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We use this protocol and it's working

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GeoMx-NGS Readout Library Preparation

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ABSTRACT

This protocols describes the preparation of a library for Illumina NGS sequencing from Nanostring GeoMx DSP aspirate samples.

MATERIALS

Equipment, Materials, and Reagents for GeoMx—NGS RNA Library Prep

Equipment

A	B	C
Equipment	Manufacturer	Part Numbers
Pre-PCR hood (or designated space)	Various	Various
ALPS 50 plate sealer (see Materials list for compatible heat- sealing foil)	Thermo Fisher	AB-1443A
Plate spinner/centrifuge with plate adapter	Various	Various
Library quantitation device - qubit or similar	Various	Various
Vortex	Various	Various
Picofuge	Various	Various
Heatblock or thermal cycler	Various, such as Bio-Rad C1000 deep-well model	Various, such as 1851197
Plate spinner/centrifuge (up to at least 2000 g)	Various	Various
Capillary Electrophoresis device (e.g., Bioanalyzer)	Agilent	G2939BA
Illumina NGS instrument	Illumina	Various

Table 1: Equipment not provided by NanoString

Materials

A	B	C
96-well PCR plates (RNase/DNase free)	Fisher Scientific	BC2496
Heat-sealing foil seals	Fisher Scientific	AB-0559
Magnetic stand (e.g., DynaMag-96)	Invitrogen	12331D
Materials	Manufacturer	Part Numbers
Microtubes (RNase/DNase free)	Sarstedt (or comparable)	72.785.005
Permeable membranes (dry-down seal)	Sigma	A9224
96 well plate stickers (freezing and storing plates)	Various	Various
Cooling block (optional)	Various	Various
DNA LoBind tubes (for library storage before sequencing)	Various	Various
Filter Tips (RNase/DNase free)	Various	Various
PCR strip tubes (12-tube or 8-strip, RNase/DNase free)	Various	Various
Two* 12-channel P20 multi-channel pipettes	Various	Various

A	B	C
Surface decontaminant (i.e., RNase Away or 10% Bleach)	Various Thermofisher	Various 7002

Table 2: Materials not provided by NanoString

* separate pipettes are needed for pre- and post PCR processes.

Reagents

Table 3: Reagents not provided by NanoString

Reagents	Manufacturer	Part Numbers
100% ethanol 	Various	Various
Agencourt AMPure XP	Beckman Coulter	A63880
Elution buffer (Tris-HCl 10 mM with 0.05% Tween-20, pH 8.0) *	Teknova	T1485
Bioanalyzer DNA High Sensitivity Kit	Agilent	5067-4626
PCR grade water	Various	Various

* Elution buffer can also be made by adding 5 mL 100mM Tris pH 8.0 and 2.5 mL 1% Tween-20 to 42.5 mL PCR-grade nuclease-free H_2O. Mix well.

REAGENTS/KITS PROVIDED BY NANOSTRING

The following kits and reagents are available from NanoString

Master Mix can be stored at -20°C (stored in glycerol so aliquoting not required).

Seq Code plates can be stored at -20°C. Once thawed, Seq Code plates can be stored up to 3 months at 4° C; alternatively, they can go through up to three freeze-thaw cycles.

Transferring the DSP Collection Plate

1h

1 PREPARING THE COLLECTION PLATE (1 HOUR)

2 Remove the collection plate from the DSP instrument by following the instructions at the end of the GeoMx DSP run or clicking the plate status icon.

3 Seal the collection plate with a permeable membrane. if delaying library preparation for over 24 hours, seal with an nonpermeable membrane and store at -20°C. If shipping to another facility, seal with a nonpermeable membrane and wrap plate in bubble wrap and ship on dry ice (direct contact with dry ice may cause the plate to crack).

4 Dry down the collection plate by incubating on a thermocycler at 65°C for up to 1 hour. If there is still liquid in some of the wells after this time, dry down until all liquid has evaporated.

5 Spin down the plate and check that there is no liquid remaining prior to rehydrating the samples in the next step.

6 Rehydrate the samples. Add 10 µL of nuclease-free water, pipette up and down 5 times and allow the collected targets to solubilize for 10 minutes at room temperature. Use

an adhesive plate seal to keep the sample from re-evaporating.

7 Pulse centrifuge the plate to 1000 x g to ensure all liquid has been collected at the bottom.

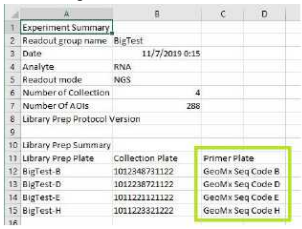
Library Preparation

2h

8 **PCR setup (2 hours)**

BEFORE YOU START

- Program a thermocycler with a 100°C heated lid according to table (see Table 4).
- Clean workspace with 10% bleach or RNase Away. Rinse with distilled H2O, followed by 70% EtOH.
- Refer to the Lab Worksheet (see Figure 4) to choose the correct letter of GeoMx Seq Code Primer Plate for your DSP collection plate. Thaw the primer plate on ice or on cooling block.



	A	B	C	D
1	Experiment Summary			
2	Readout group name	BigTest		
3	Date	11/7/2019 0:15		
4	Analyte	RNA		
5	Readout mode	NGS		
6	Number of Collection	4		
7	Number Of AOIs	288		
8	Library Prep Protocol Version			
9				
10	Library Prep Summary			
11	Library Prep Plate	Collection Plate	Primer Plate	
12	BigTest-B	1012348731122	GeoMx Seq Code B	
13	BigTest-D	1012238721122	GeoMx Seq Code D	
14	BigTest-E	1011221121122	GeoMx Seq Code E	
15	BigTest-H	1011229321222	GeoMx Seq Code H	
16				

Figure 4: Lab Worksheet

- Keep all PCR reagents as well as the PCR plate on ice or on cooling block during setup.
- Once thawed, pulse centrifuge 5x PCR Master Mix, primer plate, and DSP collection plate to 1000 X g. Do not vortex at any point.

9 This PCR protocol begins with uracil-DNA glycosylase (UDG) incubation and deactivation.

A	B	C	D
Step	Temp.	Time	Cycles
UDG incubation	37 °C	30 min	1 X
UDG deactivation	50 °C	10 min	1 X
Initial denaturation	95 °C	3 min	1 X
Denaturation Anneal Extend	95 °C 65 °C 68 °C	15 sec 60 sec 30 sec	18 X
Final extension	68 °C	5 min	1 X
Hold	4 °C	∞	1 X

Table 4: Cycling conditions

10 **PROCEDURE**

The instructions here assume a 96-well plate. If running more or fewer aspirates, adjust the procedure and volumes accordingly.

11 Prepare a new 96- well PCR plate Table 5: PCR reaction according to the table (see Table 5) and the steps below. Use of a multichannel pipette is recommended. Ensure that each component is added to the bottom of the well and that your Primer Plate , DSP collection plate and PCR plate are all in the correct orientation (well A1 at upper left).

A	B
PCR amplification reaction components	Volume (µL)

A	B
5x PCR Master Mix	2 µL
Primer mix (F & R, 2.5 µM each)	4 µL
DSP aspirate	4 µL
TOTAL VOLUME per reaction	10 µL

Table 5: PCR reaction

12 Add 2 µL of 5x PCR Master Mix to each well of the new PCR plate. It is acceptable to reuse pipette tips for each column during this step.

13 Transfer 4 µL of primer from each row/column of the GeoMx SeqCode primer plate to each row/column of the PCR plate. Use of a multichannel pipette is encouraged. Change pipette tips for every row/column.

14 Add 4 µL of DSP aspirate from each row/column of the DSP collection plate to each row/column of the PCR plate. Change pipette tips for every row/column.

15 Pipette up and down 10 times to mix.

16 Heat-seal PCR plate according to manufacturer instructions. Pulse centrifuge to 1000 x g.

17 Incubate PCR plate in a thermocycler with program specified in Table 1(see Table 4).

The PCR plate may be stored at 4°C overnight, or -20°C for up to 72 hours.

Pooling and AMPure cleanup (45 minutes)

45m

18 IMPORTANT: The following are post-PCR steps and should be performed in a separate lab space from the pre-PCR steps.

Pool and purify PCR products using the steps below. The instructions below are for a single 96-well plate.

19 Once PCR is complete, pulse centrifuge PCR plate to 1000 x g.

20 Pool 4 µL of each PCR well into one 1.5 mL tube (see Figure 6).

Using a multichannel pipette (8-channel), combine each column of samples into one 8-well strip tube. Once all columns have been combined into the strip tube, the contents of each strip tube well can then be pooled into a single 1.5 mL tube. If running multiple 96-well plates, pool each plate into its own 1.5 mL tube. However, if less than 12 reactions are on a plate, you may combine that pool with another plate's pool.

21 Measure the volume in the 1.5 mL tube using a pipette.

22 Add 1.2x volume of the measured volume of AMPure XP beads to the pooled PCR product. Mix well by pipetting up and down 10x. Pulse centrifuge.

It is important to measure the exact volume of the pool.

Example: pooling 4 µL from 96 wells of PCR product results in a ~384 µL pool. Measure the actual volume using a pipette, then add 1.2x this measured volume of AMPure XP beads to the pool.

23 Incubate 5 minutes at room temperature.

24 Prepare 20 mL fresh 80% EtOH by combining 16 mL 100% EtOH and 4 mL PCR-grade H2O.

25 Pellet beads on a magnetic stand for 5 minutes or until the solution is clear.

26 Carefully remove the supernatant. Avoid disturbing the beads, as they contain the library at this stage.

27 Wash beads with 1 mL of freshly-prepared 80% ethanol, incubating on magnetic stand for 30 seconds.

28 Discard the supernatant, being careful not to disturb the beads.

29 Wash beads a second time with 1 mL of freshly- prepared 80% ethanol, incubating on magnetic stand for 30 seconds.

30 Discard the supernatant, being careful not to disturb the beads.

31 Visually inspect the beads to ensure that as much ethanol as possible is removed without disturbing the beads. Use a low volume pipette (e.g., P20) to remove any residual ethanol, if necessary.

32 Dry beads on magnetic stand for ≤ 5 minutes. Remove from the magnetic stand.

33 Resuspend in 54 µL Elution Buffer. Mix well by pipetting.

34 Pellet beads on a magnetic stand for 5 minutes until the solution is clear.

35 Extract 50 µL of supernatant to a new tube. Leave 2–4 µL of supernatant in tube, if necessary, to avoid disturbing beads. This supernatant contains the sequencing library.

36 Add 60 µL of Ampure XP beads to the 50 µL supernatant. Mix well by pipetting up and down 10x. Pulse centrifuge.

37 Incubate 5 minutes at room temperature.

38 Pellet beads on a magnetic stand 5 minutes or until the solution is clear.

39 Carefully remove the supernatant. Avoid disturbing the beads.

40 Wash beads with 1 mL of freshly-prepared 80% ethanol, incubating on magnetic stand for 30 seconds.

41 Discard the supernatant, being careful not to disturb the beads.

42 Wash beads a second time with 1 mL of freshly- prepared 80% ethanol, incubating on magnetic stand for 30 seconds.

43 Discard the supernatant, being careful not to disturb the beads.

44 Visually inspect to ensure that as much ethanol as possible is removed without disturbing the beads. Use a low volume pipette (e.g., P20) to remove any residual ethanol, if necessary.

45 Dry beads on magnetic stand for ≤ 5 minutes. Remove from magnetic stand.

46 Resuspend beads in Elution Buffer according to the table below . Mix well by pipetting.

	A	B
	# reactions	Elution Buffer volume (µL)
	96	48 µL
	48	36 µL
	24	24 µL
	12	18 µL

Table 6: Elution Buffer volume

47 Incubate 5 minutes at room temperature.

48 Pellet the beads on the magnetic stand 5 minutes or until the solution is clear.

49 Extract the supernatant to a new tube. Leave 2 µL of supernatant in tube, if necessary, to avoid disturbing beads. This supernatant contains the sequencing library.

STOPPING POINT: The purified library may be stored in a DNA LoBind tube at -20°C until ready for sequencing.

Quality control

50 Combine 2 µL of library and 14 µL of Elution Buffer to create a 1:8 dilution .

51 Assess quality of library stock and 1:8 dilution quality using a capillary electrophoresis device such as the Agilent Bioanalyzer. Follow manufacturer instructions for use.

52 Check for the expected size of the amplicons and absence of primers, primer-dimers, or high molecular weight overamplification products (see Figure 8).

Expected library amplicon size is 162 bp. Using the Agilent Bioanalyzer, the library will appear as ~150bp.

Verify that the concentration of the ~150bp amplicon peak of either the undiluted library or the 1:8 dilution falls within the Bioanalyer assay's quantitative range (5 – 500 pg/ul). Use the molarity value (pM) from the Bioanalyzer to calculate library concentration.

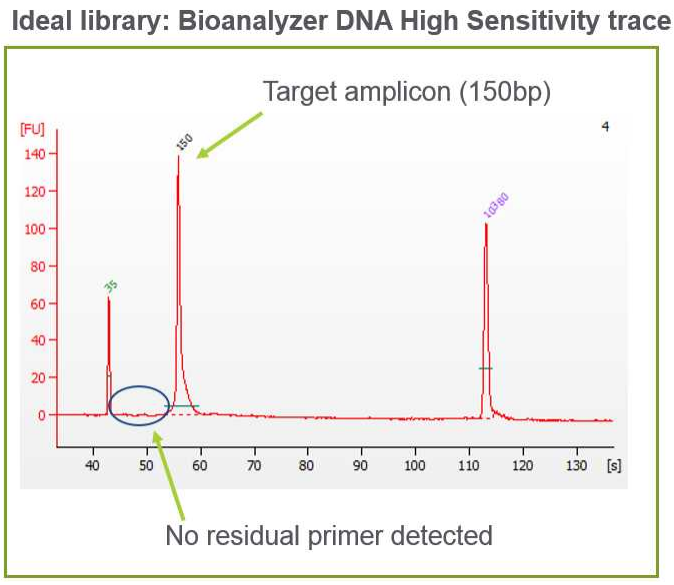


Figure 8: Library QC results

Sequencing Setup

53 Libraries should be sequenced on Illumina® sequencing platforms with the following workflow

specifications:

- Generate FASTQ
- Dual-indexing
- Paired-end with reads 2 x 27
- 5% PhiX spike-in by volume

Use the Sample Index List downloaded from the GeoMx DSP Instrument. Copy and paste the Sample Index List into your Illumina Sample Sheet. Confirm the sequencing details entered here. If corrections are needed, you may correct the sheet and re-download the readout package, which includes the Sample Index List.

Set up the run according to the respective Illumina platform instructions (see respective Illumina platform user manuals at support.illumina.com). Suggested loading concentrations are listed below (see Table 7).

Illumina platform	Flow cell	Loading concentration
MiSeq	v3	9 pM
NextSeq550	High-output	1.6 pM
NextSeq2000	P2	650 pM

Table 7: Suggested loading concentration per Illumina platform

These values may be adjusted empirically based on internal workflow/specific sites/instruments.

Transferring from the Illumina NGS Run

54 **Transferring the NGS raw data (FASTQ) files**

Sequencing output FASTQ files will need to be transferred and processed by NanoString's GeoMx NGS Pipeline (DND) software program before being uploaded back to the GeoMx DSP system for Data Analysis. See the GeoMx-NGS DND Software User Manual for additional guidance.