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# Automated MicroRNA Library Construction using randomized adapters for Illumina Sequencing

## I. Purpose

To prepare microRNA(miRNA) sequencing libraries from total RNA or FFPE total nucleic acid samples on Hamilton NIMBUS in a 96-well plate format. The protocol is capable of capturing small RNAs in the range of 20-30nt, but the analysis pipeline is reporting mainly microRNAs (miRNAs) at the moment. Randomized adapters are expected to increase ligation efficiency and reduce the biased selection of miRNA species. The randomer sequences can be used to filter duplicate reads in silico. In addition, libraries prepared using this SOP are compatible with PE sequencing primers.

## II. Scope

All procedures are applicable to the BCGSC Library TechD and Production 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 QA associate.

## IV. Responsibility

It is the responsibility of all personnel performing this procedure to follow the current protocol. It is the responsibility of the Library Core Group Leader to ensure personnel are trained in all aspects of this protocol. It is the responsibility of Quality Systems Team 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 v 1.5 protocol | Illumina © 2009 |

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## 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 |
| Quantifying DNA Samples using the Qubit Fluorometer | LIBPR.0030 |
| Operation and Maintenance of the LabChip GX for RNA Samples using the HT RNA Assay | LIBPR.0052 |
| Operation and Maintenance of the LabChip GX for DNA Samples using the High Sensitivity Assay | LIBPR.0051 |
| Span-8 Pooling of DNA Samples | LIBPR.0093 |

## 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_6N, 1µM | IDT | NA | | |
| 3' adenylated adapter_6N, 2.5µM | IDT | NA | | |
| RT primer, 20µM | IDT | NA | | |
| PE 1.0 primer, 25µM | IDT | NA | | |
| Indexed primers, 25µM | IDT | NA | | |
| T4 RNA ligase 2, truncated (200U/µL) | NEB | M0242L | | ✓ |
| 50% PEG 8000 | NEB | M0242L | | ✓ |
| 10X T4 RNA Ligase buffer | NEB | M0242L | | ✓ |
| T4 RNA Ligase (5U/µL) | Ambion | AM2141 | | ✓ |
| 10 mM ATP, molecular grade | NEB | 9804 | | ✓ |
| RNase Out | Invitrogen | 10777-109 | | ✓ |

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| | | | | |
|---|---|---|---|---|
| DEPC-treated water | Ambion | 9922 | | ✓ |
| H Minus RT, 200U/µL | ThermoFisher | EP0753 | | ✓ |
| 5X RT Buffer | ThermoFisher | EP0753 | | ✓ |
| dNTPs Mix, 10µM | Invitrogen | 46-0519 | | ✓ |
| DMSO (Dimethyl sulfoxide) | Fisher Scientific | BP231-100 | | ✓ |
| Phusion HS high fidelity DNA pol | NEB | F-540L | | ✓ |
| RNase Zap | Ambion | 9780 | | ✓ |
| DNA*AWAY* | MBS | 7010 | | ✓ |
| 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 | | ✓ |
| Bench Coat (Bench Protection Paper) | Fisher | 12-007-186 | | ✓ |
| Small Autoclave waste bags 10"X15" | Fisher | 01-826-4 | | ✓ |
| Rainin RNase free tips (1-20µL) | Rainin | RT-L10F | | ✓ |
| Rainin RNase free tips (20-200µL) | Rainin | RT-L200F | | ✓ |
| 10x BPB/XC loading buffer | In house | N/A | N/A | N/A |
| 100bp ladder | Invitrogen | 15628-019 | | ✓ |
| 25bp ladder | Invitrogen | 10597011 | | |
| SybrGreen | CAMBEX | 50513 | | ✓ |
| Dark Reader (Transilluminator) | InterSicence | DR-190M | ✓ | |
| Gel Elution buffer | In house | N/A | N/A | N/A |
| Spin-X Filter Columns | Costar | 8160 | ✓ | |
| 2mL RNase/DNase free tubes | | | | |
| Abgene 96-well plate | Thermo Scientific | FSSP974145<br>0 | | ✓ |
| P450 96-well plate | Perkin Elmer | 6008290 | | ✓ |
| AB1127 1.2 mL 96-well plate | Abgene | AB1127 | | ✓ |
| MagMAX express 2mL deep well plate | Thermo Fisher | 4388476 | | ✓ |
| Low profile reservoir | Seahorse | EK2036 | | ✓ |
| 100% Isopropanol (2-Propanol) | Fisher Science | A464-4 | | ✓ |
| 70% Ethanol | In house | | | ✓ |
| 3M NaOAc, pH 5.5 | Ambion | AM9740 | | ✓ |

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| | | | | |
|---|---|---|---|---|
| ALINE PCR DX beads<br>ALINE RNA MagClean DX beads | ALINE<br>ALINE | C-1003-450<br>C-1005-250 | | ✓ |
| Qiagen Elution buffer | Qiagen | 109086 | | ✓ |
| Hamilton NIMBUS P50 clear tips | Hamilton | 235831 | | ✓ |
| Hamilton NIMBUS P300 clear tips | Hamilton | 235832 | | ✓ |
| Peltier spring plate | In house | | N/A | N/A |
| Alpaqua Magnum EX magnet | Alpaqua | A000380 | | ✓ |
| Distriman Pipette | Gilson | GF-F164140 | | ✓ |
| VWR foil tape | VWR | 60941-126 | | ✓ |
| 3M Tape Pads | Qiagen | 19570 | | ✓ |
| Vinyl gloves | Medicom Safetouch | 361004643 | | ✓ |
| Foil Tape | Scotch/3M | 34000740 | | ✓ |
| Caliper HSDNA chip & reagents | Caliper | 760517, 760568 | | ✓ |
| Quant-iT assay 500 assays | Invitrogen | Q33120 | | ✓ |
| Ultrapure water | Invitrogen | 10977-023 | | ✓ |
| 40% Polyacrylamide (19:1 acrylamide:bis) | Bio Rad | 161-0144 | | ✓ |
| 50X TAE Buffer | In house | | | |
| 10% APS | Bio Rad | 161-0700 | | ✓ |
| Temed | Bio Rad | 161-0800 | | ✓ |
| 10bp DNA ladder | Invitrogen | 10821-015 | | ✓ |
| Mylar PET film, clear | McMaster-Carr | 8567K32 | | ✓ |
| Glass plates for PAGE gels | Owl Scientific | P1-14R/P1-14G | | ✓ |
| Bags for Gel Pouring | Fisher Scientific | GP2-100 | | ✓ |
| Casting Tray | Owl Scientific | P1-CST | | ✓ |
| Power Supply | Bio Rad | Power Pac200 | ✓ | |
| Mussel glycogen | Roche Scientific | 10 901 393 001 | | ✓ |
| Anhydrous Ethyl Alcohol (100% Ethanol) | Commercial | People Soft ID: 23878 | | ✓ |

**Oligo Sequences**

miRNA 3' Adapter , 2.5µM working stock
5' NNNNNNTGGAATTCTCGGGTGCCAAGTCG 3', N= randomized sequence

miRNA 5' Adapter, 1µM working stock
5' UUCCCUACACGACGCUCUUCCGAUCUNNNNNN 3', N= randomize sequence

RT primer for First Strand Synthesis, 20µM working stock
5' CGACTTGGCACCCGAGAATTCCA 3'

PE 1.0 primer, 25µM working stock
5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'

miRNA indexed primers, 25µM working stock
CAAGCAGAAGACGGCATACGAGATCNNNNNNATCGTACGTCGACTTGGCACCCGAGAATTCCA, underlined index sequence is unique for up to 96 samples.

**IX. Introduction and Guidelines**

**1. General Guidelines**

1.1. Ensure proper personal protective equipment is used when handling samples, reagents and equipment. Always wear gloves and a disposable lab coat when working in the RNA area. Always wear gloves when handling equipment, reagents and samples. Spray gloves with RNaseZap if you must handle dirty surfaces such as labeling
1.2. Wipe down the assigned workstation, pipettes, tip boxes and small equipment with RNase Zap. Change your gloves and then place new bench coat down in your clean workstation.
1.3. All of the steps in this protocol up to and including first strand synthesis are to be carried out in the designated RNA working spaces. First strand purification and PCR set up are performed in the designated Pre-PCR spaces. Post PCR purification is performed in the designated post-PCR designated spaces.
1.4. Discuss your results with your APC/PC/designated trainer after each QC step. Report and record equipment failures and/or malfunctions and any noted variations in reaction well volumes.

**2. General Plate Guidelines**

2.1. One plate can be processed at a time by one technologist using the NIMBUS.

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2.2. To avoid cross-contamination, reaction plates should not be vortexed and plate seals should not be reused. Use the NIMBUS robot for all mixing steps.
2.3. Use VWR foil seals for short term storage and tetrad incubations/PCR and 3M aluminum foil seal for long term storage.
2.4. Quick spin your plate at 4°C for one minute at 2000 x g after each incubation step.
2.5. Sample plates can be stored at -80°C after 2X bead clean after the 3' ligation step or at -20°C after first strand synthesis or PCR. Safe stopping steps are noted where applicable.

**3. Input requirements and controls**

3.1. The starting material for this procedure is total RNA, flow-through RNA collected after mRNA isolation using the MultiMACs isolation system or FFPE total nucleic acid.
3.2. The minimum starting amount is 250 ng of total RNA in 8μL of DEPC-treated water, however we recommend using 500 ng of total RNA if possible. The maximum starting amount is 1000 ng of total RNA in 8μL of DEPC-treated water. For FFPE total nucleic acid, the recommended starting amount is 1ug RNA (based on RNA QC of untreated total nucleic acid).
3.3. Evaluate total RNA, flow through fraction or FFPE total nucleic acid on the Caliper following LIBPR.0052 or alternatively using Agilent RNA total eukaryotic RNA assay following LIBPR.0018.
3.4. For this procedure RNA should have a RIN value equal or greater to 7 or RQS value equal or greater to 6. If the RNA fails this criterion or if there is no detectable miRNA on either assay discuss with your supervisor to determine if the samples can be processed.
3.5. No need to treat total RNA sample with DNase I prior to starting the procedure.
3.6. The positive control template for this pipeline is FG032A Placental total RNA. The input amount should be equal to that of the collaborator's samples.
3.7. The negative control templates are DEPC-treated water for library construction and ultrapure water for PCR.

**4. General brew prep guidelines**

4.1. Double check the QA release and expiry dates of each reagent and enzyme.
4.2. Thaw required reagents and then place them on ice. Enzymes should be stored in the freezer until required for use. PEG-8000 must reach room temperature prior to use and must be carefully pipetted using the reverse pipetting technique.
4.3. Reagents and enzymes should be well mixed, the former by pulse-vortexing and the later by tapping and inverting the tube. Quick spin tubes in a mini-centrifuge after mixing.

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4.4. All prepared brews should be well mixed by gentle, repeated pulse-vortexing to ensure equal distribution of all components and thus uniformity of enzymatic reactions across a plate. Please note the ligation reactions are extremely viscous due to a high content of PEG-8000.
4.5. Prepare brew reactions in the PCR clean room on the 5th floor in the designated laminar flow hood or from one-use aliquots at a designated RNA area work station.

**5. Hamilton NIMBUS Guidelines**

5.1. The following steps are followed to log into a program on the NIMBUS

5.1.1. Log into the desktop with your user name and password.
5.1.2. Double click on the Hamilton Run Control icon.
5.1.3. Select the appropriate file: File>Open>Production>Small RNAseq First Strand Synthesis >Small RNAseq First Strand Synthesis.med
5.1.4. Select the 'play' icon to initiate the instrument.
5.1.5. Select the appropriate step, i.e. "3' Adapter Ligation"

5.2. Once a program is initiated you will be prompted to specify the number of columns to process and the starting column (default=1).
5.3. Follow the prompts to set up the deck layout.
5.4. Remember to lock down the plate adapter to the deck.
5.5. After the program is complete, clear the deck and log out of the desk top.
5.6. Wipe down the instrument and pedestals with 70% Ethanol after use.

## X. Procedure

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

**1. Template Transfer/Sample Normalization**

1.1. Retrieve the plate or tubes containing the total RNA or flow through fraction from -80°C and thaw on ice. Do not remove the cover. Once thawed, quick spin the plate and then return the plate to ice.

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1.2. Use the following program to normalize input for miRNA sequencing. This pipeline has been validated to use a minimum of 250 ng in 8 µL (37.25ng/µL) and a maximum of 1000 ng in 8 µL (125 ng/µL) depending on starting material.

| NIMBUS> Hamilton Run Control>File>Open>Production> ssRNAseq> **Total RNA Normalization** |
|---|

1.3. Return the stock plate to -80°C and place the working plate on ice.

1.4. Add FG032A Placental total RNA (positive control) and 8 µL of DEPC-treated water (negative controls) to two empty wells. Ensure that the input amount of FG032A matches the minimum input amount of the collaborator's samples. This pipeline has been validated to use a minimum of 250 ng of FG032A input. Lower input amounts may require more PCR cycles.

## 2. 3' Adapter Ligation

2.1. Retrieve an aliquot of 2.5 µM 3' adapter from -20°C storage. Allow the DNA adapter to thaw at room temperature and then return the adapter tube to ice. Aliquots are intended for one time use, do not freeze-thaw DNA adapters. Throw out any remaining adapter.

2.2. Retrieve reagents required for 3' adapter ligation. Allow PEG 8000 to reach room temperature before use. Allow buffers to reach room temperature and then return to ice. Enzymes should be stored at -20°C until required to prepare brew.

2.3. Gently vortex adapter and then quick spin. Aliquot adapter into one column of a 96-well plate and quick spin and then return to ice. Carefully dispense 2 µL of adapter to wells of an empty AB1000 plate using a multichannel pipette. Quick spin the plate and then store it on ice.

2.4. Set a tetrad to hold at 70°C and place a label on the tetrad to show that it is in use.

2.5. Log into the following NIMBUS program.

| NIMBUS> Hamilton Run Control >File>Open>Production> Small RNAseq First Strand Synthesis >Small_RNAseq_FirstStrandSynthesis.med>**3' Adapter Ligation** |
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2.6. Follow the prompts and once the deck layout is correct, press okay. The robot will transfer the entire total RNA template to the plate containing 2 µL of 3' adapter. After the sample has been mixed you will be prompted to cover the plate with foil tape and then quick spin the plate. Double check that volumes are consistent across the plate. Check the source plate to ensure that all templates have been transferred.

2.7. The NIMBUS will keep the tips loaded for the following transfer step.

2.8. Set a timer for two minutes and then incubate the RNA template and 3' adapter at 70°C for 2 minutes in a tetrad to break down the RNA secondary structures. Snap chill on ice for 1 minute and then quick spin plate and return to ice.

2.9. Prepare 3' ligation brew. The volume requirement for 1 reaction set up is as follows:

| Solution | 1 rxn (µL) |
|---|---|
| RNA+3' Adapter | 10 |
| 50% PEG-8000 | 5 |
| 10X T4 RNA ligase buffer | 2 |
| DEPC-treated water | 1 |
| Rnase Out (40 U/uL) | 0.5 |
| T4 RNA ligase 2 (truncated) (200 U/uL) | 1.5 |
| **Brew total** | **10** |
| **Reaction volume** | **20** |

miRNA4_3pLigBrew (10µL)

2.10. Generate the 3' ligation Brew Mix calculator using LIMS:

LIMS: Mix Standard Solutions > **miRNA4_3pLigBrew**

2.11. Obtain the 1D large Solution/Box/Kit Label and Chemistry Label. Prepare the brew in an appropriate sized tube according to the chemistry calculator.

2.12. Prepare brew according to the LIMS calculator. Add PEG-8000 to an empty tube first using the reverse pipetting technique due to the viscosity of the reagent.

2.13. Dispense 10 µL of brew using a Distriman pipette to empty wells of an AB1000 plate stored on ice. Cover the plate with tape seal and quick spin the plate. Double check that the volumes are consistent between wells.

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2.14. Return to the NIMBUS and select 'okay' if you have denatured the template and 3' adapter. Follow the prompts.

2.15. The NIMBUS will transfer all of the template plus adapter to the 3' ligation brew and mix. You will be prompted to cover your plate with VWR foil seal and quick spin the plate.

2.16. Double check that the volumes are consistent in the destination plate and that no template remains in the source plate.

2.17. Incubate plate for one hour at 22°C using the following tetrad protocol. The total volume of the reaction is 20 µL/well.

**MIRNA>3LIG**

2.18. Pre-warm ALINE beads and 70% Ethanol during the 3' ligation protocol.

2.19. Once the program is finished, remove the plate from the tetrad and quick spin the plate. Double check that volumes are consistent across the plate. Store the plate on ice.

2.20. Proceed to 2X bead purification. This is not a safe stopping point.

## 3. 2X Bead Clean

3.1. This step is essential to remove excess 3' adapter.

3.2. Bead purification has been optimized for removing the 29 bp adapter while retaining the adapter ligated template, 55 bp. **NaOAc, ALINE beads and isopropanol must be added stepwise, and not premixed together prior to addition.** Premixing results in a significant reduction in yield.

3.3. Prepare the following reagents as described below using a Distrimann or multichannel pipette. Cover with tape seal until ready for use. Pour 100% isopropanol and DEPC-treated water into low profile reservoirs immediately prior to use.

| Reagent | Labware | Volume/well (µL) |
|---|---|---|

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| 70% Ethanol | 2 mL MagMAX deep well plate | 400 *2 blocks |
|---|---|---|
| 3M NaOAc | P450 Storage plate | 75 |
| RNA MagClean Beads | AB1127 plate | 130 |
| 100% Isopropanol | EK 2036 Low Profile Reservoir | Fill to line |
| DEPC treated water | EK 2036 Low Profile Reservoir | Fill to line |

3.4. Ensure that MagClean beads have reached room temperature prior to starting 2X bead clean up of excess 3' adapters.

3.5. Log into the following NIMBUS protocol:

NIMBUS> Hamilton Run Control >File>Open>Production> Small RNAseq First Strand Synthesis >Small_RNAseq_FirstStrandSynthesis.med>**2X Bead Clean**

3.6. If required, samples can be stored temporarily on ice after the first bead clean or stored overnight at -80°C.

3.7. After 2 rounds of bead clean proceed immediately to 5' adapter ligation or alternatively, store 3' ligated template at -80°C overnight.

## 4. 5' Adapter Ligation

4.1. Retrieve 1 µM 5'miRNA adapter from -80°C and thaw at room temperature and then immediately store on ice.

4.2. Retrieve reagents required for 5' adapter ligation. Allow PEG 8000 to reach room temperature before use. Allow buffers to reach room temperature and then return to ice. Enzymes should be stored at -20°C until required to prepare brew.

4.3. Set a tetrad to hold at 70°C.

4.4. Gently vortex, quick spin and then dispense adapter into one column of a 96-well plate, cover with VWR foil and then quick spin the plate. Break down the secondary structures of the 5' adapter by incubating at 70°C for 2 minutes in a tetrad. Immediately snap chill the adapter for 1 minute. Quick spin plate and return to ice.

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4.5. Transfer 2 µL of denatured 5' adapter to appropriate columns of an AB1000 plate using a multichannel pipette. Cover with tape seal and quick spin the plate. Double check that the volume is consistent across the plate. Store the plate on ice.

4.6. Prepare 5' ligation brew. The volume requirement for 1 reaction set up is as follows:

| Solution | 1 rxn (µL) |
|---|---|
| 3'Ligated template + 5' adapter | 11 |
| 50% PEG-8000 | 3.5 |
| 10X T4 RNA ligase buffer | 2 |
| 10 mM ATP, molecular grade | 2 |
| Rnase Out (40 U/uL) | 0.5 |
| Ambion T4 RNA ligase (5U/uL) | 1 |
| **Brew total** | **9** |
| **Reaction volume** | **20** |

miRNA4_5pLigBrew (9µL)

4.7. Generate the 5' Ligation Brew Mix calculator using LIMS:

4.8. Obtain the 1D large Solution/Box/Kit Label and Chemistry Label. Prepare the brew in an appropriate sized tube according to the chemistry calculator.

LIMS: Mix Standard Solutions > **miRNA4_5pLigBrew**

4.9. Prepare brew according to the LIMS calculator. Add PEG 8000 to any empty tube first using the reverse pipetting technique.

4.10. Carefully dispense 9 µL of brew to empty wells of an AB1000 plate stored on ice using a Distriman pipette. Cover the plate with tape seal and quick spin the plate. Double check that the volumes are consistent between wells.

4.11. Log into the NIMBUS workstation as described below.

NIMBUS> Hamilton Run Control >File>Open>Production> Small RNAseq First Strand Synthesis >Small_RNAseq_FirstStrandSynthesis.med>**5' Adapter Ligation**

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4.12. Follow the prompts. Once the deck is correct, press okay. The NIMBUS will transfer the entire 3' ligation template to the plate containing 2 µL of denatured 5' adapter. After the sample has been mixed you will be prompted to cover the plate with tape seal and then quick spin the plate. Double check that volumes are consistent across the plate. Check the source plate to ensure that all templates have been transferred.

4.13. Follow the prompts for the new deck layout. The NIMBUS will transfer the template + 5' adapter to the destination plate containing brew. After the run has completed cover the plate with VWR foil and quick spin the plate. Check that the volumes are consistent across the plate and double check that the source plate is empty.

4.14. Incubate the plate for one hour at 37°C using the following tetrad protocol. The total volume of the reaction is 20 µL/well.

**MIRNA>37LIG**

4.15. Proceed immediately to first strand synthesis or alternatively, store samples at -80°C overnight.

## 5. First Strand Synthesis

5.1. Retrieve 20 µM RT primer from -20°C and thaw at room temperature, and then immediately store it on ice.

5.2. Program a tetrad to hold at 65°C.

5.3. Gently vortex and quick spin and then divide RT primer into 1 column of an AB1000 plate. Use a multichannel pipette to transfer 2 µL of primer into empty wells of an AB1000 plate.

5.4. Cover plate with VWR foil and quick spin plate. Double check that volumes are consistent across the plate and then store the plate on ice.

5.5. Log into the NIMBUS workstation as described below.

| <u>NIMBUS</u>> Hamilton Run Control >File>Open>Production> Small RNAseq First Strand Synthesis >Small_RNAseq_FirstStrandSynthesis.med **>1st Strand Synthesis** |
|---|

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5.6. Follow the prompts. Once the deck is correct, press okay. The NIMBUS will transfer the entire ligated template to the plate containing 2 µL of RT primer per well.

5.7. After the sample has been mixed you will be prompted to cover the plate with VWR foil tape and then quick spin the plate. Double check that volumes are consistent across the plate. Check that the source plate is empty.

5.8. The NIMBUS will keep the tips loaded for the following transfer step.

5.9. Incubate RT primer and ligated template at 65°C for 10 minutes on a tetrad. Set a timer. Snap chill plate on ice immediately after incubation. Incubate on ice for at least one minute and then quick spin the plate and return it to ice.

5.10. During the 10 minute incubation prepare first strand brew. The volume requirement for 1 reaction set up is as follows:

| Solution | 1 rxn (µL) |
|---|---|
| Denatured Template + RT primer | 22 |
| 5X First Strand buffer (contains DTT) | 8 |
| 10mM dNTPs | 2 |
| Nuclease free water | 6 |
| Rnase Out | 1 |
| Maxima H minus RT (200U/µL) | 1 |
| **Brew total** | **18** |
| **Reaction volume** | **40** |

miRNA4_1stStrand (18µL)

5.11. Generate the first strand brew mix calculator using LIMS:

LIMS: Mix Standard Solutions > **miRNA4_1stStrand**

5.12. Obtain the 1D large Solution/Box/Kit Label and Chemistry Label. Prepare the brew in an appropriate sized tube according to the chemistry calculator and store brew on ice.

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5.13. Use a Distriman pipette to carefully aliquot 18 µL of brew into empty wells of an AB1000 plate stored on ice. Cover the plate with tape seal and then quick spin plate. Double check that volumes are even and then store on ice until it is required.

5.14. Return to the NIMBUS and follow the prompts. You will place the denatured template and primer on the Peltier block and the RT brew plate on the destination pedestal as shown in the deck layout.

5.15. The NIMBUS will transfer all of the template plus primer to the first strand brew and mix. You will be prompted to cover the destination plate with VWR foil seal and quick spin the plate.

5.16. Double check that the volumes are consistent in the destination plate and that no template remains in the source plate.

5.17. Incubate plate for one hour at 44°C using the following tetrad protocol. The total volume of the reaction is 40 µL/well.

**MIRNA>44Max**

5.18. Proceed immediately to first strand purification or alternatively store the first strand template at -20°C.

## 6. First Strand Purification (Upper/Lower cut)

6.1. This step is essential to remove the RT primer and reduce non-target products prior to PCR enrichment.

6.2. Prepare the following reagents as described below using a distrimann or multichannel pipettor. Cover with tape seal until ready for use. Pour 100% isopropanol and 70% ethanol into low profile reservoirs immediately prior to use.

| Reagent | Labware | Volume/well (µL) |
|---|---|---|
| 70% Ethanol | 2 mL MagMAX deep well plate | 400 |
| 3M NaOAc | P450 Storage plate | 60 |
| ALINE PCRClean DX Beads | AB1127 plate | 90 |
| 100% Isopropanol | EK 2036 Low Profile Reservoir | Fill to line |
| Elution buffer | EK 2036 Low Profile Reservoir | Fill to line |

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6.3. Ensure that ALINE beads have reached room temperature prior to starting bead purification of first strand template.

6.4. Log into the following NIMBUS program:

NIMBUS> Hamilton Run Control >File>Open>Production > Small RNAseq PrePCR **>** Small_RNAseq_PrePCR.med>**Clean 1st Strand Synthesis Product**

Proceed immediately to PCR enrichment, or alternatively store the first strand template at -20°C for up to one week.

## 7. Indexed PCR enrichment and QC confirmation

7.1. Thaw the PE PCR primer 1.0 and miRNA indexing primer plate at a designated Pre-PCR work bench and then immediately place them on ice. Quick spin the indexing primer plate prior to removing the cover.

7.2. To keep track of freeze-thaw cycles, mark off the indexing primer plate each time the plate is thawed even if it is not used. The indexing primer plate can be freeze-thawed up to 5 times.

7.3. iPCR brew (minus the primers) must be in a designated Pre-PCR laminar flowhood. Add PE PCR primer 1.0 to the brew at a designated pre-PCR work bench or the laminar flowhood in the Tissue Culture room 511.

7.4. The volume requirement for 1 reaction set up is as follows:

| Solution | 1 rxn (µL) |
|---|---|
| First Strand Template | 19 |
| Ultrapure water | 12.75 |
| 5X Phusion HF buffer | 10 |
| 10 mM dNTPs | 1.25 |
| DMSO | 1.5 |
| Phusion hot start | 0.5 |
| PE 1.0 primer (25µM) | 2.5 |
| **Brew total** | **28.5** |
| Index Primer (25µM) | 2.5 |

miRNA4_iPCR (28.5µL)

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| Solution | 1 rxn (µL) |
|---|---|
| **Reaction volume** | **50** |

7.5. Generate the PCR Brew Mix calculator using LIMS.

LIMS: Mix Standard Solutions > **miRNA4_iPCR**

7.6. Obtain the 1D large Solution/Box/Kit Label and Chemistry Label. Prepare the brew in an appropriate sized tube according to the chemistry calculator. Carefully aliquot 28.5 µL of PCR brew to a new AB1000 plate using a Distriman pipette.

7.7. Log into the NIMBUS workstation and select the following program:

NIMBUS> Hamilton Run Control >File>Open>Production > Small RNAseq PrePCR **>** Small_RNAseq_PrePCR.med>**>PCR Enrichment**

7.8. Follow the prompts. The NIMBUS will first transfer indexed primers directly to the brew and then mix. You will be prompted to quick spin the plate if bubbles are introduced during primer addition. Tips will be held for the second transfer.

7.9. The NIMBUS will add the entire template to the destination plate containing primer and PCR brew. Cover the plate with VWR foil and quick spin the plate. Double check that the volume is consistent across the plate and that there is no template remaining in the source plate.

7.10. Run PCR program MIR15.4 specified in the table below. Use a rubber pad on top of the reaction plate. The total reaction volume is 50 µL.

PCR parameters

- 98˚C 1 min
- 98˚C 15 sec
- 65˚C 30 sec
- 72˚C 15 sec
- 72˚C 5min
- 4˚C ∞

(98˚C 15 sec, 65˚C 30 sec, 72˚C 15 sec: Total of 15 cycles)

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7.11. Run enriched samples on Caliper HS DNA assay (5X dilution) or Agilent DNA 1000 assay (2X dilution) to confirm that miRNA is present in each sample prior to pooling and gel purification. A miRNA peak will run at ~159bp on the DNA 1000 chip (Figure 1).

7.12. To make a dilution for Caliper log into the following program using the dedicated post-PCR NIMBUS:

| NIMBUS> Hamilton Run Control >File>Open>Production > Transfer to AB1000 >Transfer to AB1000.med |
|---|

7.13. Follow the prompts to dilute 4 µL of PCR product in 16 µL of Qiagen EB to make a 1 in 5 dilution for Caliper QC.

7.14. Note that you will first run the program to transfer 16 µL of EB from a low profile reservoir to a new AB1000 plate. Select the appropriate number of columns so that the EB plate matches the sample source plate.

7.15. Open the program a second time to transfer 4 µL of PCR product in an AB1000 plate to the AB1000 plate containing 16 µL of EB.

7.16. Run the diluted plate on Caliper as described in LIBPR.0051.

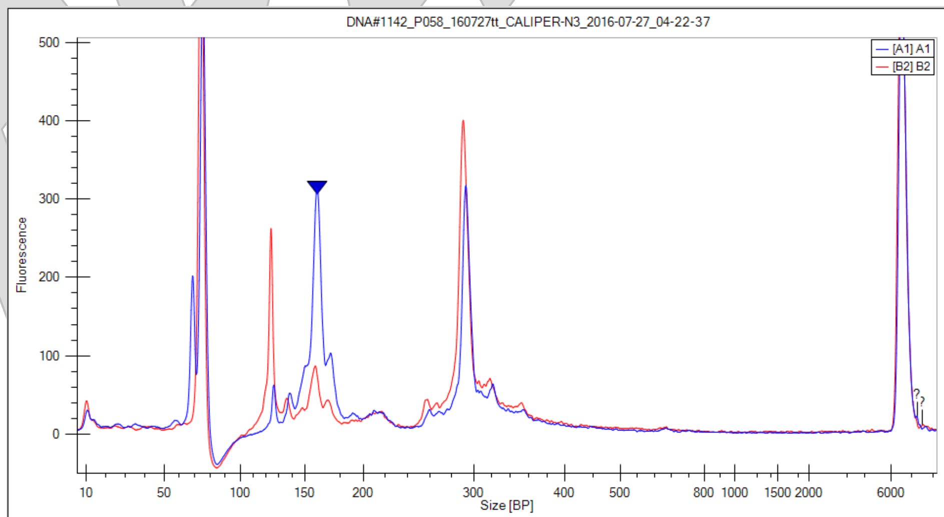


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Figure 1. iPCR product prepared from 1 ug of human placental total RNA (blue) and 1 ug of CEMT-9 total RNA (red).

7.17. Under the 'Analysis' tab select smear analysis and 154:168 for the range. Select Molarity (nmol/l) as the property to be displayed in a well table. Apply this to all wells. Export the well table to R:\Library Core\QC\Caliper\PCR.

7.18. Follow the instructions in Appendix C to create a file for equimolar pooling using Span-8 (LIBPR.0093). Use program Pool_Samples_from_AB1000_to_microfuge Tubes 17mm. The samples will be pooled to a 1.7mL tube into destination A1.

7.19. After QC confirmation proceed immediately to PAGE size selection or alternatively store the indexed PCR product at -20°C.

## 8. Manual Size Selection Using PAGE

**Note: the 12% PAGE gel should be poured the day before size selection as the gel needs to polymerize for 40minutes before use and the total gel running time is 6 hours.**

8.1. 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.

**12% POLYACRYLAMIDE GEL:**

| REAGENT | Per GEL |
|---|---|
| Ultrapure $dH_20$ | 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 |

8.2. Let the gel polymerize for **at least 40 minutes**. Place a paper towel soaked in 1x TAE buffer on the combs to keep the gel moist. Store gel wrapped in saran wrap in 4C fridge.

8.3. 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.

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8.4. Load 20µl of the 10bp DNA Ladder (20ng/µL) into one well and 20µL of 25bp 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.

8.5. Add 10X Bromophenol blue loading dye to each of the pools, i.e 1µL dye/9µL template..

8.6. Split each pool over many wells – one pool per gel. On each gel load up to 40µL/well into wells on both sides of the ladders. Leave 1 empty lane between sample and ladder. Avoid the very edge wells if possible.

8.7. Immediately after loading, **run the gel @ 200 V for 6 hours**. Change the running buffer half way through the run.

8.8. Using colored tape, attach a label to the gel apparatus which states the Pool name, start time, finish time, date, and your initials. Attach a note to the tap for the water to stay running.

8.9. Dispose of all waste.

## 9. Gel Scan and Cutting DNA Fraction from PAGE

9.1. Put on a clean pair of gloves.

9.2. Set up sets of 0.5mL and 2mL tubes for shearing the gel slices: Make a hole through the bottom of 0.5mL tubes with an 18 gauge needle. Place each 0.5mL tube into a 2mL tube. You will one set of tubes per each pool.

9.3. Label each 2mL tube on the side with the pool, name, size fraction, date, and initials.
9.4. Cover the Dark Reader screen with a fresh sheet of plastic wrap.

9.5. Prepare fresh stain: 10µL stock in 100mL 1x TAE. Minimize exposure to light.

9.6. Stop the gel run after 6 hours and dismantle the PAGE apparatus.

9.7. Using a clean post PCR dedicated tray, stain the gel for at least 3 minutes.

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9.8. 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.

9.9. 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_150606.gel

9.10. Print the image and paste it into your lab notebook.

9.11. Lay the Mylar sheet with the gel on top onto the Dark Reader and cut out the region containing miRNA which runs to ~150bp (See Figure 2).

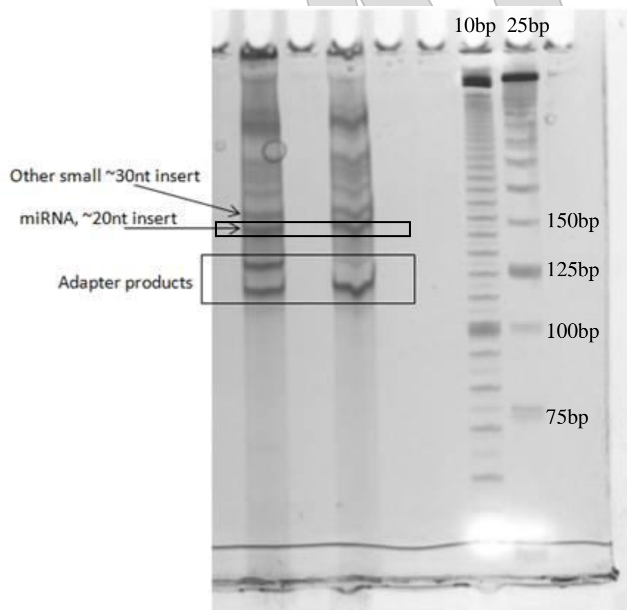


**Figure 2. Polyacrylamide gel electrophoresis of a miRNA library pool.**

9.12. Each well should look as above. Combine gel slices of each pool into separate 0.5mL tube prepared and labeled earlier for shearing.

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9.13. 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.

9.14. 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.

9.15. 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.

9.16. 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.

9.17. 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.

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

9.19. Vortex and pulse spin.

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

9.21. 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.

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

**10. Precipitate and Purify miRNA products**

10.1. Pool samples for precipitation as described by your APC.

10.2. The total volume of eluate will vary depending on the number of wells run per pool. Each well produces 200 µL of eluate. Adjust 3M Sodium acetate and ethanol according to the volume of the eluate.

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| Reagent | Volume |
|---|---|
| Eluate | 400 µL |
| 3 M Sodium Acetate (0.1X Eluate) | 40 µL |
| Mussel Glycogen (20 mg/mL) | 3 µL |
| 100 % Ethanol (2.5x Eluate + Acetate) | 1110 µL |
| **Total volume** | **1553µL** |

10.3. Vortex and pulse spin. Chill the tubes at -20°C for at least 30 minutes.

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

10.5. 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.

10.6. Repeat the 70% EtOH wash.

10.7. 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.

10.8. 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.

10.9. Resuspend each sample in a total volume of 12 µL Qiagen EB buffer.

## 11. QC final product

11.1. Run the Agilent DNA 1000 Series II assay of size selected product by following LIBPR.0017.

11.2. Check the Agilent profile for the correct size fraction. The expected product of 155bp runs on Agilent Bioanalyzer at ~159bp on DNA 1000 (Figure 2). Discuss your results with your APC.

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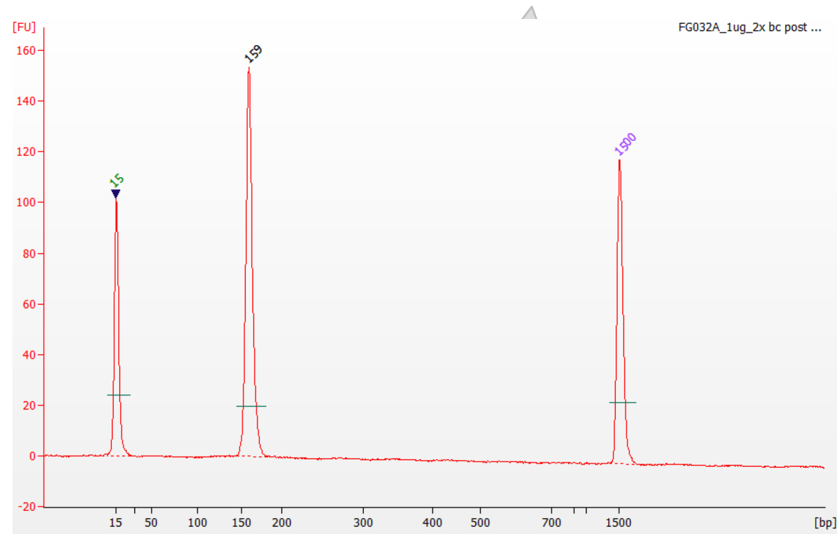

Figure 2. Final QC of PAGE-purified pooled miRNA libraries.

11.3. If samples are ready for submission, quantify 1µL of each sample with Qubit as per LIBPR.0030.

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**Appendix A: LIMS Solutions**

1. Log into aldente>Lab>Solutions
2. Select the number of samples to be run.
3. The following brews will be prepared using the LIMS calculators:
   a. miRNA4_3'LigBrew
   b. miRNA4_5'LigBrew
   c. miRNA4_1stStrand
   d. miRNA4_iPCR
4. Select 'Mix Standard Solution'
5. **Ensure that 1 well is selected for "# of BrewSourceWells" because the NIMBUS program does not require dead volume.**
6. Scan solution numbers of reagents into the calculator
7. Select Group:Lib Construction, Barcode Label: 1D large label
8. Select 'Save Standard Mixture'
9. Use the brew checklist to prepare brews and store brew solution barcode for LIMS tracking. **Note that the volume aliquoted is noted as 'manual' because the NIMBUS does not require dead volume in the brew plate.**

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**Appendix B: LIMS Protocols**

1) Start of Plate Library Construction
2) miRNA4_cDNA. Pipeline: MIR_4.0: miRNA 4.0
3) Plate_Indexed_PCR. Pipeline: MIR_4.0: miRNA 4.0
4) Caliper Run
5) Action: Aliquot volume to be pooled into a new 96-well ABgene plate. Use this TRA for pooling. Pipeline: MIR_4.0: miRNA 4.0
6) Rearray/Pool
7) Plate_Manual_PPGP. Pipeline ISE:ISET
8) Create Bioanalyzer run
9) Final_Submission ISE:ISET

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**Appendix C: Preparing a CSV file for equimolar pooling**

1) Open the Caliper smear analysis file.

2) Copy columns A-F and paste them into columns A-F in the 'Smear Analysis' tab of the following worksheet template:

   R:\Library Core\Work Sheets and Calculators\miRNA\version4\Template\miRNA4_pooling

3) Save the file as described here: TRA_Collaborator_PoolX_date_Analysis.

4) Delete any wells that will not be included in the pool. You may need to create two or more files (pools) per plate as defined by your APC.

5) The total volume of the pool will calculated automatically. If the total volume is less than the volume required it will be flagged as red. Increase the 'Factor' number to increase the total volume of the pool until it is no longer flagged as too low.

6) Select the "Span-8 Pooling" tab. This tab will automatically populate with the necessary information for equimolar pooling.

7) Copy the entire Span-8 Pooling tab data and save in a new excel file. Save as MS DOS.CSV and save it into the Span-8 PPGP file: R:\Library Core\Span 8 PPGP.

8) Repeat the process for additional pools.

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