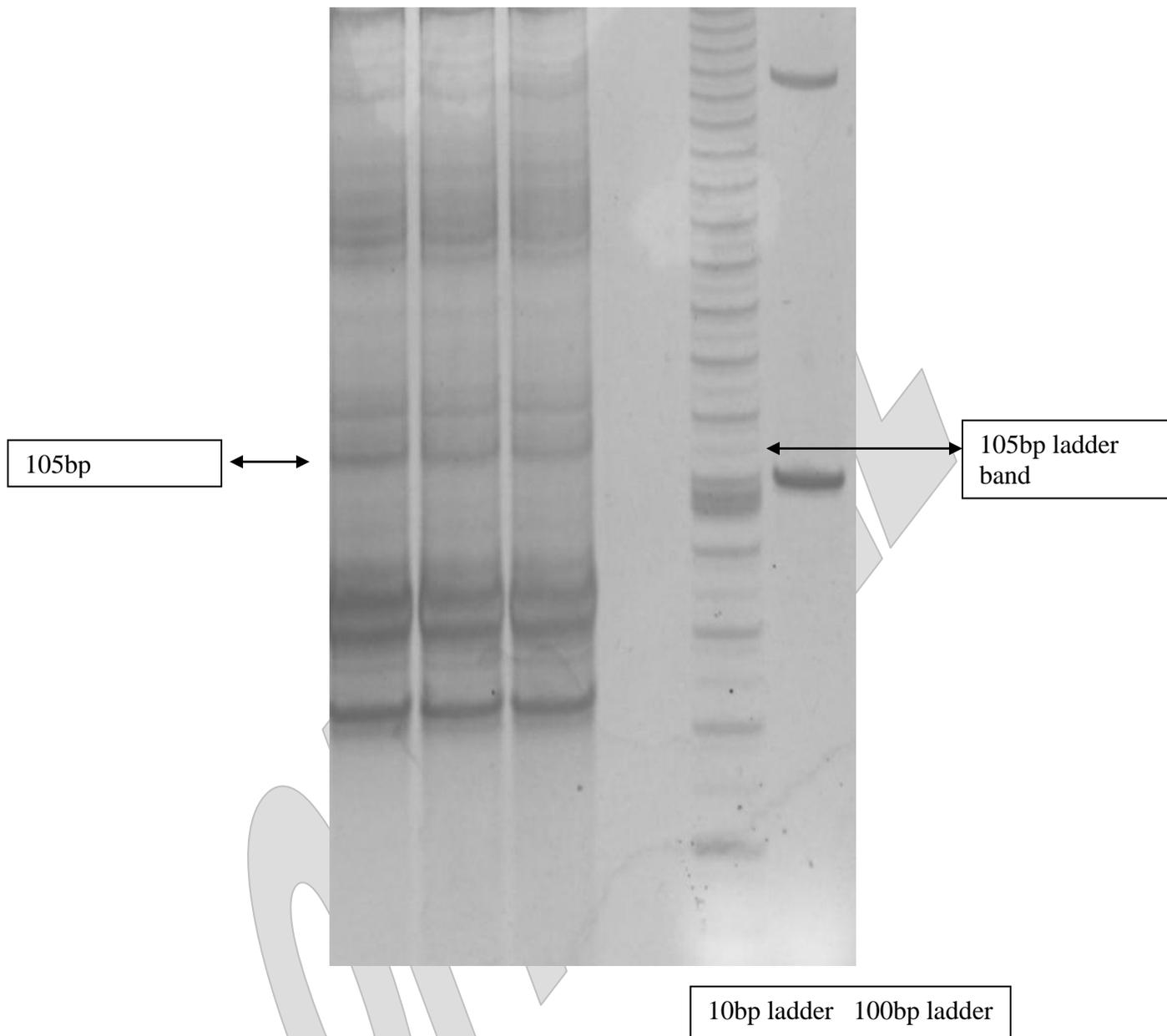


Figure 4: Gel Scan and Cutting DNA Fraction from PAGE

- 2.12. Each well should look as above. If any wells are not resolved and have smears that mix adapter band edges (running at about 85bp) with miRNA band, exclude that well. Combine gel slices of each pool into separate 0.5mL tube prepared and labeled earlier for shearing.

miRNA3 – Plate Format miRNA Library Construction	
Document #: LIBPR.0054	Supersedes: Version 14
Version: 15	Page 24 of 26

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

- 2.13. Discard stain, spray with water, and wipe down tray. Discard Mylar sheet and remainder of the gel. Discard used blade in sharps container. Tidy up area. Change gloves.
- 2.14. Spin tubes at 12,000 rpm @ RT for 3 minutes. The gel slices should shear through the holes and collect into the bottom of the 2mL tubes.
- 2.15. After shearing check that all of the gel has cleared the 0.5 ml tubes. If no gel remains, discard the 0.5 mL tubes and add 500 µL of Elution buffer (5:1, LoTE:7.5M Ammonium Acetate) to each gel slurry. Mix well by vortexing. Pulse-spin.
- 2.16. If time permits, incubate for 1 hour at 65°C to elute DNA. If there is insufficient time to continue, incubate overnight at 4°C.
- 2.17. Clean PAGE apparatus: Run tap water over PAGE apparatus for 2 minutes; wipe down with 2% micro90; run water over PAGE apparatus for another 2 minutes. Wipe down the PAGE workstation.
- 2.18. *Please complete LIMS, “Plate_Manual_Size_Selection”*

3. Precipitate and Purify miRNA products

- 3.1. Put on a clean pair of gloves.
- 3.2. Retrieve the gel slurries from the previous day’s PAGE gel from 4°C.
- 3.3. Vortex and pulse spin.
- 3.4. Heat the gel slurries at 65°C for 15 minutes in the preheated heat block.
- 3.5. 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.
- 3.6. Check each Spin Column tube and ensure that all of the buffer has spun through the filter. Re-spin the tubes if there is still liquid trapped in the gel material.

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

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:

Table 4: Reagents for EtOH Precipitation

REAGENT	VOLUME
Eluate	500 µL
3 M Sodium Acetate	50 µL
Mussel Glycogen (20 mg/mL)	3 µL
100 % Ethanol	1250 µL
TOTAL VOLUME	1803µL

- 3.7. Vortex and pulse spin. Chill the tubes at -20°C for a minimum of 30 minutes.
- 3.8. Spin at 14,000 rpm at 4°C for 30 minutes.
- 3.9. Wash the pellet with 1 mL of 70% EtOH by adding the EtOH solution and inverting the tube. Make sure 70% EtOH is premade and QC'd. Spin at 14,000 rpm / 4°C for 2 minutes. Discard the supernatant as previously.
- 3.10. Repeat the 70% EtOH wash.
- 3.11. 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.
- 3.12. Allow the tube to air-dry for approximately 5 to 10 minutes at room temperature, until the white precipitate becomes translucent and is no longer visible.
- 3.13. 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. The APC will advise if changes to the volume are necessary.

3.14. *Please complete LIMS, "Plate_Manual PPGP".*

3.15. Please continue on to Section 10 (Agilent/Qubit area).

miRNA3 – Plate Format miRNA Library Construction	
Document #: LIBPR.0054	Supersedes: Version 14
Version: 15	Page 26 of 26

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

Appendix C – ALPS 50V Microplate Heat Sealer

NOTE: The seals should be stored in the foil seal packaging to maintain proper orientation. Failure to orient the foil seal with the adhesive side down in the plate sealer will result in the seal adhering to the instrument rather than the plate.

1. Turn on the ALPS 50V heat sealer and allow the instrument to warm up. The Heat on/off LED will flash during this time and stay on once the desired temperature is reached. The sealer should be pre-set for 165°C, 3 second seal time.
2. Place the foil seal on top of the input plate (shiny side up).
3. Place the plate on the plate carrier so that well A1 is in the back left corner. Avoid touching the heating surface while loading the sample plate to prevent injury.
4. Grasp the handle and lower to thermally compress the foil seal onto the input plate. Do NOT apply more pressure to the handle than necessary. When the correct pressure is achieved, an audible tone will sound and the timer will count down to zero.
5. Once the timer reaches zero, another audible tone will sound. Raise the handle to release the heater plate.
6. Rotate the plate so that well A1 is in the front right corner (H12 will be in the back left corner), and repeat the sealing steps 4 and 5.
7. Use a roller seal to ensure that all wells are properly sealed.
8. Put a thermal pad on top of the output plate, then close and tighten the lid.