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Magnetic bead-based mRNA isolation

I. Purpose

To capture poly (A) mRNA from Total RNA using mRNA isolation kit from New England Biolabs (NEB). This process represents mRNA isolation V2.0 and replaces the process of MultiMACs RNA isolation described in LIBPR.0057 and LIBPR.0118.

II. Scope

All procedures are applicable to the BCGSC Library Core and the Library 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 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 Group Leader 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

Document Title	Document Number
NEBNext Poly(A) mRNA Magnetic Isolation Module	E7490L

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 LabChipGX for RNA samples using the HT RNA Assay	LIBPR.0052
Total RNA Normalization on Hamilton Nimbus	LIBPR.0121
DNase I treatment of RNA/Total Nucleic Acid	LIBPR.0143

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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 sheets (MSDS) for additional information.

VIII. Materials and Equipment

Name	Supplier	Number
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
RNAse free 1.5 mL eppendorf tube	Ambion	12400
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
Mandel P200 DF200 tips	Mandel	GF-F171503
Mandel P1000 DF1000 tips	Mandel	GF-F171703
VX-100 Vortex Mixer	Rose Scientific	S-0100
200µL Rainin tips	Rainin	RT-L200F
20µL Rainin tips	Rainin	RT-L10F
200µL Pipet-Lite	Rainin	L12-200
20µL Pipet-Lite	Rainin	L12-20
1250 µL pipette tip, 96tips/rack, 480 tips/cs filter sterile	Mandel Scientific	TM-4445
Pipette-VIAFLO 8 Channel	Mandel Scientific	TM-4124
Large Kimwipes	Fisher	06-666-117
Black ink permanent marker pen	VWR	52877-310
Bench Coat (Bench Protection Paper)	Fisher	12-007-186
Small Autoclave waste bags 10"X15"	Fisher	01-826-4
DNAse1 Amplification Grade 100U	Invitrogen	18068-015
DEPC water	Ambion	9922
Mini-centrifuge	Eppendorf	5417R
Thermo Scientific 0.2mL Ultra Rigid Skirted 96-well	Thermoscientific	AB1000-150s
Deep-well, 96-well, 1.2 mL, U bottom, low pro, 50/cs	Fisher Scientific	AB1127
NEBNext Poly(A) mRNA Magnetic Isolation Module	New England Biolabs	E7490L
RNA MagClean DX	ALINE Biosciences	C-1005
Sterile Filtered Conductive 50**µL** Tips in Frames	Hamilton	235979
Sterile Filtered Conductive 300**µL** Tips in Frames	Hamilton	235938
Tape Pads	Qiagen	19570
Foil Tape	VWR	60941-126

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IX. GENERAL GUIDELINES

1 General guidelines and input material

1.1 The recommended input material for this procedure is 100-1000ng Total RNA. Input volume to be requested from collaborators should be a maximum of 20µL to have suitable concentration for QC. The actual input volume for the first reaction is 35µL/well in DEPC H_20 in a 96-well plate.

1.2 The positive control for this procedure is 500 ng Universal Human Reference RNA (UHR or FG031). Please also add a positive control (UHR) that is the same amount as the rest of the normalized plate. The negative control is DEPC H_2O.

1.3 The beads (**RNA MagClean DX**) to be used in this protocol are different from those used for other protocols as they are certified to be RNase-free.

1.4 Ensure proper personal protective equipment is used when handling sample plates, reagents and equipment. Treat everything with, and adhere to, strict RNA handling techniques.

1.5 Wipe down the assigned workstation, pipetman, tip boxes and small equipment with RNAse Zap (Ambion) followed by DEPC-treated water. Ensure you have a clean working surface before you start.

1.6 Double check the QA release and/or expiry date of each reagent and enzyme.

1.7 Reactions in plates should never be vortexed and plate covers are never to be re-used.

1.8 Retrieve and thaw all reagents at room temperature. Once thawed, pulse-vortex, quick spin and keep reagents on ice. Enzymes should be left in the freezer until ready to use.

1.9 Ensure the waste bag for the Nimbus is empty.

1.10 **Brews are prepared and dispensed manually**. Note that what is dispensed into the final brew plate is the actual volume of the specified volume for each step below **without any dead volume.**

1.11 Aline RNA MagClean DX Beads are manually dispensed into wells in a 1.2 mL plate (AB1127). 20 µL dead volume should be factored in. Ensure that you include 25 mL dead volume for 70% ethanol and DEPC H_2O on top of what is required for the actual washes and elution, respectively.

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1.12 The Nimbus adds sample in a given reaction to the brew and bead cleanups are performed on Nimbus. Follow the prompts and lay out from the Nimbus programs to execute a particular step.

1.13 The Nimbus mixes at 80% of total volume 10 times.

1.14 Note that where it is specified that you proceed immediately to the next step, plates can be briefly placed on ice (not more than 30min) in the case of emergency. Make an active attempt to proceed as immediate as possible.

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. Upstream Preparation

1.1 Retrieve the plate containing Total RNA. If stored in -80°C, thaw it on ice followed by a quick spin at 4°C, 700g for 1min. Place it on ice.

2. Input QC:

2.1 For total nucleic acid as input, it is recommended that contaminating gDNA is first quantified using Qubit (LIBPR.0030) or Quant-IT (LIBPR.0108). RNA QC can be skipped at this stage (200-400ng gDNA should generally give more than 200ng RNA after DNase treatment). The RNA/DNA mixture entering DNase treatment should contain <400ng gDNA. Normalization to 200-400ng gDNA should be performed using Nimbus (LIBPR.0121) or manually if deemed appropriate. Following DNase treatment, RNA is assayed (see step 3.2).

2.2 For purified RNA as input, RNA is quantified using Agilent RNA Nano (LIBPR.0018) or Caliper RNA (LIBPR.0052) assays.

2.2.1 For Caliper QC on Standard Assay, use 2 µL of sample and 46 µL of buffer.

2.2.2 For Caliper QC on HiSens Assay, use 2 µL of sample diluted with 4 µL DEPC water (6 µL total) and 19 µL buffer. Also, select “AB1000_2.5mm” as the total volume is lower for the Caliper plate. For Caliper QC:

Open file: **Production** > toggle to workflow > **mRNA Isolation** > **mRNA Isolation – *Scheduler.wfl** > **> Caliper Transfer > Standard or High Sensitivity**

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"*" Every time you see "**Scheduler.wfl**" addition to the name of the program, it means that you will have to select "work flow" option from the drop down menu at the bottom right corner of the window.

2.3 Normalize samples from purified RNA to the same RNA amount within 100-1000ng using Nimbus (LIBPR.0121) or manually if deemed appropriate.

LIBPR.0121	Total RNA Normalization on the Hamilton Nimbus

3. 1st DNase I Treatment: Remove contaminating gDNA in samples

3.1 All samples should be DNase treated according to LIBPR.0143 (with 1U for purified RNA and 5U for total nucleic acid).

3.2 **For Total Nucleic Acid only**, DNase-treated RNA should be quantified using Agilent RNA Nano (LIBPR.0018) or Caliper RNA (LIBPR.0052). Skip this QC for purified RNA input.

3.2.1 For Caliper QC on HiSens Assay, use 2 µL of sample diluted with 4 µL DEPC water (6 µL total) and 19 µL buffer. Also, select "AB1000_2.5mm" as the total volume is lower for the Caliper plate. The resulting concentrations are considered as they are without any dilution factor. Log into Nimbus Program as follows to transfer 2 µL of the RNA for Caliper QC:

Open file: **Production** > *toggle to workflow* > **mRNA Isolation** > **mRNA Isolation – Scheduler.wfl** > **> Caliper Transfer > Standard** ***or*** **High Sensitivity**

3.3 DNase-treated RNA from Total nucleic Acid should be normalized to 100-1000ng at this stage using Nimbus according to LIBPR.0121.

4. mRNA capture; manual preparation

4.1 RNA volume is 50 µL.

4.2 Aliquot 15 µL of NEBNext Oligo d(T)$_{25}$ beads into 96-well plate (ABGENE). Label the plate as "**Beads**".

4.3 Aliquot 350 µL of RNA Binding Buffer into Plate, 96-well v bottom plate (P-96-450V-C). Label the plate as "**Binding Buffer**".

4.4 Aliquot 900 µL of Wash buffer into Deep-well, 96-well, 1.2 mL, U bottom, low profile (AB1127). Label the plate as "**Wash Buffer**".

4.5 Aliquot 90 µL of Tris Buffer into 96-well plate (ABGENE). Label the plate as "**Tris Buffer**".

5. mRNA capture; on nimbus

5.1 Log into Nimbus Program as follows and follow the prompts to perform the mRNA isolation steps including incubation steps:

Open file: **Production** > *toggle to workflow* > **mRNA Isolation** > **mRNA Isolation – Scheduler.wfl** > **> mRNA Capture**

The detailed steps, which the Nimbus will be performing within the program selected as above are described in Appendix A. Remember **DO NOT** spin down plate before the Denature, Elute 1 and Elute 2 steps on Tetrad.

6. The mRNA is now ready for cDNA synthesis.

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Appendix A: Detailed steps performed by Nimbus within the program "mRNA Capture"

1. Add100 µL of RNA Binding Buffer from the "**Binding Buffer**" plate to the "**Beads**" plate. Mix (80% Total volume=92 µL) 10 times.
2. Place the beads plate on magnet for 2 minutes.
3. Remove the supernatant.
4. Remove the plate from the magnet.
5. Repeat steps 1–4.
6. Add 50 µL of RNA Binding Buffer from the "**Binding Buffer**" plate.
7. Add 50 µL of **total RNA sample** using same tips. Mix (80% Total volume=80 µL) 10 times.
8. *Place the plate on a thermal cycler (tetrad: MRNA>DENATURE) and incubate 65°C for 5 minutes and hold at 4°C to denature the RNA and facilitate binding of the poly-A mRNA to the beads.*
9. *Remove the plate when the temperature reaches 4°C (2min).*
10. Mix (80% Total volume=80 µL) 10 times.
11. Incubate at room temperature for 5 minutes to allow the mRNA to bind to the beads.
12. Place the plate on the magnet at for 2 minutes.
13. Remove the supernatant.
14. Remove the plate from the magnet.
15. Wash the beads by adding 180 µL of Wash Buffer from the "**Wash Buffer**" plate. Mix (80% Total volume=160 µL) 10 times.
16. Place the plate on the magnet for 2 minutes.
17. Remove the supernatant.
18. Remove the plate from the magnet.
19. Repeat steps 15–18.
20. Add 50 µL of Tris Buffer from the "**Tris Buffer**" plate. Mix (80% Total volume=40 µl) 10 times.
21. *Place the plate on the thermal cycler (tetrad: MRNA>ELUTE1) and incubate at 80°C for 2 minutes, then hold at 25°C to elute the Poly-A mRNA from the beads.*
22. *Remove the plate from the thermal cycler when the temperature reaches 25°C (2minutes).*
23. Add 50 µL of RNA Binding Buffer from the "**Binding Buffer**" plate to allow the mRNA to re-bind to the beads. (80% Total volume=80 µL) 10 times.
24. Incubate the plate at room temperature for 5 minutes.
25. Place the plate on the magnetic rack at room temperature for 2 minutes.
26. Remove the supernatant.
27. Remove the plate from the magnet.
28. Wash the beads by adding 180 µL of Wash Buffer from the "**Wash Buffer**" plate. Mix (80% Total volume=160 µL) 10 times.
29. Place the plate on the magnet for 2 minutes.
30. Remove the supernatant.

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31. Remove the plate from the magnet.
32. Repeat steps 28–31.
33. Elute mRNA from the beads by adding 36 µL of the Tris Buffer from the the "**Tris Buffer**" plate. Mix (80% Total volume=29 µL) 10 times.
34. *Incubate the plate in a thermocycler (tetrad: MRNA>ELUTE2) at 80°C for 2 minutes.*
35. Place the plate on magnet.
36. Transfer the supernatant (36 µL) into a clean 96-well plate (ABGENE) ("**mRNA plate**")

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Appendix B: LIMS SOP

1. Start of Plate Library Construction

2. Bioanalyzer Run / Caliper Run. QC Category: Total RNA QC

3. Mag Bead Based mRNA Isolation . RNA_strategy is "strand specific" and the pipeline is Strand Specific Transcriptome 3.1: SSTRA_3.1

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