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****Note: controlled versions of this document are subject to change without notice.***

Magnetic Bead-based mRNA isolation v2

I. Purpose

To capture poly (A) mRNA from Total RNA using mRNA isolation kit from New England Biolabs (NEB).

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 Quality Systems 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
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
Automated DNA Quantification using the dsDNA Quant-iT High Sensitivity Assay Kit and VICTOR3V	LIBPR.0108
Total RNA Normalization on the Hamilton Nimbus	LIBPR.0121

Document Title	Document Number
DNase I treatment of RNA/Total Nucleic Acid	LIBPR.0143
Quantifying DNA samples using the Qubit 4 Fluorometer	LIBPR.0153

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 safety data sheets (SDS) 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
Gilson P20 pipetman	Mandel	GF23600
Gilson P200 pipetman	Mandel	GF-23601
Gilson P1000 pipetman	Mandel	GF-23602
P20 DF30 tips	Mandel	GF-F171303
P200 DF200 tips	Mandel	GF-F171503
P1000 DF1000 tips	Mandel	GF-F171703
VX-100 Vortex Mixer	Rose Scientific	S-0100
200µL Rainin tips	Rainin	RT-L200F
200µL Pipet-Lite	Rainin	L12-200
Large Kimwipes	Fisher	06-666-117
Black ink permanent marker pen	VWR	52877-310
Bench Coat (Bench Protection Paper)	Fisher	12-007-186
Small Autoclave waste bags 10”X15”	Fisher	01-826-4
DEPC water	Ambion	9922
Mini-centrifuge	Eppendorf	5417R
Thermo Scientific 0.2mL Ultra Rigid Skirted	Thermoscientific	AB1000-150s
Deep-well, 96-well, 1.2mL, U bottom, low pro,	Fisher Scientific	AB1127
NEBNext Poly(A) mRNA Magnetic Isolation	New England Biolabs	E7490L

PCRmax Alpha Cycler 4	Froggabio/Cole Parmer	AC496
MagMax express 96 Deep Well plates	Invitrogen	4388476
96 well V bottom storage plate	Ultident	24-P96-450V-C
Rainin AutoRep E	Mettler Toledo	17000723
1.25mL Encode syringe tip	Mettler Toledo	17001874
12.5mL Encode syringe tip	Mettler Toledo	17001876
25mL reagent reservoir	VWR	89094-664
NIMBUS Liquid Handling Workstation	Hamilton	
CO-RE 50µL Tips in Frames	Hamilton	235831
Sterile Filtered Conductive 300µL Tips in	Hamilton	235832
Tape Pads	Qiagen	19570
Foil Tape	VWR	60941-126

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 500ng 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. Ensure proper personal protective equipment is used when handling sample plates, reagents and equipment. Treat everything with, and adhere to, strict RNA handling techniques.

1.4. 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.

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

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

1.7. 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.

1.8. Ensure the waste bag for the Nimbus is empty.

1.9. 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.

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

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

X. PROCEDURE

Note: If you are unsure of which Nimbus protocol version to use, please 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 1 minute. Place the plate 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.0153) or Quant-IT (LIBPR.0108). RNA QC can be skipped at this stage (200-500ng gDNA should generally give more than 200ng RNA after DNase treatment). The RNA/DNA mixture entering DNase treatment should contain <500ng gDNA. Normalization to 200-500ng 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 2µL of sample and 46µL of buffer.

2.2.2. For Caliper QC on HiSens Assay, use 2µL of sample diluted with 4µL DEPC water (6µL total) and 19µL 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 v2** > **mRNA Isolation Scheduler v2.wfl** > **> Caliper Transfer > Standard or High Sensitivity**

"*****" 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. 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 2µL of sample diluted with 4µL DEPC water (6µL total) and 19µL 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 2µL of the RNA for Caliper QC:

Open file: **Production** > *toggle to workflow* > **mRNA Isolation v2** > **mRNA Isolation Scheduler v2.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 50µL.

4.2. Aliquot 15µL of NEBNext Oligo $d(T)_{25}$ beads into a 96-well plate (AB-1000). Label the plate as "**Beads**".

4.3. Aliquot 300µL of RNA Binding Buffer into a 96-well v bottom plate (P-96-450V-C). Label the plate as "**Binding Buffer**".

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

4.5. Aliquot 90µL of Tris Buffer into a 96-well plate (AB-1000). 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 v2** > **mRNA Isolation Scheduler v2.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.

Quick spin plate for 10 seconds at 4°C only AFTER denaturation on the tetrad. A spindown is NOT necessary after the Elute 1 and Elute 2 steps on the tetrad.

6. The mRNA is now ready for cDNA synthesis.

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

1. Add 75µL of RNA Binding Buffer from the "**Binding Buffer**" plate to the "**Beads**" plate. Mix (80% Total volume = 72µL) 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 50µL of RNA Binding Buffer from the "**Binding Buffer**" plate.
7. Add 50µL of **total RNA sample** using same tips. Mix (80% Total volume = 80µL) 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 (2 minutes).*
10. Mix (80% Total volume = 80µL) 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 180µL of Wash Buffer from the "**Wash Buffer**" plate. Mix (80% Total volume = 160µL) 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 = 40µL) 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 (2 minutes).*

23. Add 50µL of RNA Binding Buffer from the "**Binding Buffer**" plate to allow the mRNA to re-bind to the beads. (80% Total volume = 80µL) 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 180µL of Wash Buffer from the "**Wash Buffer**" plate. Mix (80% Total volume = 160µL) 10 times.

29. Place the plate on the magnet for 2 minutes.

30. Remove the supernatant.

31. Remove the plate from the magnet.

32. Repeat steps 28–31.

33. Elute mRNA from the beads by adding 20µL of the Tris Buffer from the the "**Tris Buffer**" plate. Mix (80% Total volume = 16µL) 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 (20µL) into a clean 96-well plate (ABGENE) ("**mRNA plate**").

Appendix B: LIMS SOP

1. If **DNase Treatment** Protocol has not been run already do so here.
2. Start of Plate Library Construction
3. Bioanalyzer Run / Caliper Run. QC Category: Total RNA QC
4. Mag Bead Based mRNA Isolation. RNA_strategy is “Strand_specific - Positive” and the pipeline is Strand Specific Transcriptome 4.0: SSTRA_4.0.