GeoMx® DSP
NGS Readout
User Manual

Library Preparation & GeoMx NGS Pipeline

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nanoString®

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Changes in this Revision

Changes in this manual revision include:

- Added information to support the new Immuno-oncology Proteome Atlas (IPA) with Pro Code indices. Please refer to the following page for a summary of key information and workflow changes for this assay.
- Added to Lab Worksheet description, which now includes suggested sequencing depth, on page 18 and on page 61
- Updated storage guidelines for PCR products before AMPure cleanup (4°C for up to 3 days) to align with Illumina® library preparation best practices, on page 28
- Added guidance for situations where the No-Template Control (NTC) well is excluded from collection on page 29. For collections in which some slides use an NTC and some don't, the NTC may be in a well other than A1. Check the Lab Worksheet to confirm the location of the NTC.
- Added additional incubation time prior to eluting library from beads on page 30 (step 16) for optimal yield
- Added to Troubleshooting section on page 75
- Updated typeface for improved readability

Key Considerations for the Immuno-oncology Proteome Atlas (IPA) with Pro Code Indices

NanoString's **Immuno-oncology Proteome Atlas (IPA)** covers 570+ protein targets across dozens of pathways and is the first GeoMx assay to use **Pro Code** i5 and i7 indices (sequences important in Next-Gen Sequencing (NGS)). Pro Code indices are 2 nucleotides longer than **Seq Code** indices, which are used in all other GeoMx NGS assays. For more information on i5 and i7 indices, please refer to the GeoMx DSP NGS Readout User Manual (MAN-10153).

When running a Pro Code assay such as the Immuno-oncology Proteome Atlas, please pay attention to Pro Code-specific instructions throughout the user manuals, summarized here:

- GeoMx DSP software v3.1 or later is required to run Pro Code assays such as IPA.
- The IPA probe mix is packaged as two tubes: core and module. The concentration of the module is different than NanoString's other Protein-NGS Assays with Seq Code indices, so the Antibody Working Solution is prepared differently. See details in the GeoMx DSP Manual Slide Preparation User Manual (MAN-10150) or Automated Slide Preparation User Manual (MAN-10151).
- Generally, assays with Pro Code indices such as IPA cannot be combined in the same plate or readout group as assays with Seq Code indices. Compatibility rules are detailed in the GeoMx DSP Instrument User Manual (MAN-10152). An exception is the Spatial Proteogenomic Assay in which RNA and Protein are collected from the same slide; refer to the GeoMx DSP Spatial Proteogenomic Assay User Manual (MAN-10158).
- In NGS library preparation, tags with Pro Code indices such as IPA are PCR-amplified with Pro Code primer plates, rather than Seq Code primer plates. Less DSP aspirate is required in the PCR amplification step for Pro Code assays compared to Seq Code assays (2 µL vs 4 µL). See details in the GeoMx DSP NGS Readout User Manual (MAN-10153). Note that 2 Pro Code primer plates are available (Y and Z), allowing multiplexing of up to 192 wells.
- Pro Code assays can generally be sequenced at less depth than Seq Code assays. See details in the GeoMx DSP NGS Readout User Manual (MAN-10153).
- At this time, Proteogenomic Assays that include IPA can only be processed on the standalone GeoMx NGS Pipeline software version 3.1, not on the GeoMx NGS Pipeline on DRAGEN via BaseSpace Sequencing Hub. Obtain GeoMx NGS Pipeline software version 3.1 and installation instructions at https://nanostring.box.com/v/GeoMxNGSPipeline. Refer to the GeoMx DSP Spatial Proteogenomic Assay User Manual (MAN-10158).
- There are no changes in the Data Analysis workflow for Pro Code assays.

Conventions

The following conventions are used in the GeoMx DSP user manuals and are described for your reference.

Bold text is typically used to highlight a specific button, keystroke, or menu option. It may also be used to highlight important text or terms.

Blue underlined text is typically used to highlight links and/or references to other sections of the manual. It may also be used to highlight references to other manuals or instructional material.

A gray box indicates general information that may be useful for improving assay performance. These notes aim to clarify other instructions or provide guidance to improve the efficiency of the assay workflow.

IMPORTANT: This symbol indicates important information that is critical to ensuring a successful assay. Following these instructions may help improve the quality of your data.

WARNING: This symbol indicates the potential for bodily injury or damage to the instrument if the instructions are not followed correctly. Always carefully read and follow the instructions accompanied by this symbol to avoid potential hazards.

For NGS readout: Content in blue boxes denotes steps or information specific to NGS readout of GeoMx DSP. Follow these instructions if using Illumina® NGS to read out GeoMx DSP counts.

For nCounter readout: Content in green boxes denotes steps or information specific to nCounter readout of GeoMx DSP. Follow these instructions if using nCounter® Pro, MAX/FLEX or SPRINT to read out GeoMx DSP counts.

GeoMx DSP Workflow

The GeoMx Digital Spatial Profiler (DSP) is a novel platform developed by NanoString. Antibody or nucleic acid probes are coupled to photocleavable oligonucleotide tags. After probes hybridize to targets in slide-mounted tissue sections, the oligonucleotide tags are released from discrete regions of the tissue via UV exposure. Released tags are quantified by nCounter technology or Illumina Next Generation Sequencing (NGS). Counts are mapped back to tissue location, yielding a spatially resolved digital profile of analyte abundance (Figure 1).

- **Day 1: Slide Staining.** Prepare slides and incubate biological targets with UV-cleavable probes. Prepare manually or using the BOND RX/RX^{m} Fully Automated IHC/ISH Stainer from Leica Biosystems®.
- **Day 2: Process Slides on GeoMx DSP.** Load prepared slides into the GeoMx DSP instrument. Slides are scanned to capture fluorescent images used to select regions of interest (ROIs). ROIs may be segmented into discrete compartments or areas of illumination (AOI). The instrument collects UV-cleaved oligos from the AOIs into the wells of a collection plate.

For NGS readout:	For nCounter readout:
Day 3: Transfer the collected aspirates to a PCR plate and perform **Library Prep** with Seq Code or Pro Code primers. Pool and purify the products, then **Sequence** on an Illumina NGS instrument. **Day 4**: Process FASTQ sequencing files into digital count conversion (DCC) files using **GeoMx NGS Pipeline** with NanoString's standalone software or Illumina's DRAGEN™ platform accessed via BaseSpace™ Sequence Hub (cloud) or NextSeq 1000/2000 (local).	**Day 2, continued**: Transfer the collected aspirates to a hybridization plate along with GeoMx Hyb Code reagents. Hybridization occurs overnight. **Day 3**: Pool wells and **Process on an nCounter Pro or MAX/FLEX Analysis System or SPRINT Profiler** to generate reporter count conversion (RCC) files.

- **Day 4 or 5: Upload counts** to GeoMx DSP and **create a study** in the Data Analysis Suite. Perform quality-control checks and data analysis, and generate analysis plots.

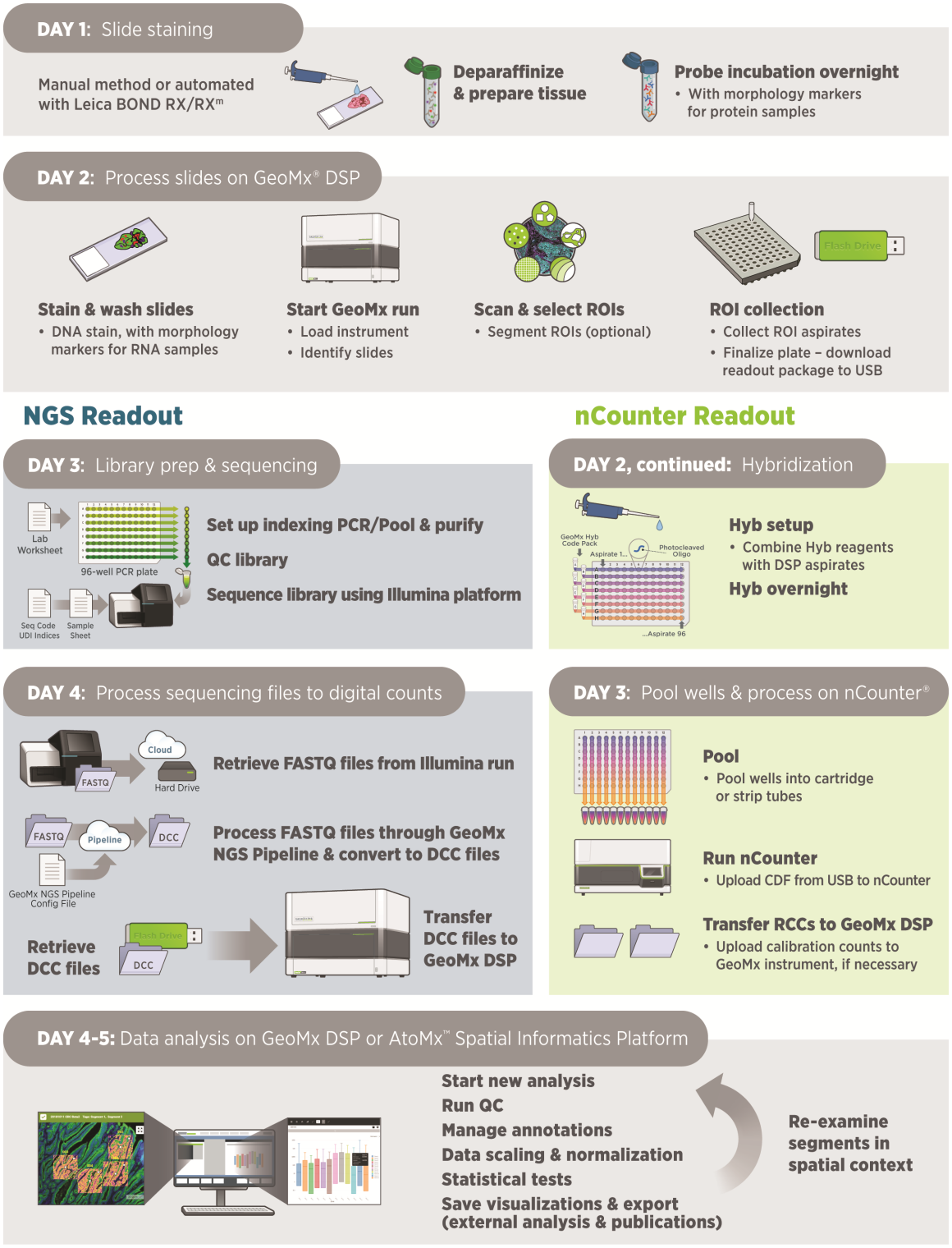


Figure 1: GeoMx DSP workflow summary

User Manuals and Resources

The GeoMx DSP workflow is divided into the following user manuals:

Workflow Step 1	GeoMx DSP Manual Slide Preparation User Manual MAN-10150 GeoMx DSP Automated Slide Preparation User Manual MAN-10151	
Workflow Step 2	GeoMx DSP Instrument User Manual MAN-10152	
Workflow Step 3	**For NGS readout:** GeoMx DSP NGS Readout User Manual MAN-10153	**For nCounter readout:** GeoMx DSP nCounter Readout User Manual MAN-10089
Workflow Step 4	GeoMx DSP Data Analysis User Manual MAN-10154	

User manuals and other documents can be found online in the NanoString University Document Library at https://university.nanostring.com. Instrument and workflow training courses are also available in NanoString University.

For NGS readout:
For documentation specific to the Illumina platform, see https://support.illumina.com.

For nCounter readout:
For documentation specific to the nCounter Pro, MAX/FLEX, and SPRINT instruments, visit the NanoString University Document Library at https://university.nanostring.com.

For the GeoMx DSP Spatial Proteogenomic Protocol (RNA and Protein on the same slide), please refer to the GeoMx DSP Spatial Proteogenomic Assay User Manual (MAN-10158).

Introduction to GeoMx NGS Readout

GeoMx DSP assays with NGS readout use next-generation sequencing of molecular barcodes to quantify gene and protein expression in spatial context. Figure 2 provides an overview of the workflow for NGS readout.

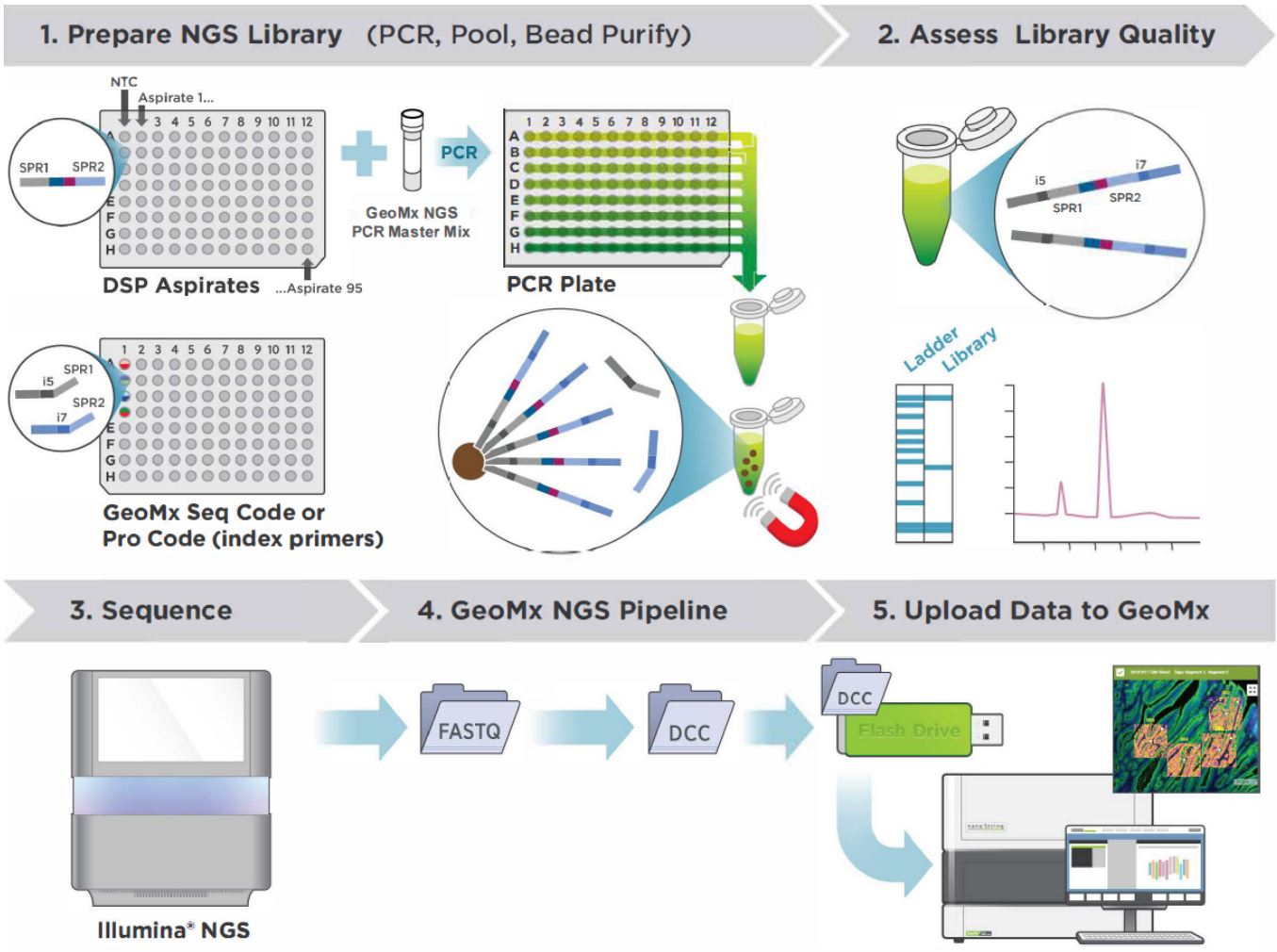


Figure 2: GeoMx DSP NGS readout overview

NGS Library Preparation and Sequencing

Following collection on the GeoMx DSP instrument, the 96-well collection plate contains aspirates from the experimental regions of interest. The aspirates are photocleaved oligos, each containing a **readout tag sequence identifier (RTS ID)** to identify the biological target (Figure 3), and a **unique molecular identifier (UMI)** enabling removal of PCR duplicates in the subsequent data processing pipeline. Finally, **SPR1** (sequencing primer read 1) and **SPR2** (sequencing primer read 2) sequences allow hybridization to **GeoMx Seq Code** or **Pro Code primers** in PCR amplification. Note that SPR1 and SPR2 sequences are different in Seq Code versus Pro Code tags.

During PCR amplification, the GeoMx Seq Code or Pro Code primers add **i5** and **i7 indexing sequences** (unique dual indexes) for multiplexing many GeoMx DSP aspirate sequences into a single sequencing run, as well as **P5** and **P7 sequences** for binding and amplification on Illumina flow cells.

Following a pooling, purification, and quality control process, the final NGS library is ready for sequencing on an Illumina NGS platform.

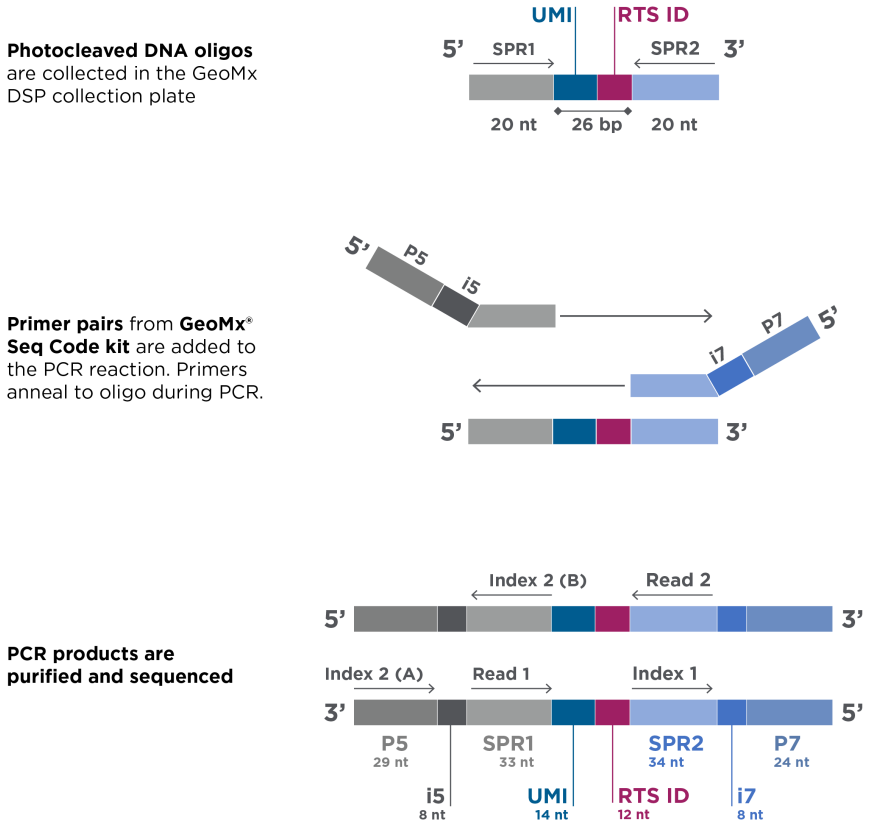


Figure 3: Seq Code tag structure and PCR product.
In Pro Code tags, the i5 and i7 are 10 nt in length, and the sequences of SPR1 and SPR2 are different.

Depending on the sequencing platform used, the **i5 index** will be read in either the forward or reverse direction. Table 1 indicates the i5 sequence orientation to be selected at the step **Finalize Plate** on the GeoMx DSP, depending on the Illumina platform.

Table 1. i5 Index Read Direction

i5 Sequence Orientation	Workflow	Illumina Platform
Forward	Forward Strand (Index 2 – Workflow A)	• HiSeq® 2000/2500 • MiSeq™ • NovaSeq™ 6000 with v1.0 reagent kits
Forward	Reverse Complement (Index 2 – Workflow B)	• NextSeq™ 500/550 where local run manager software is used to set up sequencing run • NextSeq 1000/2000
Reverse	Reverse Complement (Index 2 – Workflow B)	• HiSeq 3000/4000/X • NovaSeq 6000 with v1.5 reagent kits • NextSeq 500/550 without local run manager – sample sheet directly inputted into NextSeq 500/550 control software • NextSeq 1000/2000 set up with custom pipeline for BCL conversion (rare)

NOTE: "Workflow A" is Illumina's forward strand workflow and "Workflow B" is the reverse complement workflow. Read more about Illumina terminology in the Indexed Sequencing Overview Guide (Illumina Document # 15057455).

GeoMx NGS Pipeline

Following sequencing on the Illumina platform, the sequencing results are run through the GeoMx NGS Pipeline. This bioinformatic processing pipeline was developed by NanoString to convert FASTQ files from Illumina sequencers according to parameters defined in the configuration file generated by the GeoMx DSP. The pipeline computationally processes these files to output DCC files, which are uploaded to the GeoMx DSP system for data analysis.

The automated data processing pipeline depicted here (Figure 4) illustrates the steps of the GeoMx NGS Pipeline.

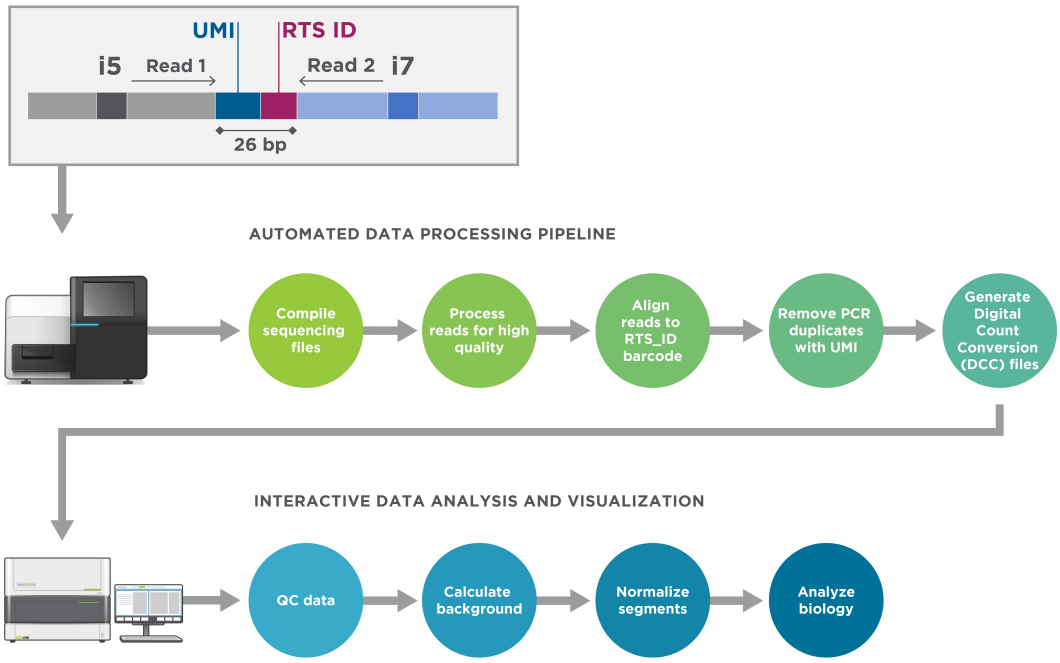


Figure 4: GeoMx NGS Pipeline

In the first step, the **raw reads** (raw sequencing FASTQ files) are compiled with the configuration file, which specifies the processing parameters. Next, the raw reads are processed by computationally removing the adapter sequences (resulting in **trimmed reads**), and merging the overlapping paired-end reads (resulting in **stitched reads**). In the third step, the stitched reads are aligned to the RTS-ID barcodes in the reference assay, creating **aligned reads** and assigning raw counts to biological target names. Then, PCR duplicates are removed using the Unique Molecular Identifier in each read, resulting in **deduplicated reads**. The DCC files are created and presented as a .zip file in a folder which you designate and can then be uploaded into the DSP Control Center for study creation in the DSP Data Analysis Suite, or loaded into R for data processing with the GeomxTools open software package.

Two Ways to Run the GeoMx NGS Pipeline

1. Run GeoMx NGS Pipeline software, installed on a computer, server, or cloud.
    - Control data and workflows in your IT environment.
    - Requires Windows OS computer, Linux server on-premises or Amazon Web Services® (AWS).
2. Run GeoMx NGS Pipeline on DRAGEN via BaseSpace Sequence Hub (cloud) or NextSeq 1000/2000 (local).
    - Pay-for-usage alternative to NanoString's standalone GeoMx NGS Pipeline software.
    - Ideal for users who want to start processing data quickly without IT infrastructure.
    - For cloud option, requires a subscription to BaseSpace Sequence Hub. Free trial and low-cost subscriptions are available; see https://support.illumina.com for more information.
    - For local option, requires a subscription to DRAGEN license with NextSeq 1000/2000.

To evaluate the pipeline option that is best for you, refer to Figure 10 on page 35 and the instructions for the different options (starting on page 36). Additional resources include the GeoMx DSP IT Specifications and Requirements guidelines, your NanoString Applications Scientist, and technical support from Support@nanostring.com.

> **IMPORTANT:** GeoMx NGS Pipeline on DRAGEN has been validated to produce concordant results to the standalone NanoString GeoMx NGS Pipeline. For best data processing practices, it is recommended that studies be processed in one pipeline or the other.

> **IMPORTANT:** Sequencing data from spatial proteogenomic assays (RNA + Protein) that include NanoString's Immuno-oncology Proteome Atlas can only be processed on the standalone NanoString GeoMx NGS Pipeline (not GeoMx NGS Pipeline on DRAGEN via BSSH) at this time. Refer to the GeoMx DSP Spatial Proteogenomic Assay User Manual (MAN-10158) for assay information.

File Inputs and Outputs

File inputs and outputs are illustrated below (Figure 5).

After the GeoMx DSP run, you will download a zipped readout package containing the files:

- **SeqCodeIndices.csv**, a file with sample information needed by the Illumina software. Use the contents to create a **SampleSheet.csv** for input into demultiplexing the Illumina run. In GeoMx software v3.1, the filename is the same even if it is a Pro Code readout group. (NextSeq 1000/2000 users download a SampleSheet.csv and whitelist.txt instead of an Indices.csv.)
- **LabWorksheet.txt**, a spreadsheet for reference during library prep, including ROI and segment details, ROI coordinates in XYZ, scan details, biological target details, and recommended sequencing depth.
- **Configuration (.ini) file** containing pipeline processing parameters.

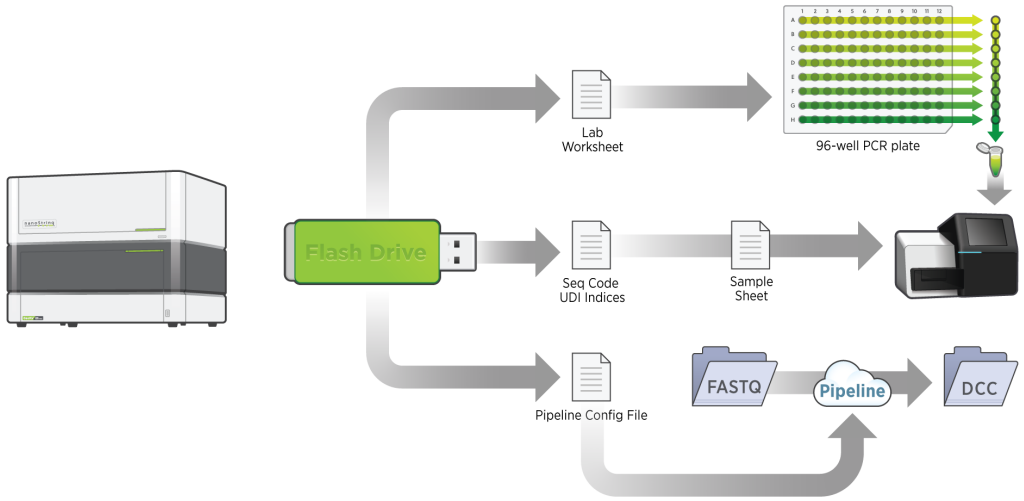


Figure 5: Output files from GeoMx run

The file input to demultiplex the Illumina NGS run is the **SampleSheet.csv**.

The file output from BCL conversion and demultiplexing the Illumina NGS run is the compressed **FASTQ.gz files,** which contain sequencing data relating to each well of the collection plate. Depending on your GeoMx NGS Pipeline configuration, the FASTQ files may be generated in the BaseSpace Sequence Hub or you will download them from the sequencer-networked local server and save in an accessible location for input to the GeoMx NGS Pipeline.

The file input to the GeoMx NGS Pipeline is the set of **FASTQ.gz** compressed files from the sequencer and the **configuration (.ini) file** from the GeoMx DSP readout package. A Sample ID Translator file is an optional input to use when the FASTQ files have a different filename structure than the defaults from the GeoMx; see information on page 38).

The file output from the GeoMx NGS Pipeline is a zipped folder of **DCC files** representing the count data from each well of the collection plate, or segment from your study. Depending on the pipeline workflow used, the zipped folder of DCC files is .zip format or output.tar.gz format. The folder is uploaded to the GeoMx DSP Data Analysis Suite for analysis or export (for analysis using customized bioinformatics tools).

The GeoMx NGS Pipeline also generates a **summary.txt file** with the number of paired or single-end reads for each pipeline processing step. The GeoMx NGS Pipeline on DRAGEN also generates Dragen_Files, metric files that may be used for pipeline troubleshooting.

GeoMx NGS Readout Site Readiness

NanoString recommends three distinct, physically separated areas for containment, each with its own set of consumables (pipette tips, tubes) and equipment (pipettes, centrifuges, pens, lab coats, etc.):

1. Slide preparation area for handling concentrated probe pool:
   - Concentrated probe pool used for overnight *in situ* hybridization should be kept isolated from pre-PCR and post-PCR areas.
   - Negative air pressure is ideal.
2. Pre-PCR area for PCR setup:
   - PCR setup should take place in a separate room or containment area, ideally in a dedicated PCR setup laminar flow hood.
   - Positive air pressure is ideal.
3. Post-PCR area for handling amplified PCR products:
   - Amplified PCR products should be handled in a separate room or containment area away from the slide preparation area or pre-PCR area.

GeoMx NGS Readout Equipment, Materials, and Reagents

The following tables list equipment, materials, and reagents **not supplied by NanoString**.

Table 2: Equipment for NGS readout not supplied by NanoString

Equipment	Source, Catalog No.
PCR set-up hood (or designated space)	Various
Vortex	Various
Picofuge	Various
Plate spinner/centrifuge with plate adapter (up to at least 2000 x *g*)	Various
Heated plate sealer and foil seals (optional alternative to adhesive foils)	Various
Thermal cycler	Various, such as Bio-Rad® 1851197
Qubit™ or similar device for library quantitation	Various, such as Thermo Fisher® Q33238
Agilent® Bioanalyzer®, TapeStation®, Fragment Analyzer™ or similar capillary electrophoresis device for nucleic acid analysis	Agilent G2939BA or G2992AA or M5310AA
Illumina® NGS instrument	Illumina, Various

Table 3: Materials for NGS readout not supplied by NanoString

Materials	Source, Catalog No.
Pipettes for 5–1,000 µL	Various
Two* 12-channel P20 multi-channel pipettes	Various
Filter tips (DNase/RNase free)	Various
Microcentrifuge tubes (DNase/RNase free)	Various
DNA LoBind® tubes (for library storage before sequencing)	Various
96-well PCR plates (DNase/RNase free) to match thermal cycler specs	Various
Magnetic stand (e.g. DynaMag-2™ Magnet)	Thermo Fisher, 12321D
Permeable membranes (included in GeoMx Training Kit)	Sigma®, A9224
96-well PCR plate adhesive foil seals	VWR™, 60941-076 or Thermo Fisher, AB0626 (or comparable)
PCR strip tubes (12-tube or 8-tube strip, DNase/RNase free)	Various
RNase AWAY® or 10% Bleach (RNaseZap® is not a substitute)	Thermo Fisher, 7002
USB drive v3.0, 64 GB or higher (able to be NTFS formatted)	SanDisk, SDCZ800-128G-G46 (or comparable)

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

Table 4: Reagents for NGS readout not supplied by NanoString

Reagents	Source, Catalog No.
Nuclease-free water	Various
100% ethanol 	Various
Agencourt® AMPure XP®	Beckman Coulter®, A63880
Elution buffer (Tris-HCl 10 mM with 0.05% Tween-20, pH 8.0)[1]	Teknova®, T1485
High sensitivity kit for nucleic acid analysis on capillary electrophoresis device	Agilent, 5067-4626; 5067-5584 and 5067-5585; or DNF-477-0500
Reagents for Qubit or similar device for NGS library quantitation	Various, such as Thermo Fisher Q32851
Illumina Sequencing Reagent Kit (sufficient for ~75 cycles)[2]	Illumina, various options

[1] 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 water. Mix well.

[2] Reagent kit must accomodate 70 cycles for assays using Seq Code indices (e.g. Whole Transcriptome Atlas) and 74 cycles for assays using Pro Code indices (e.g. Immuno-oncology Proteome Atlas).

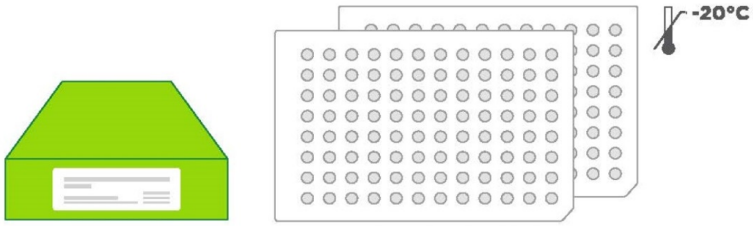
NanoString Reagents

Contact your NanoString Sales Representative to use our reagent planning tools to calculate required quantities.

5X GeoMx NGS Master Mix

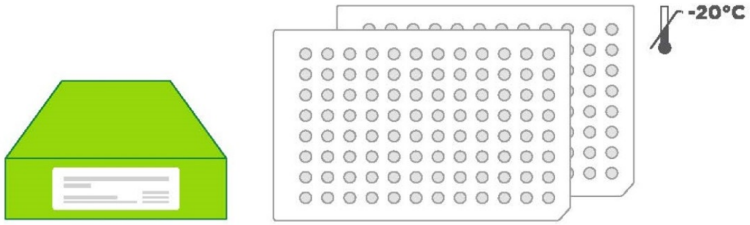
Master Mix should be stored at -20°C (aliquoting not required).
Older tubes labeled "GeoMx Seq Code Master Mix" can be used for Seq Code or Pro Code assays.

For assays with Seq Code indices (including Whole Transcriptome Atlas, Cancer Transcriptome Atlas, and Seq Code Protein Core/Modules):



Seq Code primer plates (available as A & B, C & D, E & F, or G & H)

For assays with Pro Code indices (Immuno-oncology Proteome Atlas):



Pro Code primer plates (available as Y & Z)

Store Seq Code or Pro Code plates at -20°C. Once thawed, store at 4°C up to 3 months, or freeze again. Plates can go through 3 freeze-thaw cycles.

> **IMPORTANT:** Seq Code plates (A-H) allow multiplexing of up to 768 wells. Pro Code plates (Y-Z) allow multiplexing up to 192 wells.

Transferring from the GeoMx DSP run

Finalize the GeoMx DSP Collection Plate(s)

Refer to the GeoMx DSP Instrument User Manual (MAN-10152) for instructions on finalizing the collection plate(s). Finalizing the plate(s) sets the readout group, or group of samples that will be processed together in the subsequent NGS readout step.

Download Files for Illumina Processing

After finalizing the plate(s) into a readout group, download the zipped readout package containing the following files. See File Inputs and Outputs on page 18 for details on file contents.

- **SeqCodeIndices.csv** (or SampleSheet.csv and whitelist.txt for NextSeq 1000/2000 users) (In GeoMx software v3.1, the filename is the same even if it is a Pro Code readout group.)
- **LabWorksheet.txt**
- **Configuration (.ini) file**

NextSeq 1000/2000 users who run the cloud integrated workflow (Option 4 on page 52) send their readout group files to BaseSpace Sequence Hub directly from the GeoMx DSP. They may still download the readout group in order to use the Lab Worksheet for library preparation. (Please note that this workflow is not available for Spatial Proteogenomic (RNA+Protein) assays which include the Immuno-oncology Proteome Atlas.)

Prepare the GeoMx DSP Collection Plate for Library Prep

1. **Remove the collection plate** from the GeoMx DSP instrument by following the instructions at the end of the GeoMx DSP run. Refer to the GeoMx DSP Instrument User Manual (MAN-10152) as needed.
2. **If processing immediately**, seal with a permeable membrane and proceed to drying (step 3).

   If storing plate before processing for readout, seal plate with adhesive foil to prevent contamination. Store plate following these guidelines:

   - If stored 24 hours or less: store at 4°C.
   - If stored between 24 hours and 30 days: store at -20°C.
   - If stored longer than 30 days: store at -80°C.

When ready to process plate, thaw (if necessary), centrifuge briefly, replace foil with a permeable membrane, and proceed to step 3.

The GeoMx DSP aspirates, PCR products, or prepared NGS library can be transferred to a sequencing core facility. Ship a plate by drying down the plate, foil-sealing, and shipping at 4°C; or do not dry down the plate, but foil-seal and ship on dry ice. To ship the NGS library, use a DNA LoBind tube and ship on dry ice. When shipping on dry ice, protect the plate or tube from direct contact with the dry ice, which can cause the plastic to crack.

3. **Dry down the collection plate** by leaving on the bench top overnight **OR** incubating on a thermocycler at 65°C for 1 hour. The lid of the thermocycler needs to be in the open position to allow evaporation to occur. Visually check that there is no liquid remaining in the plate wells. If there is still liquid in any of the wells after this time, incubate for another 30 minutes.
4. After dry-down, **carefully remove the permeable membrane sticker,** ensuring not to contaminate the plate with any remaining water condensed on the membrane. It is not uncommon to observe condensed water on the membrane.
5. **Seal the collection plate** with a **new permeable membrane** sticker and **spin down**. Check that there is no liquid remaining prior to rehydrating the samples in the next step. If there is liquid, dry down until all liquid is evaporated.
6. **Rehydrate the samples**. Depending on the analyte targeted, add the volume of nuclease-free water indicated (Table 5), 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. **NOTE:** For proteogenomic assays (RNA+Protein), refer to the GeoMx DSP Spatial Proteogenomic Assay User Manual (MAN-10158), rather than this user manual.

Table 5: Rehydration volumes

Analyte	Rehydration Volume
RNA	10 µL
Protein	80 µL

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

Library Preparation

PCR setup (2 hours)

Before you begin, review **PCR setup best practices**:

- Decontaminate working surfaces before and after use with 10% bleach, followed by distilled water and 70% ethanol.
- Keep consumables (tubes, plates, pipette tips) covered when not in use.
- Limit handling of consumables—don't touch tips, the inside of tubes, plates, etc.
- Use filter pipette tips.
- Pulse centrifuge all tubes and plates before opening.
- Open tubes and plate seals carefully to prevent aerosolization of contents.
- Clearly label all tubes and plates.
- Perform a visual check of pipette tip volume when pipetting small volumes, especially when using a multichannel pipette.

These instructions assume a full 96-well plate. If processing more or fewer aspirates, adjust the procedure and volumes accordingly.

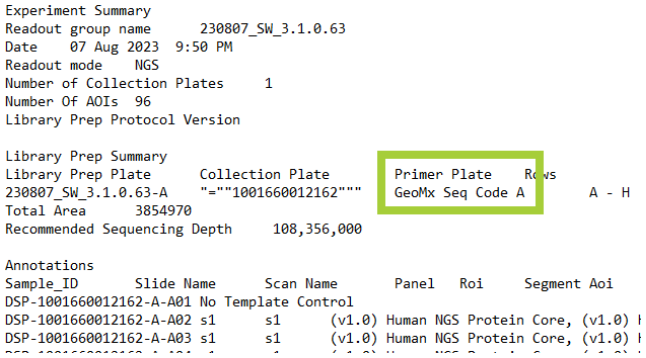
1. **Clean workspace** with 10% bleach or RNase AWAY. Rinse with **distilled water**, followed by **70% ethanol**. If possible, expose workspace to UV light source for 15 minutes to disinfect.
2. **Program a thermocycler** with a 100°C heated lid (Table 6).

Table 6: Cycling conditions

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	95°C	15 sec	18 X
Anneal	65°C	60 sec	
Extend	68°C	30 sec	
Final extension	68°C	5 min	1 X
Hold	4°C	∞	1 X

3. **Keep GeoMx NGS Master Mix on ice** or cooling block during setup. (Older tubes labeled GeoMx Seq Code Master Mix can be used for Seq Code or Pro Code assays).

4. Refer to the Lab Worksheet (Figure 6) to **choose the correct letter of GeoMx Seq Code or Pro Code Primer Plate** for each DSP collection plate. **Thaw the primer plate** at room temperature. Note that primer plates are **for single-use only** to minimize risk of cross-contamination and impact on data integrity.
5. **Pulse centrifuge** thawed primer plate, GeoMx NGS Master Mix, and DSP collection plate to 1000x*g*.



```
Experiment Summary
Readout group name      230807_SW_3.1.0.63
Date    07 Aug 2023  9:50 PM
Readout mode    NGS
Number of Collection Plates     1
Number Of AOIs  96
Library Prep Protocol Version

Library Prep Summary
Library Prep Plate      Collection Plate        Primer Plate    Rows
230807_SW_3.1.0.63-A    ""="1001660012162""     GeoMx Seq Code A        A - H
Total Area      3854970
Recommended Sequencing Depth     108,356,000

Annotations
Sample_ID       Slide Name      Scan Name       Panel   Roi     Segment Aoi
DSP-1001660012162-A-A01 No Template Control
DSP-1001660012162-A-A02 s1      s1      (v1.0) Human NGS Protein Core, (v1.0) H
DSP-1001660012162-A-A03 s1      s1      (v1.0) Human NGS Protein Core, (v1.0) H
```

Figure 6: Lab Worksheet

6. **Set up the PCR reactions in a new 96-well PCR plate** as in Table 7 (also described in steps a.-c.). Ensure the **primer plate**, **DSP collection plate** and **PCR plate** are in the correct orientation (well A1 at upper left).

Table 7: PCR reaction

Step	Assays with Seq Code indices (WTA, CTA, Seq Code Protein Core/Module)	Assays with Pro Code indices (Immuno-oncology Proteome Atlas)
Aliquot from GeoMx NGS Master Mix tube to PCR plate	2 µL Master Mix	2 µL Master Mix
Transfer from primer plate to PCR plate	4 µL Seq Code Primer Mix	4 µL Pro Code Primer Mix
Transfer from DSP collection plate to PCR plate	4 µL DSP aspirate	2 µL DSP aspirate
Nuclease-free water	—	2 µL nuclease-free water
Total PCR volume	10 µL	10 µL

   a. Using a multichannel pipette, aliquot **2 µL of GeoMx NGS Master Mix** to the bottom of each well of the new PCR plate. Changing pipette tips is not required.

   b. Using a multichannel pipette and changing pipette tips for every row/column, transfer **4 µL of primer** from each well of the Seq Code or Pro Code Primer Plate to the bottom of the corresponding well of the PCR plate.

   c. Using a multichannel pipette and changing pipette tips for every row/column, transfer **DSP aspirate** from each well of the DSP collection plate to the bottom of the corresponding well of the PCR plate:

      - **4 µL aspirate** for assays with Seq Code indices (including Whole Transcriptome Atlas, Cancer Transcriptome Atlas, and Seq Code Protein Core/Module), OR
      - **2 µL aspirate** for assays with Pro Code indices (Immuno-oncology Proteome Atlas), **plus 2 µL** nuclease-free water.

For assays with Pro Code indices, the volume of DSP aspirate to use in the PCR may be increased to 4 µL depending on empirical testing and optimization by the user. Exclude the nuclease-free water to keep the total PCR volume at 10 µL.

7. **Pipette** up and down 10 times to mix.
8. **Seal the PCR plate** using PCR plate stickers or a heat-sealer according to manufacturer instructions. **Pulse centrifuge** to 1000 x *g*.
9. Incubate the PCR plate in a thermocycler with program specified (Table 6).

After the PCR run is complete, the PCR plate may be stored at 4°C for up to 3 days before AMPure cleanup.

Instructions to set up a PCR positive control reaction are included in Appendix I: PCR Positive Control on page 58. Including a positive control reaction in the same plate runs the risk of cross-contamination into experimental wells. It is recommended only as needed for training or troubleshooting.

Pooling and AMPure cleanup (45 minutes)

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

IMPORTANT: Ensure that AMPure beads are at room temperature **and** thoroughly vortexed before adding to the pooled PCR product.

Pool and purify PCR products using the steps below. A visual diagram of this process is provided on page 32.

1. Once PCR is complete, **pulse centrifuge PCR plate** to 1000 x *g*.
2. Pool PCR products as follows (Figure 7). Note that depending on how the collection was set up, the **NTC may not be in well A1**, or it may not have been included. Check the Lab Worksheet file to know if/where the NTC is located in the plate. Please note that without an NTC, library QC and initial data QC are limited, and NanoString's ability to troubleshoot problems is limited.

   If running multiple 96-well plates, pool each plate into its own 1.5 mL tube. If any plate has less than 12 reactions, you may combine that pool with another plate's pool.

   a. Pool **4 µL of each PCR product,** including the NTC but excluding any PCR Positive Control, into **one 1.5 mL tube**. This is the **Sample Pool.**

   b. Pipette remainder of **NTC PCR product** into its own 1.5 mL tube.

   c. **If you set up a PCR Positive Control,** pipette the 10 µL PCR Positive Control to its own (i.e. a third) 1.5 mL tube.

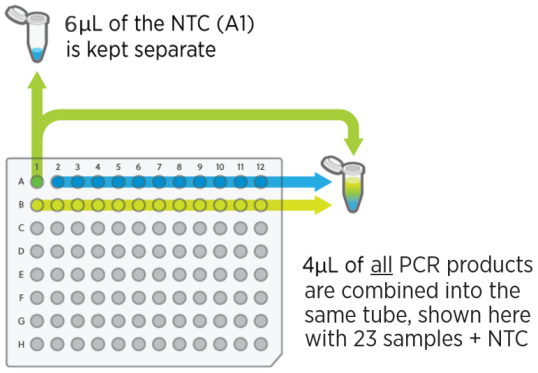


Figure 7: Pooling PCR reactions. Note that the NTC may not be in well A1, depending on how the collection was set up. Refer to the Lab Worksheet to check.

3. **Take all the tubes (Sample Pool, NTC, and optional PCR Positive Control) through the following AMPure cleanup protocol.** In the subsequent section, you will check the quality and size of the purified pooled library against the purified NTC and the optional PCR Positive Control.
4. **Measure the exact volume in each tube** using a pipette. **Do not skip this step.**
5. **Multiply by 1.2x to determine the volume of AMPure XP beads to add to each tube. Mix well** by pipetting up and down 10x. **Pulse centrifuge**.
6. **Incubate 5 minutes** at room temperature.
7. **Prepare 20 mL fresh 80% ethanol** by combining 16 mL 100% ethanol and 4 mL PCR-grade water.
8. **Pellet beads on a magnetic stand** for **5 minutes** or until the solution is clear.
9. **Carefully remove the supernatant**. Avoid disturbing the beads, as the library is bound to them at this stage.
10. **Wash beads** with **1 mL** of freshly prepared **80% ethanol,** incubating on **magnetic stand** for **30 seconds**.
11. **Discard the supernatant**, being careful not to disturb the beads.
12. **Wash beads** a second time with **1 mL** of freshly-prepared **80% ethanol,** then incubate on **magnetic stand** for **30 seconds**.
13. **Discard the supernatant**, being careful not to disturb the beads.
14. **Visually inspect the beads** to ensure that as much ethanol as possible is removed. Use a low volume pipette (e.g., P20) to remove any residual ethanol, if necessary.
15. **Dry beads on magnetic stand for ≤5 minutes**. Remove from the magnetic stand.
16. **Resuspend beads in each tube in 54 µL Elution Buffer**. **Mix well** by pipetting. **Incubate** 5 minutes at room temperature.
17. **Pellet beads on a magnetic stand for 5 minutes** or until the solution is clear.
18. **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.
19. **Add 60 µL of Ampure XP beads** to the **50 µL supernatant**. **Mix well** by pipetting up and down 10x. **Pulse centrifuge**.
20. **Incubate 5 minutes** at room temperature.
21. **Pellet beads on a magnetic stand 5 minutes** or until the solution is clear. The library is on the beads.

22. **Carefully remove the supernatant**. Avoid disturbing the beads.
23. **Wash beads** with **1 mL** of freshly-prepared **80% ethanol,** incubating on **magnetic stand** for **30 seconds**.
24. **Discard the supernatant**, being careful not to disturb the beads.
25. **Wash beads** a second time with **1 mL** of freshly-prepared **80% ethanol,** incubating on **magnetic stand** for **30 seconds**.
26. **Discard the supernatant**, being careful not to disturb the beads.
27. **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.
28. **Dry beads** on magnetic stand for **≤5 minutes**. Remove from magnetic stand.
29. **Resuspend beads in Elution Buffer** according to Table 8. **Mix well** by pipetting.

Table 8: Elution Buffer volume

# reactions	Elution Buffer volume
96	48 µL
48	24 µL
24	16 µL
12	12 µL
(Purified NTC)	5 µL
(optional PCR Positive Control)	5 µL

NOTE: Reduce the elution buffer volume for standalone Custom RNA-NGS Panels (run without CTA or WTA). NanoString recommends eluting in 12 µL the first time a low-plex library is prepared, increasing to 25 µL thereafter or at your discretion.

30. **Incubate 5 minutes** at room temperature.
31. **Pellet the beads on the magnetic stand 5 minutes** or until the solution is clear.
32. **Extract the supernatant to a new tube**. Leave 1 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.

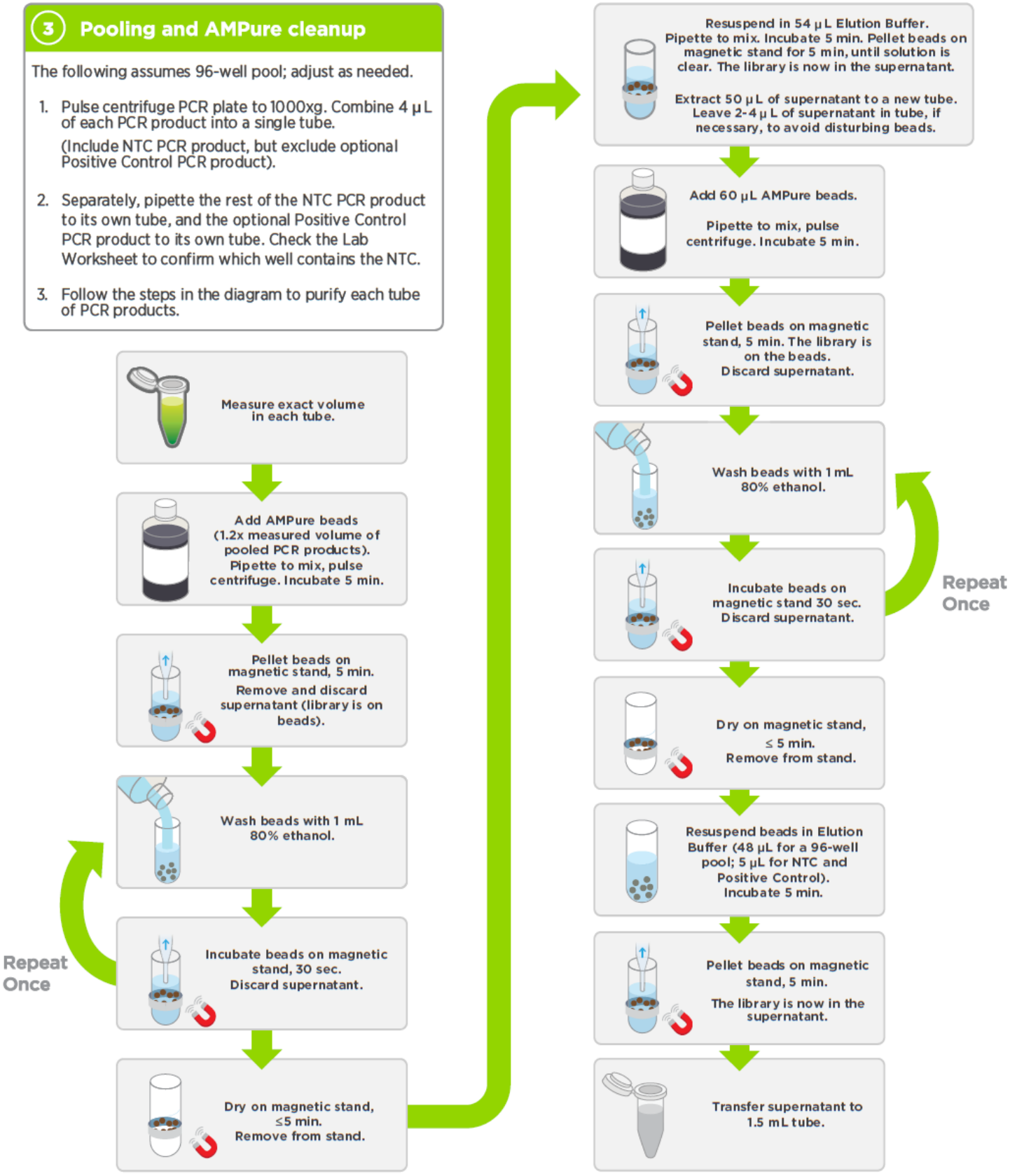


Figure 8: AMPure purification

Assessing library quality and quantity

1. Dilute the library by combining 2 µL of library and 14 µL of Elution Buffer. For a standalone Custom RNA-NGS Panel of low plex, combine 2 µL of library and 6 µLof Elution Buffer.
2. Assess quality of library stock and dilution, as well as purified NTC, using a capillary electrophoresis device such as the Agilent Bioanalyzer or similar. Follow manufacturer instructions for use.
3. Check for the expected size of the amplicons and absence of primers or high molecular weight over-amplification products (Figure 9):

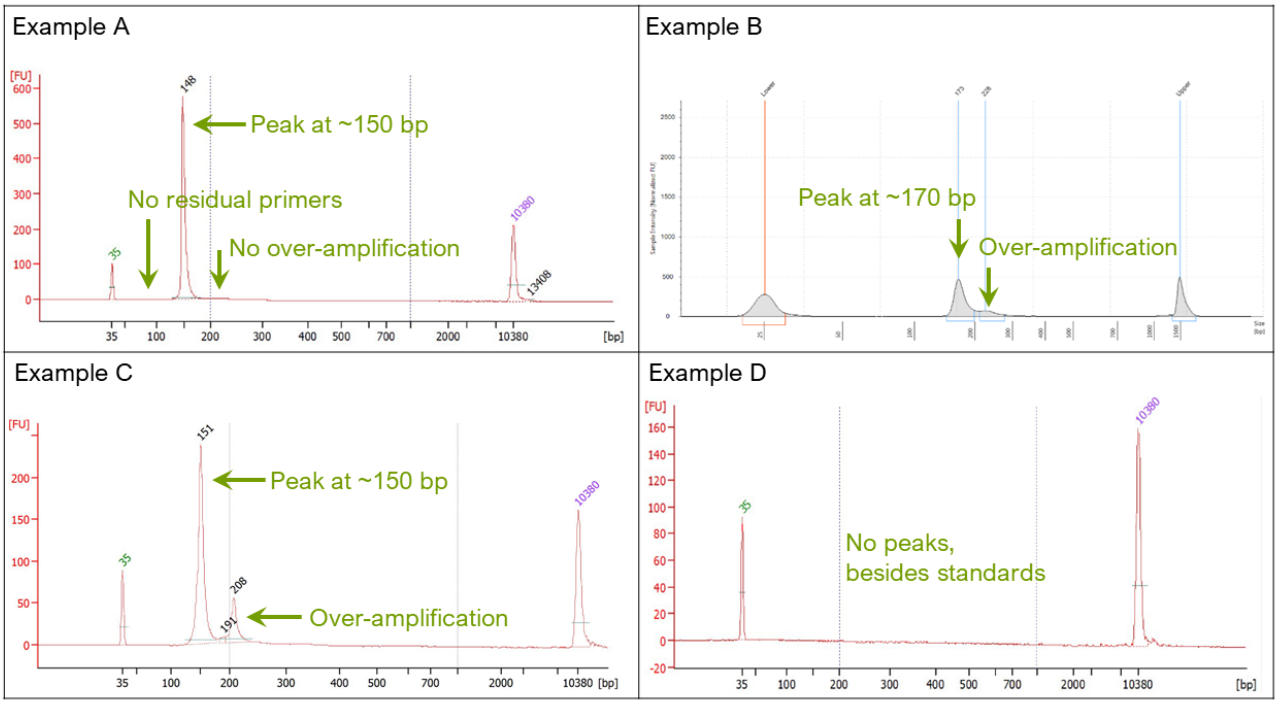


Figure 9: Library QC examples

- Example A. Bioanalyzer trace of a high-quality library.
- Example B. TapeStation trace (note different y-axis) of a mildly-over-amplified library. Most likely can be quantified, and still worth sequencing.
- Example C. Bioanalyzer trace of an over-amplified library. Quantification may be difficult. Still worth sequencing.
- Example D. Bioanalyzer trace indicating no amplification - represents the ideal NTC.

Expected library amplicon size is 162 bp (Seq Code products) or 166 bp (Pro Code products). Using the Bioanalyzer, the library will appear as ~150 bp. Using the TapeStation, the library will appear as ~170 bp.

Verify that the concentration of the ~150 bp amplicon peak of either the undiluted library or the 1:8 dilution falls within the Bioanalyzer assay's quantitative range (5 – 500 pg/µL).

4. Quantify library stock concentration using Qubit or qPCR.
5. If combining more than one collection plate (Sample Pool) into one sequencing run (Sequencing Pool), refer to Appendix II: Strategies for Pooling before Sequencing on page 59.
6. **Refer to Illumina recommendations for loading concentration** of the library, based on the platform and flow cell. Some examples are listed in Table 9. These values may be adjusted based on empirical testing. It is recommended to include 1-5% PhiX spike-in by volume for potential troubleshooting of sequencing runs. Refer to Appendix III: Sequencing Depth on page 61 for guidance in planning the sequencing depth.

Table 9: Suggested loading concentration per Illumina platform

Illumina Platform	Flow Cell	Loading Concentration*
MiSeq	v2, v3	12 pM
NextSeq 550	High-output	1.6 pM
NextSeq 1000/2000	P1, P2, P3	650 pM
NovaSeq 6000	SP, S1, S2, S4	250 pM for standard loading (100 pM for Xp loading)

*Refer to the **Dilute and Denature Guide** provided by Illumina for your specific sequencing platform. Depending on the platform and workflow, the Loading Concentration listed above may describe the solution that is pipetted into the cartridge or the final concentration of what is sequenced after onboard dilution. Consult Illumina documentation or your Illumina FAS for additional support.

Plan Your Sequencing and Pipeline Processing

Use Figure 10 to plan your sequencing and pipeline processing. Instructions for the four configurations (Option 1 - Option 4) are detailed in the pages that follow.

IMPORTANT: GeoMx NGS Pipeline on DRAGEN has been validated to produce concordant results to the standalone NanoString GeoMx NGS Pipeline. For best data processing practices, it is recommended that studies be processed in one pipeline or the other.

IMPORTANT: Sequencing data from spatial proteogenomic assays (RNA + Protein) that include NanoString's Immuno-oncology Proteome Atlas can only be processed on the standalone NanoString GeoMx NGS Pipeline (not GeoMx NGS Pipeline on DRAGEN via BSSH) at this time. Refer to the GeoMx DSP Spatial Proteogenomics Assay User Manual (MAN-10158) for assay information.

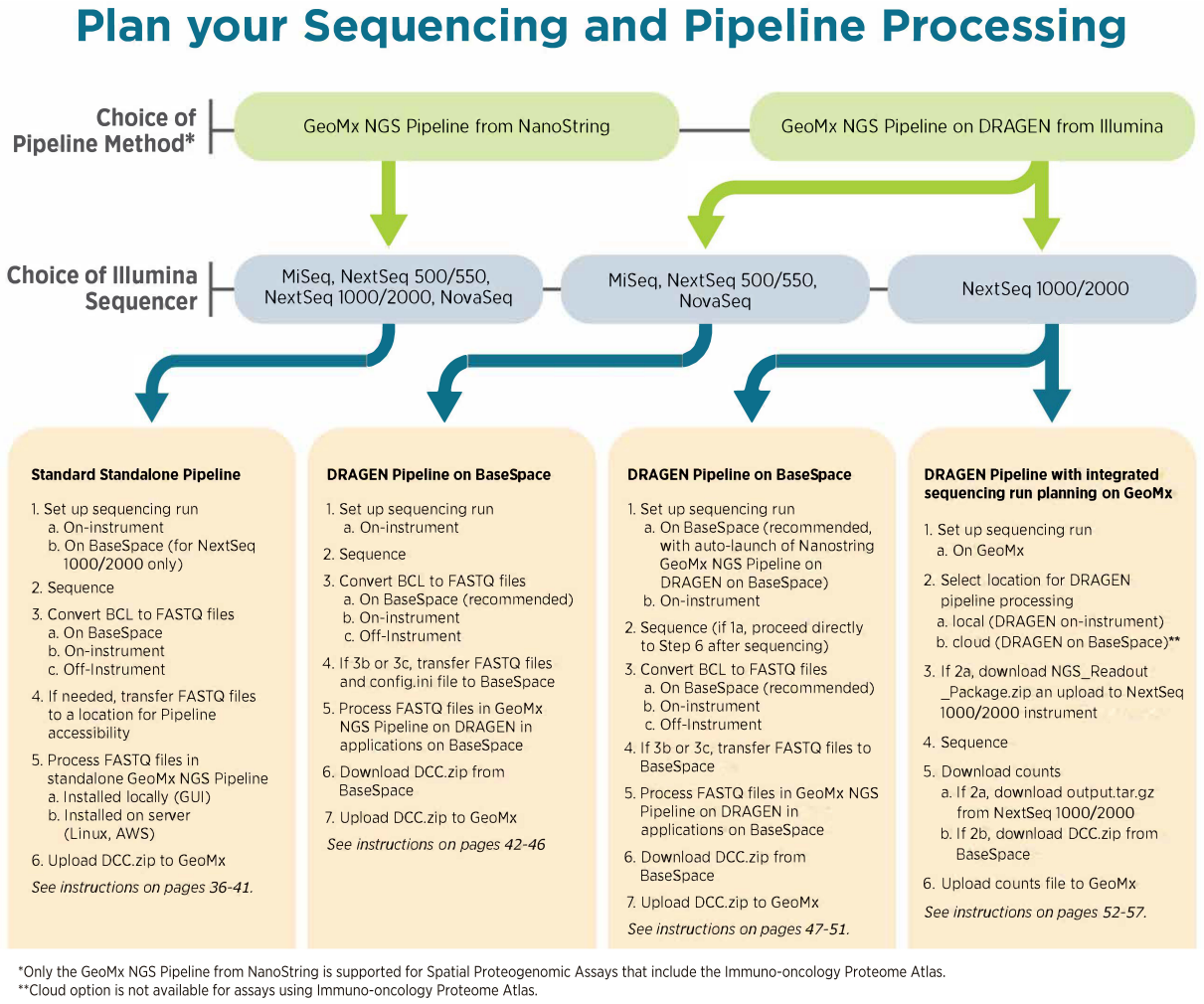


Figure 10: Plan your Sequencing and Pipeline Processing diagram

Option 1: Standard Standalone Pipeline

This option is suitable for any Illumina sequencing platform in combination with the standalone NanoString GeoMx NGS Pipeline software. To install the standalone NanoString GeoMx NGS Pipeline software, see Appendix V: Installing the GeoMx NGS Pipeline Software on page 66.

1. Set up sequencing run

Prepare the **Illumina Sample Sheet** (SampleSheet.csv) by copying and pasting contents of the SeqCodeIndices.csv file downloaded in the readout package from the GeoMx DSP instrument. (In GeoMx software v3.1, the filename is the same even if it is a Pro Code readout group.) (NextSeq 1000/2000 users download an already-prepared SampleSheet.csv from the GeoMx DSP.) Confirm the sequencing details in the Sample Sheet. If corrections are needed, make changes on the GeoMx DSP instrument for the readout group, then re-download the readout package.

2. Sequence

Start the run according to the platform instructions (see Illumina user manuals at support.illumina.com). Enter the following workflow specifications as needed:

- Library prep kit: NanoString GeoMx Seq Code, or (for Pro Code) Not Specified
- Index adapter kit: NanoString GeoMx Seq Code, or (for Pro Code) Not Specified
- Index reads: 2 indexes
- Index mismatch: 1 (default)
- Read type: Paired-end
- Read length: Read 1, 27 bp; Read 2, 27 bp
- Index 1 (i7) and Index 2 (i5): 8 bp each for assays with Seq Code indices, such as WTA, CTA, and Seq Code Protein Core/Module; 10 bp each for the Immuno-oncology Proteome Atlas with Pro Code indices (or the Spatial Proteogenomic Assay that includes IPA).
- Generate FASTQ files *(may not be an option, depending on your workflow)*

3. Convert BCL to FASTQ files

If the run doesn't automatically generate FASTQ files, generate them following the sequencing run according to the Illumina platform, workflow, and analysis location you are using. Refer to platform-specific documentation for guidance.

The GeoMx NGS Pipeline looks for FASTQ files with a naming structure such as DSP-1001250001985-A-A02_S2_L001_R1_001.fastq.gz where DSP-1001250001985-A-A02 = Sample ID matching Configuration file, SeqCodeIndices.csv, or Sample ID Translator File. S2 = Sample sheet number. L001 = Lane number (include even if your flow cell had only 1 lane). R1 = Forward or reverse, read 1 or 2. 001 = Should always be 001. The suffix .fastq.gz indicates a compressed file.

NextSeq 1000/2000 DRAGEN 3.8 (and above) demultiplexing no longer generates FASTQs with standard filenames; lane numbers are omitted. To ensure compatibility with off-instrument instances of the GeoMx NGS Pipeline (i.e. Option 1), please use DRAGEN 3.7.4 (or below) for BCL to FASTQ conversion. For on-instrument secondary processing (i.e. Option 4, local integrated workflow), the GeoMx NGS Pipeline application can only be installed on DRAGEN 3.8.3 and above. The on-instrument application bypasses FASTQ creation.

4. Transfer files to a location for GeoMx NGS Pipeline accessibility

Save your **GeoMx NGS Pipeline Configuration (.ini) file** (from the GeoMx DSP readout package) and your **FASTQ files** (from the Illumina sequencing run) to the computer running the pipeline, or a server you can access directly. FASTQ files must be in format fastq.gz.

The pipeline is designed to handle FASTQs stored on a server to which there is direct access. If you are using a VPN or local server for storage of FASTQ files and encounter errors, move files to a direct access server to improve performance. Be sure to use best practices in big data transfer and management when moving FASTQ.gz files.

5. Process FASTQ files in the NanoString GeoMx NGS Pipeline

Running the pipeline using Graphical User Interface (GUI)

Suitable for runs of <96 segments or ROIs (with <~50 million reads per segment or ROI)

1. **Open the GeoMx NGS Pipeline software**.
2. Select **Run locally** or **Run remotely,** depending on how you plan to run the pipeline. Review System Requirements on page 66 for more details.
   - The **Resources available** section lists the processing power of your local computer (if run locally is selected) or server (if run remotely is selected).
   - The **Number of threads** drop-down at the bottom of the window indicates the number of parallel processes possible given the

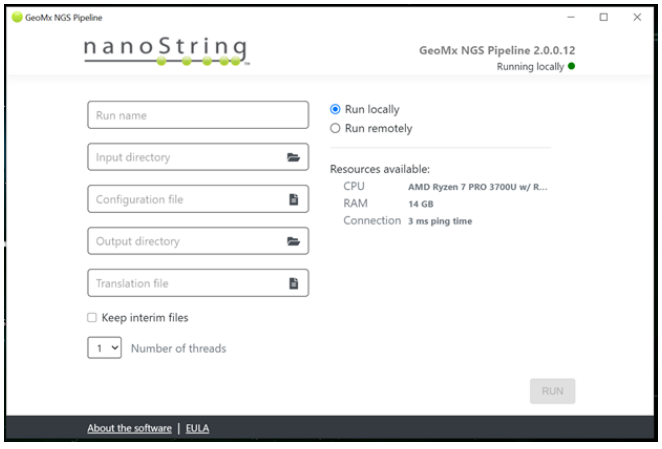


Figure 11: GeoMx NGS Pipeline run setup window

available resources. To run as fast as available resources will allow, change this number to the maximum number of threads. The default is set to 1.

- Use the gear icon or drop-down folder next to **Run on Server** to select server. The GeoMx NGS Pipeline must be installed on the remote server.

3. Create a **Run name** (Figure 11).
4. **Browse** to your **Input directory** (the folder housing your Illumina FASTQ files in format fastq.gz).
5. **Browse** to the location of your **Configuration (.ini) file**.
6. **Browse** to your **Output directory** - the location in which you would like the output files saved.
7. (Optional) **Browse** to the **Translator file**.

   A Sample ID Translator File can be used as input when FASTQ files have a different filename structure than the defaults from the GeoMx. The file lists sample names from the configuration (.ini) file next to FASTQ file root names, providing the software a key for translating. Request a Translator File template from Support@nanostring.com.

8. (Optional) Check the **Create DCC metadata** box, if desired.

   The DCC metadata file as optional output will provide additional traceability from a pipeline run. This file shows the relationships between files as they transition from FASTQ to DCC for every sample, and unique MD5 checksums for identification. This can be helpful for submitting data for publication. Keep in mind that producing this additional output will increase run execution time.

9. (Optional) Check the **Keep interim files** box, if desired. These are large intermediate files written during the pipeline processing, generally needed only for troubleshooting.
10. Click **Run**.
11. Monitor the progress (Figure 12).
    - Click the **Log**, **Error**, **Warning**, or **Processing Parameters** icon to view the respective information.
    - You may process a partial set of FASTQ files if the sequencing is not completed yet. If some FASTQ files are missing or unrecognized, you will receive a warning that the system did not find FASTQ files for all samples listed in the configuration file. You

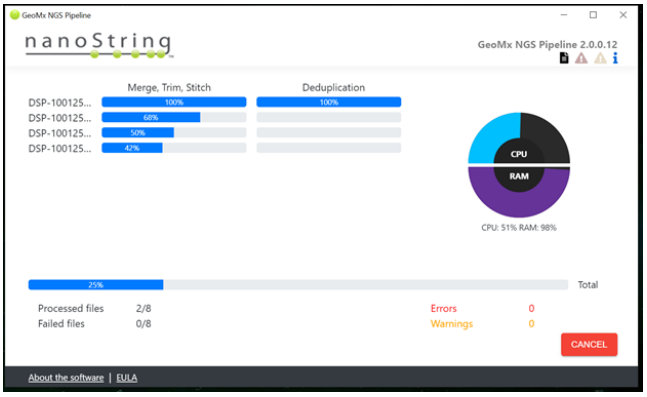


Figure 12: GeoMx NGS Pipeline run monitoring window

may proceed and the pipeline will generate "empty" DCC files (no counts for any probes) for the ROIs or segments with missing sequence data. This allows you to begin data analysis without sequencing data for all samples. You can upload an updated set of DCC files later, but you will need to create a new study to access the updated counts in the Data Analysis Suite.

12. When the process is complete, the status bar will read 100%. Click **Done**.
13. Open the output folder and locate the zipped **DCC files** subfolder. Proceed to 6. Upload DCC.zip to GeoMx DSP on page 41.

> **IMPORTANT:** Check DCC file sizes (should be >1 kb) and summary.txt to ensure files were processed as expected.

Running the pipeline using Command Line Interface (CLI) on a Linux or AWS Server
Suitable for runs of ≥96 segments or ROIs or ≥~50 million reads per segment or ROI

Running the pipeline from the CLI is similar to running it from the GUI in that you need specify three parameters: the location of the **configuration (.ini) file**, the location of the **FASTQ files**, and the location of the **output folder** where you would like to receive your DCC files.

1. Ensure your input files are copied to the server itself. If you are remotely connected to a server, the file path must be accessible to the server.

   The pipeline is designed to handle FASTQs stored on a server to which there is direct access. If you are using a VPN or local server for storage of FASTQ files and encounter errors, move files to a direct access server to improve performance.

2. Log in to the server. If you are processing on a remote server, you need to run the command from the server.
3. Create a dropoff folder on the server for your configuration file and your FASTQ files. Create an output folder where you would like your DCCs saved.
4. Copy configuration (.ini) and FASTQ files to server.
5. To be able to call this command from any place, either restart your secure shell (SSH) session (logout and login) or run the following command: `export PATH=$PATH:/var/GeoMxNGSPipeline`.

   - In the event of a permissions error: if you have already installed **GeoMxNGSPipelineAPI** on this server and you rerun the installation, check the ownership and permissions of the */var/tmp/.net/* subfolder by navigating to the above directory and type `ls -l` or `ll`.

   ```
   sudo chgrp -R ubuntu /var/tmp/.net/
   ```

```
sudo chown -R ubuntu /var/tmp/.net/
```

```
sudo chmod 777 /var/tmp/.net/
```

- The CLI processing usage command is as follows:

```
geomxngspipeline --in=INPUT_DIR_PATH --out=OUTPUT_DIR_PATH --ini=INI_
CONFIG_PATH [OPTIONS]
```

- A CLI usage example run command is as follows:

```
geomxngspipeline --in=/mnt/efs/project1/FASTQ --
out=/mnt/efs/project1/results --ini=/mnt/efs/project1/project1_config.ini -
-save-interim-files=true --threads=4
```

- To see all available run command arguments, please use the following help command:

```
geomxngspipeline --help
```

6. When you have DCC files and a summary.txt file ready, you may copy them from the server to a local folder on your computer. Copy files using your usual method for interacting with your server, such as via a shared network drive or using secure copy protocol (scp).

> **IMPORTANT:** Check DCC file sizes (should be >1 kb) and summary.txt to ensure files were processed as expected.

6. Upload DCC.zip to GeoMx DSP

1. In the GeoMx DSP Control Center, hover over the Data Collection button and select **Upload Counts**.
2. In the **Upload Count Data** window, select **Choose File**.
3. Browse to the location of your saved **zipped** DCC file and select **Open**.
4. A notification will appear under the Notifications Bell indicating that counts were uploaded successfully. This may take a few moments.

> **IMPORTANT:** If you previously uploaded counts and then upload new counts, note that the new counts will replace the old counts in slide records and any future data analysis studies. Any existing data analysis studies will remain unchanged as they were created with the old count data.

If DCC files are not accepted by the software, check that you do not have a folder within the zipped folder, and that DCC file names match SampleID names from the Sample Index List. Check that there is a DCC file for every sample in the Readout Group – partial Readout Group counts cannot be uploaded.

Proceed to the GeoMx DSP Data Analysis User Manual (MAN-10154).

Option 2. DRAGEN Pipeline on BaseSpace

This option is suitable for Illumina MiSeq, NextSeq 500/550, and NovaSeq 6000 sequencing platforms in combination with the GeoMx NGS Pipeline on DRAGEN from Illumina.

1. Set up sequencing run

Prepare the **Illumina Sample Sheet** (SampleSheet.csv) by copying and pasting contents of the SeqCodeIndices.csv file downloaded in the readout package from the GeoMx DSP instrument. (In GeoMx software v3.1, the filename is the same even if it is a Pro Code readout group.) Confirm the sequencing details in the Sample Sheet. If corrections are needed, make changes on the GeoMx DSP instrument for the readout group, then re-download the readout package.

2. Sequence

Start the run according to the platform instructions (see Illumina user manuals at support.illumina.com). Enter the following workflow specifications as needed:

- Library prep kit: NanoString GeoMx Seq Code, or (for Pro Code) Not Specified
- Index adapter kit: NanoString GeoMx Seq Code, or (for Pro Code) Not Specified
- Index reads: 2 indexes
- Index mismatch: 1 (default)
- Read type: Paired-end
- Read length: Read 1, 27 bp; Read 2, 27 bp
- Index 1 (i7) and Index 2 (i5): 8 bp each for assays with Seq Code indices, such as WTA, CTA, and Seq Code Protein Core/Module; 10 bp each for the Immuno-oncology Proteome Atlas with Pro Code indices (or the Spatial Proteogenomic Assay that includes IPA).
- Generate FASTQ files *(may not be an option, depending on your workflow)*

3. Convert BCL to FASTQ files

If the run doesn't automatically generate FASTQ files, generate them following the sequencing run according to the Illumina platform, workflow, and analysis location you are using. Refer to platform-specific documentation for guidance.

The GeoMx NGS Pipeline looks for FASTQ files with a naming structure such as DSP-1001250001985-A-A02_S2_L001_R1_001.fastq.gz where DSP-1001250001985-A-A02 = Sample ID matching Configuration file, SeqCode- or ProCodeIndices.csv, or Sample ID Translator File. S2 = Sample sheet number. L001 = Lane number (include even if your flow cell had only 1 lane). R1 = Forward or reverse, read 1 or 2. 001 = Should always be 001. The suffix .fastq.gz indicates a compressed file.

4. Transfer FASTQ files and config.ini file to BaseSpace

Unzip the GeoMx DSP readout package downloaded from the instrument. From the readout package, upload the config.ini file onto BaseSpace using the BaseSpace Web Uploader (https://help.basespace.illumina.com/articles/ tutorials/upload-data-using-web-uploader/) or command line interface (CLI). If your FASTQ files were not generated in BaseSpace, transfer them to BaseSpace now, using the Web Uploader or CLI.

5. Process FASTQ files in GeoMx NGS Pipeline on DRAGEN in BaseSpace

Before you begin

- The sample name portion of the FASTQ file must match the sample name listed in the configuration (.ini) file and cannot include underscore characters.
- All FASTQ files must have a QC status of Pass in order to be processed through the pipeline.
- GeoMx NGS Pipeline on DRAGEN has been validated to produce concordant results to the standalone NanoString GeoMx NGSPipeline. For best data processing practices, it is recommended that studies be processed in one pipeline or the other.

Launch GeoMx NGS Pipeline application via BaseSpace

1. Log into BaseSpace and select the correct workgroup (Figure 13).

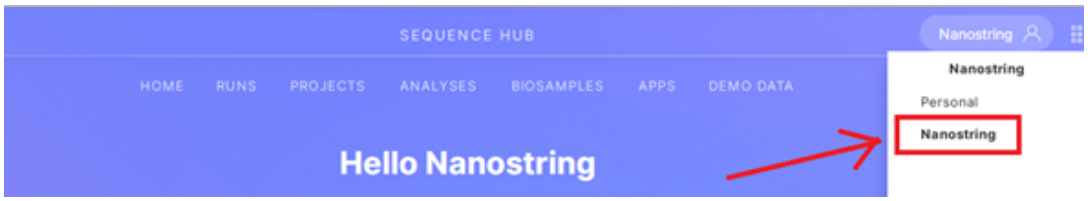


Figure 13: Select your workgroup. In this example, the workgroup is Nanostring.

2. Select Apps, then search for GeoMx and select GeoMx NGS Pipeline (Figure 14).

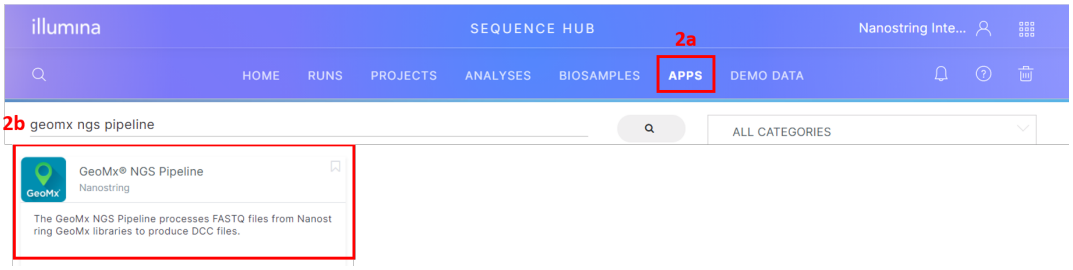


Figure 14: Find GeoMx NGS Pipeline application

3. Launch application (Figure 15).

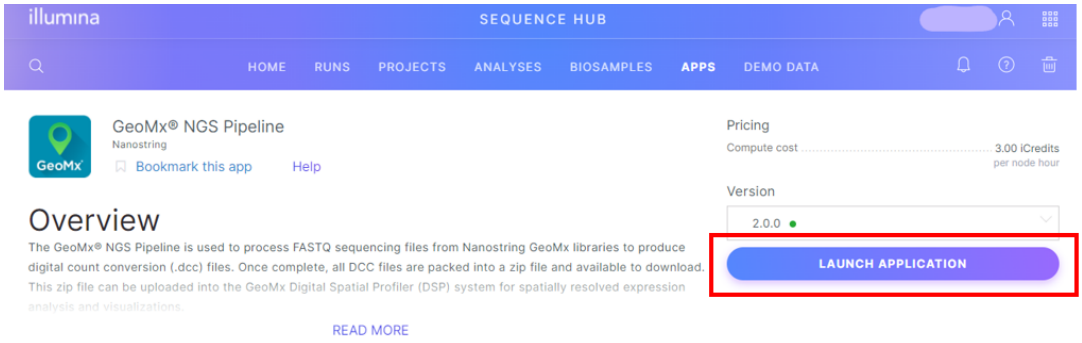


Figure 15: Launch application

4. Choose the **Start From: FASTQ files** option (Figure 16) . Under **FASTQ Files Input**, choose the configuration (.ini) file, FASTQ files, and an output project.
5. Click Launch Application.
6. Check run status by logging into BaseSpace, navigating to the workgroup, then selecting Runs.

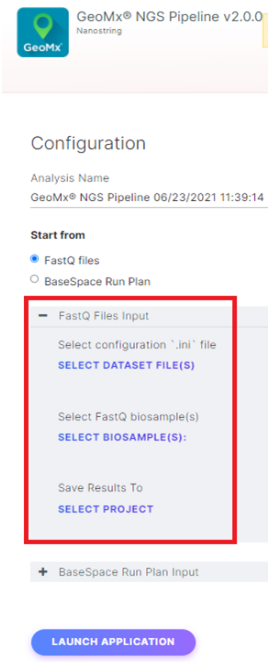


Figure 16: Choose file inputs

6. Download DCC.zip from BaseSpace

1. When the run is complete, log into BaseSpace, navigate to the workgroup, and select the **Analyses** tab (Figure 17).
2. Click on the analysis of interest (Figure 17).

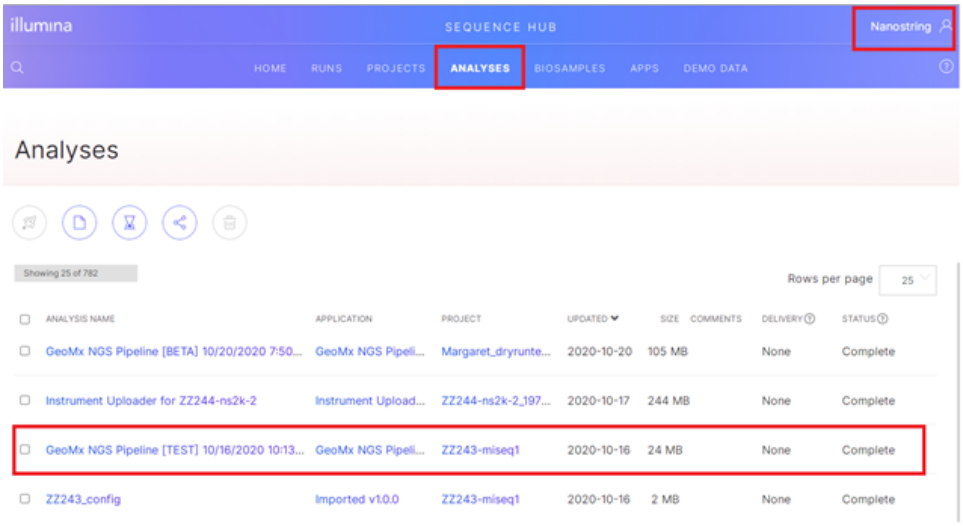


Figure 17: Analyses in BaseSpace

3. Open the folder **GeoMx_NGS_Pipeline**. Download the DCC.zip output with the **name: GeoMx_NGS_Pipeline_...** and **file type: zip** (Figure 18). (Despite its name, the folder "DCC_Files" is not the zipped DCC output for download).

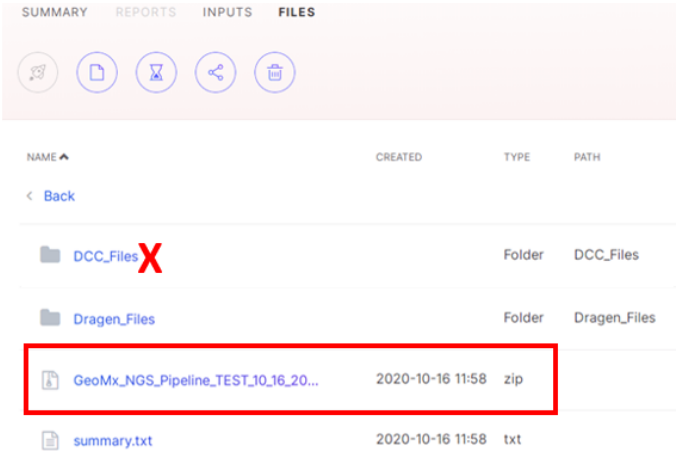


Figure 18: DCC.zip file output from GeoMx NGS Pipeline

IMPORTANT: Check DCC file sizes (should be >1 kb) and summary.txt to ensure files were processed as expected.

7. Upload DCC.zip to GeoMx

1. In the GeoMx DSP Control Center, hover over the Data Collection button and select **Upload Counts**.
2. In the **Upload Count Data** window, select **Choose File**.
3. Browse to the location of your saved **zipped** DCC file and select **Open**.
4. A notification will appear under the Notifications Bell indicating that counts were uploaded successfully. This may take a few moments.

IMPORTANT: If you previously uploaded counts and then upload new counts, note that the new counts will replace the old counts in slide records and any future data analysis studies. Any existing data analysis studies will remain unchanged as they were created with the old count data.

If DCC files are not accepted by the software, check that you do not have a folder within the zipped folder, and that DCC file names match SampleID names from the Sample Index List. Check that there is a DCC file for every sample in the Readout Group – partial Readout Group counts cannot be uploaded.

Proceed to the GeoMx DSP Data Analysis User Manual (MAN-10154).

Option 3. NextSeq 1000/2000 with DRAGEN Pipeline on BaseSpace

The configuration of the NextSeq 1000/2000 with DRAGEN Pipeline on BaseSpace enables a streamlined workflow where the sequencing run automatically launches FASTQ generation and the GeoMx NGS Pipeline on DRAGEN on BaseSpace. As described below, these users can skip from **Step 2. Sequence** to **Step 6. Download DCC.zip from BaseSpace**.

1. Set up sequencing run

(Users who do not wish to follow the streamlined workflow should set up their sequencing run on-instrument, following the guidance in Option 2 on page 42.)

1. Log into BaseSpace at https://BaseSpace.illumina.com/dashboard.
2. Select your preferred workgroup (Figure 19).

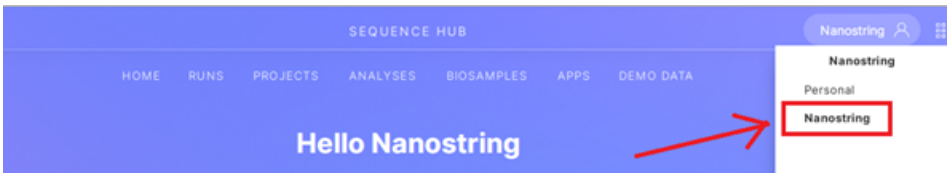


Figure 19: Select your workgroup. In this example, the workgroup is Nanostring.

3. Create a run by selecting **Runs,** then **Run Planning** or **Instrument Run Setup** (Figure 20).

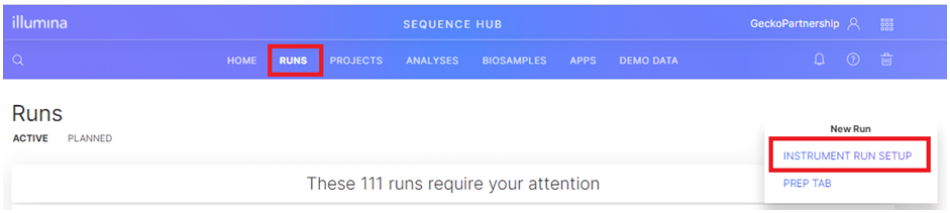


Figure 20: Instrument run setup

4. Under tab 1. Run Setup, enter the following run information and instrument settings:
    - Instrument Platform: NextSeq 1000/2000
    - Run Name: [enter a unique run identifier]
    - Analysis Location: “BaseSpace”
    - Type of Analysis: Select the latest version of the NanoString GeoMx NGS Pipeline (v2.0.21 as of Nov 2023)
    - Library prep kit: NanoString GeoMx Seq Code, or (for Pro Code) Not Specified

- Index adapter kit: NanoString GeoMx Seq Code, or (for Pro Code) Not Specified
- Index reads: 2 indexes
- Read type: Paired-end
- Index 1 (i7) and Index 2 (i5): 8 bp each for assays with Seq Code indices, such as WTA, CTA, and Seq Code Protein Core/Module; 10 bp each for the Immuno-oncology Proteome Atlas with Pro Code indices (or the Spatial Proteogenomic Assay that includes IPA).

5. Under "Sample Data" Click **Import Data** and select the **SampleSheet.csv** file from the GeoMx DSP readout package.
6. Select **Next**.
7. Under tab 2. Analysis Setup, upload the **GeoMx configuration (.ini)** file from the GeoMx DSP readout package (Figure 21).

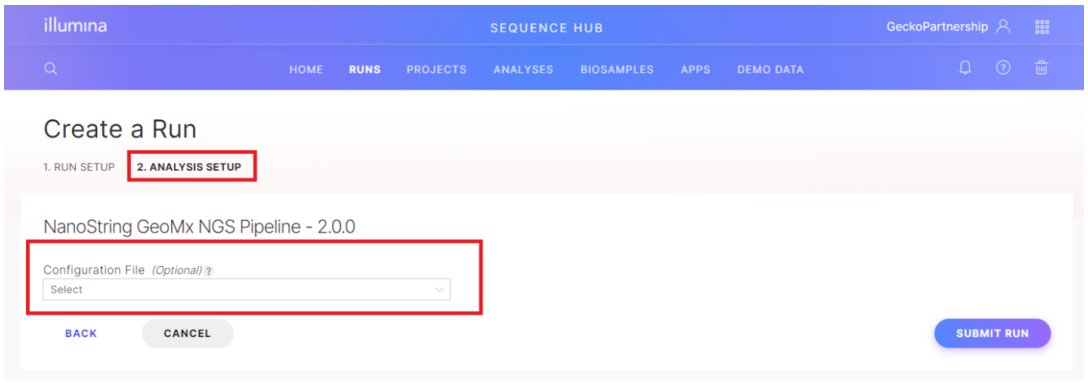


Figure 21: Upload configuration (.ini) file

8. Select **Submit Run**.

2. Sequence

(Users who do not wish to follow the streamlined workflow should sequence following the guidance in Option 2 on page 42).

1. Go to the NextSeq 1000/2000. Check Settings and ensure that **Online Run Setup** and **Proactive, Run monitoring and Storage** are selected.
2. Log into BaseSpace and select your workgroup. Select your Planned Run from the list of names.
3. Confirm the Analysis, Run Length, and Secondary Analysis version match the correct run. Select **Review**.
4. Review your run information and then select **Prep**.
5. Start the run according to the platform instructions (see Illumina user manuals at support.illumina.com).
6. Check run status by logging into BaseSpace, navigating to the workgroup, then selecting Runs.

3. Convert BCL to FASTQ files

Skip this step if you are running the streamlined workflow (i.e. if you set up Analysis in step 1.7 above, when setting up the sequencing run on BaseSpace). Users who do not wish to follow the streamlined workflow should convert BCL to FASTQ files following the guidance in Option 2 on page 42.

> The GeoMx NGS Pipeline looks for FASTQ files with a naming structure such as DSP-1001250001985-A-A02_S2_L001_R1_001.fastq.gz where DSP-1001250001985-A-A02 = Sample ID matching Configuration file, SeqCode- or ProCodeIndices.csv, or Sample ID Translator File. S2 = Sample sheet number. L001 = Lane number (include even if your flow cell had only 1 lane). R1 = Forward or reverse, read 1 or 2. 001 = Should always be 001. The suffix .fastq.gz indicates a compressed file.

4. Transfer FASTQ files to BaseSpace

Skip this step if you are running the streamlined workflow. Users who do not wish to follow the streamlined workflow should convert BCL to FASTQ files following the guidance in Option 2 on page 43.

5. Process FASTQ files in GeoMx NGS Pipeline on DRAGEN in BaseSpace

Skip this step if you are running the streamlined workflow. Users who do not wish to follow the streamlined workflow should convert BCL to FASTQ files following the guidance in Option 2 on page 43.

6. Download DCC.zip from BaseSpace

1. When the run is complete, log into BaseSpace, navigate to the workgroup, and select the **Analyses** tab (Figure 22).
2. Click on the analysis of interest (Figure 22).

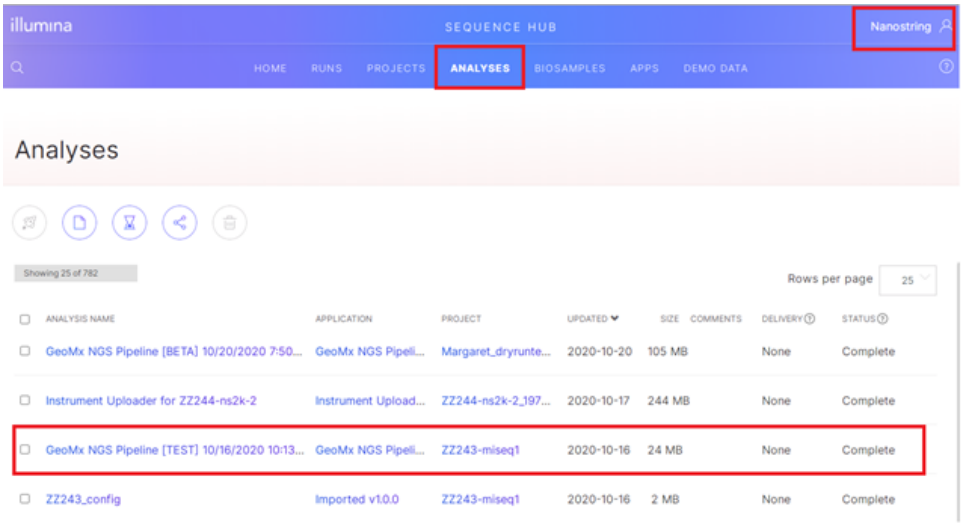


Figure 22: Analyses in BaseSpace

3. Open the folder **GeoMx_NGS_Pipeline**. Download the DCC.zip output with the **name: GeoMx_NGS_Pipeline_...** and **file type: zip** (Figure 23). (Despite its name, the folder "DCC_Files" is not the zipped DCC output for download).

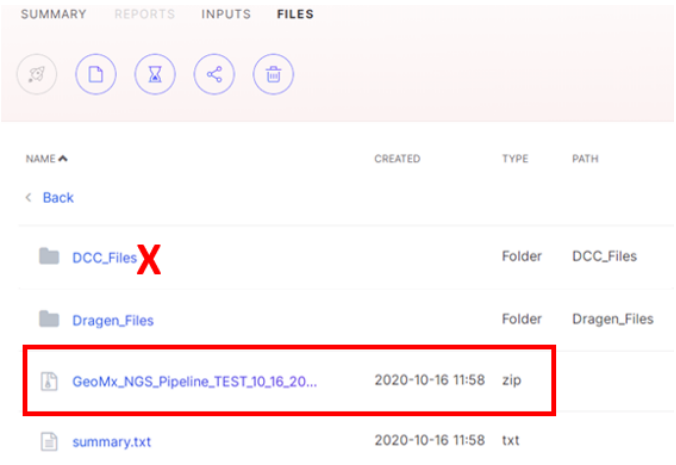


Figure 23: DCC.zip file output from GeoMx NGS Pipeline

IMPORTANT: Check DCC file sizes (should be >1 kb) and summary.txt to ensure files were processed as expected.

7. Upload DCC.zip to GeoMx

1. In the GeoMx DSP Control Center, hover over the Data Collection button and select **Upload Counts**.
2. In the **Upload Count Data** window, select **Choose File**.
3. Browse to the location of your saved **zipped** DCC file and select **Open**.
4. A notification will appear under the Notifications Bell indicating that counts were uploaded successfully. This may take a few moments.

IMPORTANT: If you previously uploaded counts and then upload new counts, note that the new counts will replace the old counts in slide records and any future data analysis studies. Any existing data analysis studies will remain unchanged as they were created with the old count data.

If DCC files are not accepted by the software, check that you do not have a folder within the zipped folder, and that DCC file names match SampleID names from the Sample Index List. Check that there is a DCC file for every sample in the Readout Group – partial Readout Group counts cannot be uploaded.

Proceed to the GeoMx DSP Data Analysis User Manual (MAN-10154).

Option 4. DRAGEN Pipeline with Integrated Sequencing Run Planning on GeoMx DSP

This option is appropriate only for Illumina NextSeq 1000/2000 users running the GeoMx NGS Pipeline on DRAGEN. In this seamless end-to-end workflow, the sequencing run is planned from the GeoMx DSP Control Center during the instrument step **Finalize Readout Group** (see details in GeoMx DSP Instrument User Manual (MAN-10152)). Choose from **local** or **cloud** integrated workflows (note, however, that the **cloud integrated workflow is not yet enabled for Immuno-oncology Proteome Atlas assays**):

- In the **local integrated workflow**, users manually transfer the readout package from the GeoMx DSP instrument to the NextSeq 1000/2000 via USB. The local integrated workflow auto-launches FASTQ conversion and GeoMx NGS Pipeline on DRAGEN, using on-instrument FPGA hardware on the NextSeq 1000/2000. NOTE: This workflow produces an output.tar.gz. It is only compatible with the GeoMx DSP Data Analysis suite for tertiary analysis. No DCC files are generated.
- The **cloud integrated workflow** sends the planned sequencing run to the NextSeq 1000/2000 instrument and automatically transfers the readout package to auto-launch FASTQ conversion and GeoMx NGS Pipeline on DRAGEN on BaseSpace. The cloud integrated workflow is only possible when both instruments are networked.

There is a one-time set-up that is needed prior to performing the integrated workflows:

- To run the local integrated workflow:
  - Obtain a DRAGEN license to run the GeoMx NGS Pipeline on DRAGEN from https://www.illumina.com/products/by-type/informatics-products/dragen-bio-it-platform.html.
  - Install the NanoString GeoMx NGS Pipeline on DRAGEN v3.9.3 on the NextSeq 1000/2000. Download the installer and release notes (with instructions) from https://sapac.support.illumina.com/downloads/dragen-geomx-ngs-installer.html.
  - On the NextSeq 1000/2000, go to **Settings** and select **Local Run Setup** and **Proactive and Run Monitoring**. Save settings.
- To run the cloud integrated workflow:
  - Connect the GeoMx DSP to the BaseSpace Workgroup. Follow the instructions in Appendix VI: Connect GeoMx DSP to BaseSpace Workgroup on page 72.

• On the NextSeq 1000/2000, go to **Settings** and select **Online Run Setup** and **Proactive, Run Monitoring, and Storage**. Save settings.

1. Set up sequencing run from the GeoMx DSP instrument

At the instrument step **Finalize Readout Group**, enter the parameters for the sequencing run (Figure 24).

- Counting Device Model: NextSeq 1000/2000
- Read Strategy: Paired
- Read Length 1: 27
- Read Length 2: 27
- i5 Sequence Orientation: Forward
- Illumina BaseSpace Account: select the appropriate, linked workgroup from the drop-down menu. (Users who run the local integrated workflow will not select a workgroup at this step).

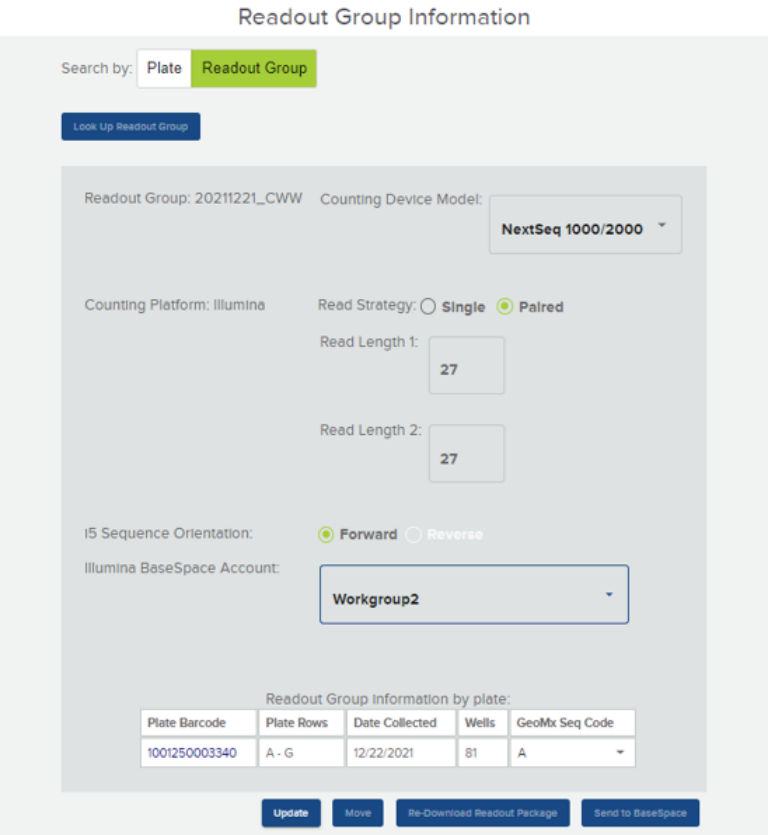


Figure 24: Finalize Readout Group window

2. Select location for DRAGEN pipeline processing

- **Local integrated workflow**: GeoMx NGS Pipeline runs on the NextSeq 1000/2000 instrument. Proceed to Step 3 Download Readout Package.
- **Cloud integrated workflow**: GeoMx NGS Pipeline runs on DRAGEN on BaseSpace. From the GeoMx DSP **Finalize Readout Group** window (Figure 24) , click **Send to BaseSpace**. Proceed to Step 4 Sequence.

3. Download readout package

From the **Finalize Readout Group** window (Figure 24), download the readout package to a USB drive. (For users of the cloud integrated workflow, the readout package is sent to BaseSpace directly, but can still be downloaded in order to refer to the Lab Worksheet during library preparation.)

4. Sequence

- **Local integrated workflow:**
    1. To transfer the readout package to the NextSeq 1000/2000:
        a. From the NextSeq 1000/2000 Start screen, click the **Menu icon**, then **Minimize Application**.
        b. Insert the USB drive and transfer the **zipped** readout package to the **Documents** folder on the NextSeq 1000/2000.
        c. Click on the minimized NextSeq 1000/2000 Control Software application at the bottom of the screen to return to the application.
    2. To start the run, select **Start**. Select the option **Skip BaseSace Sequence Hub Sign In** at the bottom of the screen.
    3. Select **Start With Sample Sheet**. Navigate to the zipped readout package in the Documents folder and click **Open**.
    4. If the DRAGEN software version is not v3.9.3 or later, an instrument warning will appear. Select **Switch DRAGEN Version**.
    5. The SampleSheet.csv within the zipped readout package will be selected. Select **Review** to review your run information and then select **Prep**.
    6. Start the run according to the platform instructions (see Illumina user manuals at support.illumina.com).

- **Cloud integrated workflow:**
    1. Log into BaseSpace and select your workgroup. Select your Planned Run from the list of names.
    2. Confirm the Analysis, Run Length, and Secondary Analysis version match the correct run. Select **Review**.
    3. Review your run information and then select **Prep**.
    4. Start the run according to the platform instructions (see Illumina user manuals at support.illumina.com).
    5. Check run status by logging into BaseSpace, navigating to the workgroup, then selecting Runs.

5. Download counts

- **Local integrated workflow**:
    1. The output file can be found in the local run folder on the NextSeq 1000/2000 instrument. Within the folder specific to this run, open **Analysis**, then **1**, then **Data**.
    2. Locate the file **output.tar.gz** and save to a USB for transfer to the GeoMx DSP. **Do not unzip** the file.

       NOTE: This workflow produces an output.tar.gz. It is only compatible with the GeoMx DSP Data Analysis Suite for tertiary analysis. No DCC files are generated.
- **Cloud integrated workflow**:
    1. When the run is complete, log into BaseSpace, navigate to the workgroup, and select the **Analyses** tab (Figure 25).
    2. Click on the analysis of interest (Figure 25).

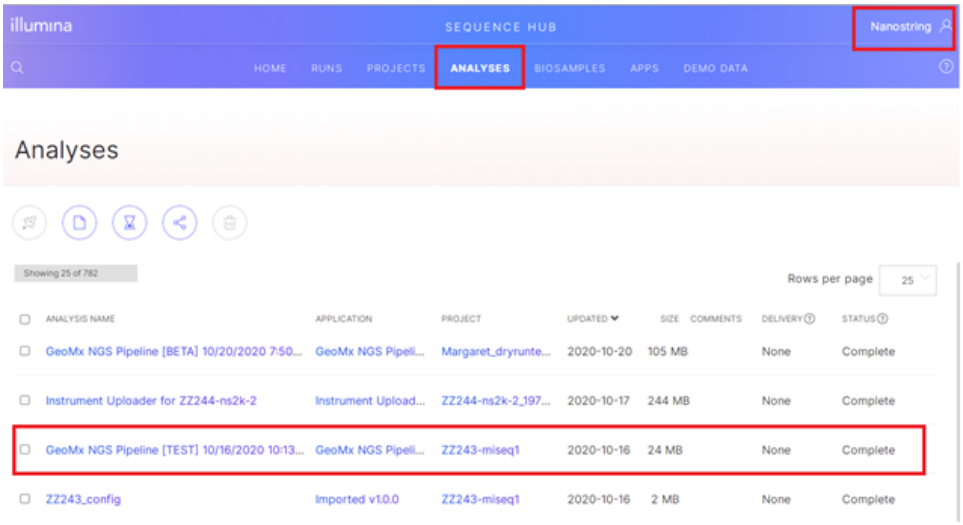


Figure 25: Analyses in BaseSpace

3. Open the folder **GeoMx_NGS_Pipeline**. Download the DCC.zip output with the **name: GeoMx_NGS_Pipeline_...** and **file type: zip** (Figure 26). (Despite its name, the folder "DCC_Files" is not the zipped DCC output for download).

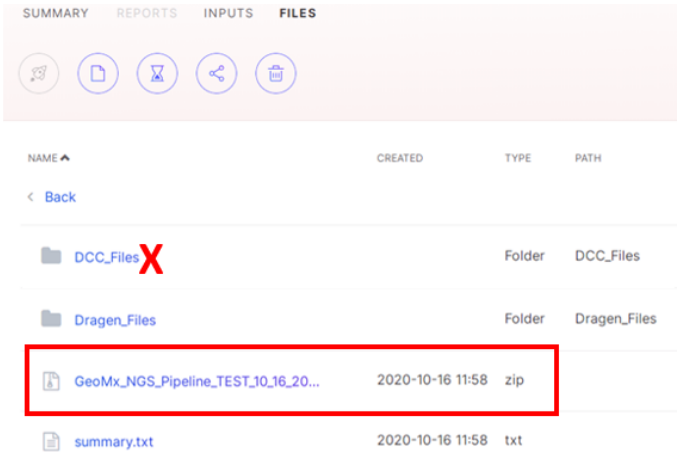


Figure 26: DCC.zip file output from GeoMx NGS Pipeline

IMPORTANT: Check DCC file sizes (should be >1 kb) and summary.txt to ensure files were processed as expected.

6. Upload counts file to GeoMx DSP

1. In the GeoMx DSP Control Center, hover over the Data Collection button and select **Upload Counts**.
2. In the **Upload Count Data** window, select **Choose File**.
3. Browse to the location of your saved **zipped** output.tar.gz or DCC file and select **Open**.
4. A notification will appear under the Notifications Bell indicating that counts were uploaded successfully. This may take a few moments.

> **IMPORTANT:** If you previously uploaded counts and then upload new counts, note that the new counts will replace the old counts in slide records and any future data analysis studies. Any existing data analysis studies will remain unchanged as they were created with the old count data.

If DCC files are not accepted by the software, check that you do not have a folder within the zipped folder, and that DCC file names match SampleID names from the Sample Index List. Check that there is a DCC file for every sample in the Readout Group – partial Readout Group counts cannot be uploaded.

Proceed to the GeoMx DSP Data Analysis User Manual (MAN-10154).

Appendix I: PCR Positive Control

A PCR positive control can be set up for training or troubleshooting purposes. Be aware that including a PCR positive control reaction in the same plate as the samples runs the risk of cross-contamination into experimental wells.

Prepare the positive control in the slide preparation area to avoid cross-contamination.

- Prepare a dilution series of the RNA-NGS or Protein-NGS probe pool to use as the PCR template:
    - Dilution 1: Take 5 µL of stock probe pool and add to 495 µL Elution Buffer.
    - Dilution 2: Take 5 µL of Dilution 1 and add to 495 µL Elution Buffer.
    - Dilution 3: Take 5 µL of Dilution 2 and add to 495 µL Elution Buffer.

    *Use 4 µL of Dilution 3 as the PCR template (instead of DSP aspirate) in a **Protein-NGS** PCR Positive Control.*

    - Dilution 4: Take 2.5 µL of Dilution 3 and add to 497.5 µL Elution Buffer.

    *Use 4 µL of Dilution 4 as the PCR template (instead of DSP aspirate) in an **RNA-NGS** PCR Positive Control.*
- Set up the PCR Positive Control reaction using Seq Code or Pro Code Primers and GeoMx NGS Master Mix, as in the main protocol, with the template prepared in the previous step instead of DSP aspirate.
- Following the PCR, take the PCR product (10 µL) and combine with 12 µL AMPure beads in the library cleanup workflow, in parallel with the pooled PCR products and NTC. Elute in 5 µL Elution Buffer. Check for PCR products using the same method used for the pooled library and NTC.
- The PCR positive control should NOT be pooled with the library and does not need to be sequenced.

Appendix II: Strategies for Pooling before Sequencing

- During PCR, products are indexed with unique dual indices and therefore can be combined (pooled) before AMPure cleanup. This pool is referred to as the Sample Pool.
- NanoString recommends all samples from a single collection plate be equivolume-pooled into a single Sample Pool, irrespective of segment size (see Figure 7 on page 30).
- If you have multiple collection plates in an experiment, and/or you are combining various experiments spanning multiple collection plates, then you will combine your Sample Pools into a single, final tube for sequencing - this is your Sequencing Pool.
- The strategy for combining Sample Pools into a Sequencing Pool is based on the total ROI area of the Sample Pools:

    a. Find the Area column of the Lab Worksheet, downloaded in the readout package from the GeoMx DSP.

    b. Sum the areas of all samples in the Worksheet to calculate the total area for that Sample Pool.

    c. Sum the total areas of all Sample Pools that will be combined in the Sequencing Pool, to calculate the grand total area of the Sequencing Pool.

    d. Use the proportion of Sample Pool area to Sequencing Pool area to determine the amount (in nmol) of each Sample Pool to add to the Sequencing Pool.

    e. See Table 10 for an example of a Sequencing Pool comprising 3 collection plates (Sample Pools).

    f. Combine the Sample Pools to form the Sequencing Pool, then measure the concentration of the Sequencing Pool on QuBit (recommended) or qPCR. Dilute the Sequencing Pool to the recommended pooled loading concentration for the Illumina sequencer and flow cell in use for the project. Include 1-5% PhiX spike-in.

Table 10: Combining Sample Pools into a Sequencing Pool

Sample Pool	Total Area of Sample Pool	Proportion of Sample Pool area to Sequencing Pool area	To Make Sequencing Pool
1	1,500,000 μm^2	15%	Combine Sample Pools at a ratio of 15:45:40 (nmol)
2	4,500,000 μm^2	45%	
3	4,000,000 μm^2	40%	
	Sum = total area of Sequencing Pool = 10,000,000 μm^2	*Sum = 100%*	

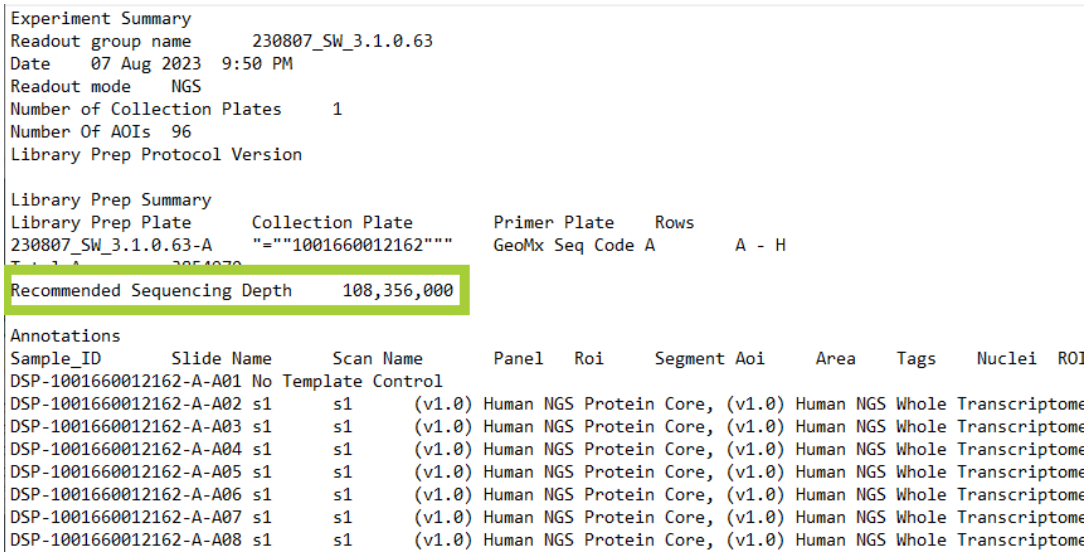
- If you need additional support to plan your library pooling strategy and sequencing, please contact Support@nanostring.com or your NanoString Field Application Scientist before your sequencing run.

Appendix III: Sequencing Depth

- Sequencing depth indicates the frequency with which a given nucleotide is read, or identified, in a given experiment. Greater sequencing depth gives confidence that low-expressing targets are identified.
- For a given GeoMx experiment, sequencing depth is dependent on numerous experimental and multiplexing factors. These include inherent factors like sample quality, tissue or cell types, and gene expression levels, as well as controllable factors like number of probes in the panel and total area-of-illumination pooled in one sequencing run.
- A single molecule present in the sequencing library corresponds to one cluster on the flow cell. The paired-end read strategy recommended by NanoString results in 2 paired-end reads, forward and reverse, per cluster. This can also be described as one read pair.
- A guide to calculate the recommended sequencing depth (number of sequenced read pairs) for different GeoMx assays is below (Table 11). In software v3.1, the recommended sequencing depth for the readout group is calculated and included on the Lab Worksheet downloaded in the readout package (Figure 27).
- If your subsequent Data QC indicates that initial sequencing depth was not sufficient, the same library can be re-sequenced and the reads combined, to increase sequencing depth.
- In general, as the expression content increases in a certain segment, the relative sequencing saturation will decrease. For example, in two different cancer datasets, the relative sequencing saturation was lower in tumor segments as opposed to tumor microenvironment segments (average of 60% versus 70-75%, respectively). Sequencing saturation was sufficient for both segments, but relative saturations were different since there were more photocleaved oligos released from the tumor than tumor microenvironment segments.
- By combining very different AOI sizes in a single experiment, it is more likely that you will under-sequence the larger AOIs. Bear this in mind in your experimental design.

Table 11: Sequencing depth recommended for different GeoMx NGS assays

Type	Assay	Sequencing Depth Calculation (number of read pairs)
RNA	Whole Transcriptome Atlas (WTA)	Total collection area (μm^2) x 100
RNA	Cancer Transcriptome Atlas (CTA)	Total collection area (μm^2) x 30
RNA	Standalone Custom RNA-NGS Panel with *n* targets	Total collection area (μm^2) x *n* x 0.05
Protein	Seq Code Protein Cores and Modules with *n* targets	Total collection area (μm^2) x *n* x 2
Protein	Pro Code Protein Core and Module (IO Proteome Atlas)	Total collection area (μm^2) x 200



Experiment Summary
Readout group name 230807_SW_3.1.0.63
Date 07 Aug 2023 9:50 PM
Readout mode NGS
Number of Collection Plates 1
Number Of AOIs 96
Library Prep Protocol Version

Library Prep Summary
Library Prep Plate Collection Plate Primer Plate Rows
230807_SW_3.1.0.63-A "=""1001660012162""" GeoMx Seq Code A A - H

Recommended Sequencing Depth 108,356,000

Annotations
Sample_ID Slide Name Scan Name Panel Roi Segment Aoi Area Tags Nuclei ROI
DSP-1001660012162-A-A01 No Template Control
DSP-1001660012162-A-A02 s1 s1 (v1.0) Human NGS Protein Core, (v1.0) Human NGS Whole Transcriptome
DSP-1001660012162-A-A03 s1 s1 (v1.0) Human NGS Protein Core, (v1.0) Human NGS Whole Transcriptome
DSP-1001660012162-A-A04 s1 s1 (v1.0) Human NGS Protein Core, (v1.0) Human NGS Whole Transcriptome
DSP-1001660012162-A-A05 s1 s1 (v1.0) Human NGS Protein Core, (v1.0) Human NGS Whole Transcriptome
DSP-1001660012162-A-A06 s1 s1 (v1.0) Human NGS Protein Core, (v1.0) Human NGS Whole Transcriptome
DSP-1001660012162-A-A07 s1 s1 (v1.0) Human NGS Protein Core, (v1.0) Human NGS Whole Transcriptome
DSP-1001660012162-A-A08 s1 s1 (v1.0) Human NGS Protein Core, (v1.0) Human NGS Whole Transcriptome

Figure 27: Lab worksheet including recommended sequencing depth (software v3.1)

Appendix IV: Requeue NextSeq 1000/2000 Run with New Sample Sheet

You may need to requeue a job on the GeoMx NGS Pipeline on DRAGEN in BaseSpace, for example if the run was originally planned with an incorrect sample sheet, or wrong configuration (.ini) file.

1. Log into BaseSpace and select the correct workgroup (Figure 28).

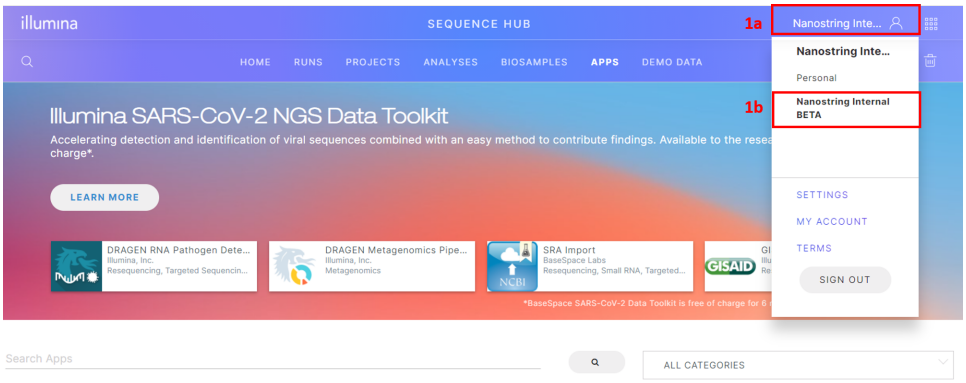


Figure 28: Select your workgroup. In this example, the workgroup is Nanostring Internal.

2. Select “Apps”, search for “GeoMx”, and select “GeoMx NGS Pipeline” (Figure 29).

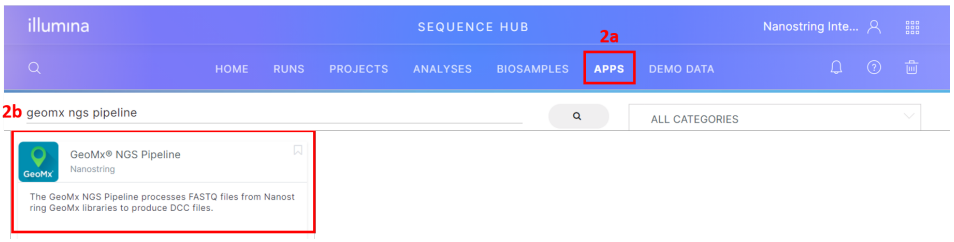


Figure 29: Locate GeoMx NGS Pipeline app

3. Launch application (Figure 30).

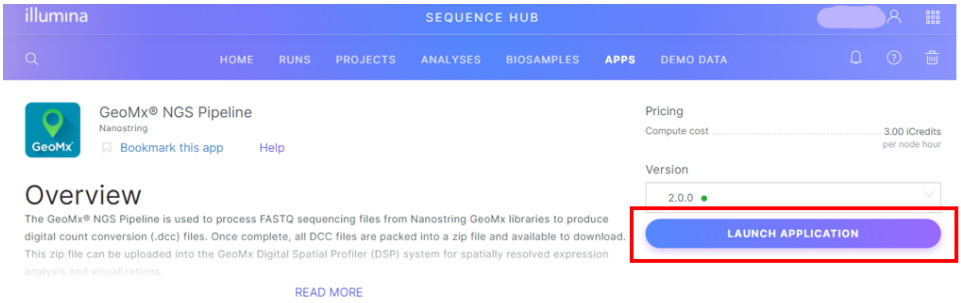


Figure 30: Launch GeoMx NGS Pipeline application

4. For **Start From**, choose **BaseSpace Run Plan**, and enter only the input files associated with BaseSpace Run Plan (Figure 31).
5. Under **Select BaseSpace Run**, choose the run to requeue, and select an override sample sheet and/or an override configuration file.
6. Click **Launch Application**.

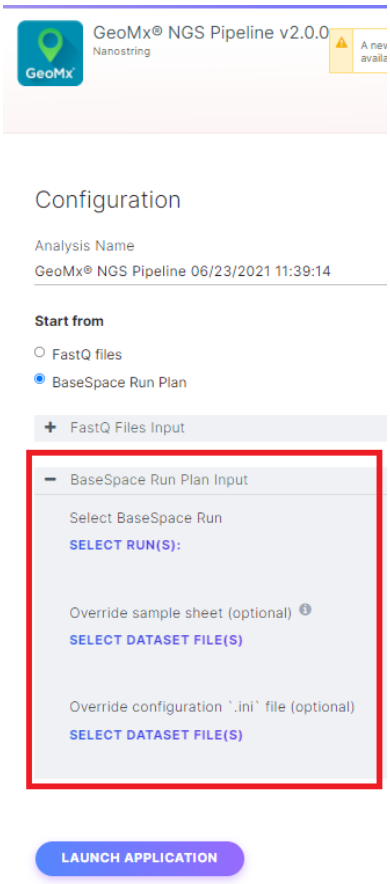


Figure 31: Start From option

7. Check run output by logging into BaseSpace, navigating to Workgroup, and selecting the **Analyses** tab (Figure 32).

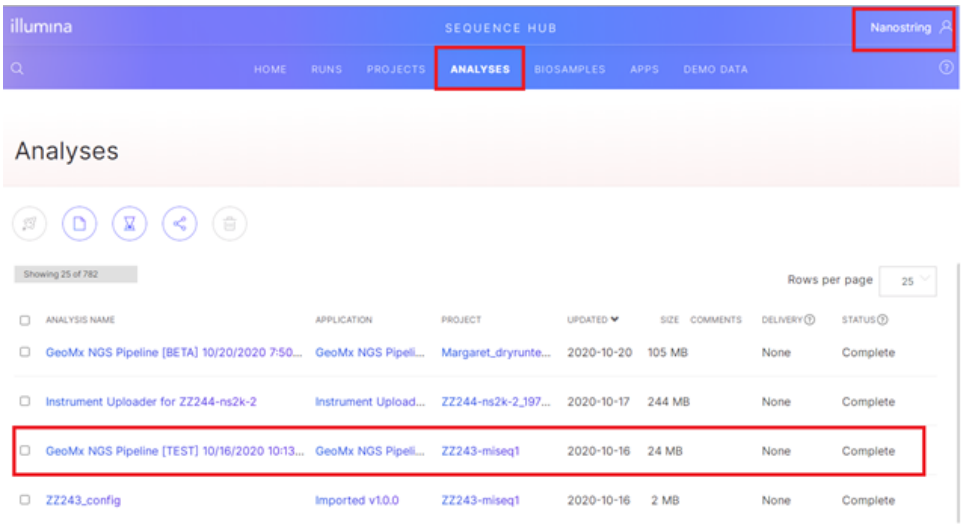


Figure 32: Analyses in BaseSpace

8. Within **Analyses**, open folder **GeoMx_NGS_Pipeline**. Download the DCC.zip output with the **name: GeoMx_NGS_Pipeline_...** and **file type: zip** (Figure 33). (Despite its name, the folder "DCC_Files" is not the zipped DCC output for download).

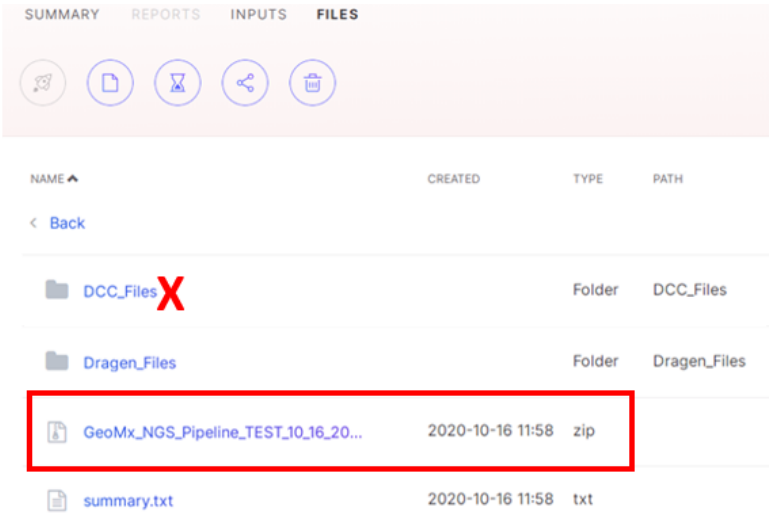


Figure 33: DCC.zip file output from GeoMx NGS Pipeline

IMPORTANT: Check DCC file sizes (should be >1 kb) and summary.txt to ensure files were processed as expected.

Appendix V: Installing the GeoMx NGS Pipeline Software

The GeoMx NGS Pipeline software is one of two methods to process NGS sequencer files into GeoMx DSP counts. This section does not apply to users of the GeoMx NGS Pipeline on DRAGEN via Illumina BaseSpace Sequence Hub.

Version 3.1 of the GeoMx NGS Pipeline Software is required to process Spatial Proteogenomic data that include the Immuno-oncology Proteome Atlas, since both Seq Code index libraries and Pro Code index libraries are sequenced. Links to the software and installation instructions are below (see **Installing the Pipeline**). Refer to the Spatial Proteogenomic Assay User Manual (MAN-10158) for more information.

System Requirements

The GeoMx NGS Pipeline software can be run on a **Linux** or **Amazon Web Services® (AWS)** server, or **locally** (only for small studies, defined below). To interact with the GeoMx NGS Pipeline Software, users have two options: **graphical user interface (GUI)** or **command line interface (CLI).** The specifications below (Table 12) reflect the needs of a pipeline run and may be impacted by other programs running on the same machine.

Table 12: System requirements for GeoMx NGS Pipeline Software

Interface and Pipeline Location	Supported OS*	Minimum CPU	Memory (GB)**	Suitable for
GUI and Pipeline on Windows (local install)	Windows 10	Intel® Core™ i5-4750 3.20 GHz	16	Small studies, <96 segments or ROIs (with <~50 million reads per segment or ROI)
GUI on Windows with Pipeline on Linux/AWS server	GUI: Windows 10 Pipeline: see specs below	Intel® Core™ i5-4750 3.20 GHz	16	All studies
CLI with Pipeline on Linux server	Linux Ubuntu® 18.04 or 20.04	Intel® Core™ i5-4750 3.20 GHz	16	All studies
CLI with Pipeline on AWS t2.xlarge instance server	AWS Linux 2 (EC2)	vCPU 4	16	All studies

* Beginning with GeoMx NGS Pipeline Software v3.1, MacOS® is no longer a supported operating system.

** In addition, ensure adequate storage for FASTQ files locally, or if they are on a network share, be sure to mount that location.

Choosing the option that is right for you

Features of AWS

- While you will be billed for services used on AWS, depending on the amount of processing you need, these costs should be considerably less than buying your own hardware.
- You will need to go through a one-time setup process to prepare an AWS environment for your data processing; see Setting up AWS to run the GeoMx NGS Pipeline Software (MAN-10167).
- You will need an AWS account, the GeoMx NGS Pipeline installed on an AWS virtual machine, a file transfer protocol client (FTP Client) software (such as WinSCP) or a way for your computer to communicate with and send your data to and from AWS.

Features of Windows GUI connected to Linux server

- Connecting to a Linux server using the Windows GUI provides a few advantages: it is easier for users who are not familiar with command line to submit their data for processing and it allows users to specify the amount of parallel processing threads for more control over the server.
- You will need a server with the GeoMx NGS Pipeline installed, and the PC to run the GUI must have the GeoMx NGS Pipeline GUI installed (instructions below in Installing the Pipeline to Run on a Server with GUI on Windows on page 68).

Features of CLI control of remote Linux/AWS server

- Using a remote connection to the server may be more convenient if the server already has direct access to your data.
- You will need a server with the GeoMx NGS Pipeline installed and with access to your data and a way to remotely log in to the server.

Features of running locally

- For smaller datasets (defined in Table 12) or if you have a fast CPU and a lot of RAM, you can use the Windows or Linux versions to process files locally on your computer. This may consume much of your system resources and we do not recommend any multitasking while processing.
- You will need the GeoMx NGS Pipeline installed and the GeoMx NGS Pipeline file inputs saved to or accessible from your computer.

Installing the pipeline

NOTE: The zipped software package includes more detailed Installation Instructions in a Word document. Please refer to that document to install the pipeline.

Installing the Pipeline to Run Locally on Windows

Suitable for studies of <96 segments or ROIs (with <~50 million reads per segment or ROI)

1. **Download** the GeoMx NGS Pipeline Software installation file from https://nanostring.box.com/v/GeoMxNGSPipeline.
2. Right-click on the installation file (.exe) and select **Extract Here.**
3. Double-click on the resulting **installer application**.
   - Follow the instructions in the Wizard to install the GeoMx NGS Pipeline software.
   - Read and accept the terms of GeoMx NGS Pipeline and wait until the Pipeline sets up the environment.
   - Check the box **GeoMxNGSPipeline Local Server**.
4. Once the GeoMx NGS Pipeline software has been installed, **open the application**.

Installing the Pipeline to Run on a Server with GUI on Windows

1. **Download** the GeoMx NGS Pipeline Software installation file from https://nanostring.box.com/v/GeoMxNGSPipeline to the Windows environment.
2. Right-click on the installation file (.exe) and select **Extract Here.**
3. Double-click on the resulting **installer application**.
   - Follow the instructions in the Wizard to install the GeoMx NGS Pipeline GUI.
   - Read and accept the terms of GeoMx NGS Pipeline and wait until the Pipeline sets up the environment.
   - DO NOT check the box **GeoMxNGSPipeline Local Server**.
4. Once the GeoMx NGS Pipeline software has been installed, **open the application**.
5. Next, **install the GeoMx NGS Pipeline on your server.** See section Installing the Pipeline on Linux or AWS Server on page 69.

6. Once installed, add the server in the GUI by clicking **new server**, entering the Public IPv4 address of the server (four integers separated by periods followed by **:5000**), then clicking **Add** (Figure 34).
7. Enter **API server address**, including port (insert your server name in lieu of the red text): `http://<remote server address>:5000`
8. In the main GeoMx NGS Pipeline menu, ensure the toggle **Run locally** is switched to **Run remotely**, and the server you saved is selected from the adjacent drop- down menu (if not already by default).

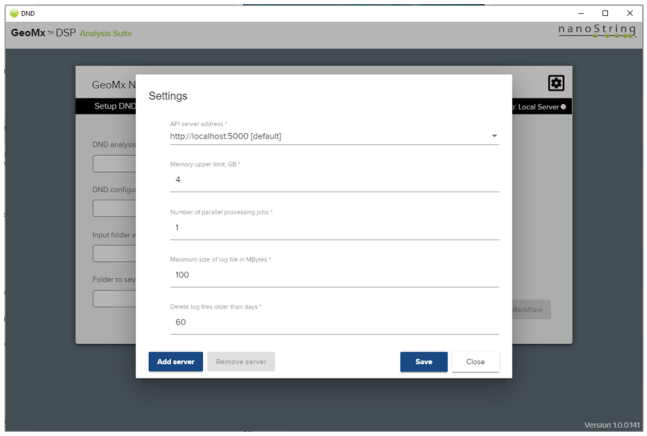


Figure 34: Adding a server

- For all GeoMx NGS Pipeline runs moving forward, you can click the gear icon and select the server from server address drop down, click save, then move slider and run on the server.

The GeoMx NGS Pipeline running on Linux only listens on port 5000 and on Windows on port 7000. This may cause a problem if there are other applications on server or network limiting the availability of that port. Contact Support@nanostring.com for support.

Installing the Pipeline on Linux or AWS Server

To set up an AWS server for the first time, refer to the **AWS Tutorial: Get started with Amazon EC2 Linux instances** at https://docs.aws.amazon.com/AWSEC2/latest/UserGuide/EC2_ GetStarted.html or **User Guideline: Set up AWS to run GeoMx NGS Pipeline Software** at https://university.nanostring.com/geomx-ngs-pipeline-software-on-aws.

Obtain the installation files from https://nanostring.box.com/v/GeoMxNGSPipeline:

- GeoMxNGSPipeline_Linux_3.1.x.x.sh – the installation script
- GeoMxNGSPipeline.tgz – the installation package file

During the installation, you will execute the installation script file. It will unpack API server files to the proper location, update configuration, and start the service.

You will first need to have access to a Linux server with **Ubuntu** or **Amazon Linux 2 (EC2)** distributive as well as **sudo** user privileges on that server.

1. **Unzip the installation archive** and use secure copy protocol (SCP) (such as WinSCP) to **copy** *GeoMxNGSPipeline_Linux_3.1.x.x.sh* and *GeoMxNGSPipeline.tgz* files to the home folder on the target server.
2. Using secure shell (SSH) client (PuTTY), **connect to the server**. Make sure you are connecting with user who has sudo privileges on that server.
3. Sometime during SCP (secure copy) the execute (x) permission may be lost.
   - To check this, navigate to your home folder (`cd /home/<your user name>`) and execute the following command: `ls - l` or `ll` and check that you have **execute** or **x** permissions for the *GeoMxNGSPipeline_Linux_3.1.x.x.sh* file.
   - If the **x** permission is missing, you will see something like: `-rw-rw-r—` (which means no one can execute this script and you will get 'permission denied' error).
   - Run the following command: `sudo chmod +x GeoMxNGSPipeline_Linux_3.1.x.x.sh`. This will add execute **x** permission and the permission set will look like: `-rwxrwxr-x`.
4. **Run the installation script**: `sudo ./GeoMxNGSPipeline_Linux_3.1.x.x.sh`.

   The installation script will ask you to specify the port. You can either specify the port under which the application will be running or leave the default port – 5000. To keep the default port, click Enter.

   If you already have installed GeoMxNGSPipeline API on this server and want to rerun the installation, the system will ask whether you would like to override existing settings and whether you want to override folder mappings settings. In both cases type **Y** to confirm or any other character to reject.
5. After installation, check if the service is running and if the port you have specified during installation is listening. To do this, run the following command: `sudo netstat -tulpn`. In the output, check that port 5000 is listening to GeoMxNGSPipeline service.

   > **IMPORTANT:** Depending on which platform you are using (Ubuntu or Amazon Linux) the output of this command may be slightly different.

6. Configure folder mapping (required if you plan to use the GUI to connect to a remote server for running the pipeline).
   - Folder mapping enables you to navigate to and view folders and folder contents on the server.
   - Folder mapping is discretionary, based on what you want to have visible in the GUI, and most likely where the data resides (i.e., if FASTQ files are in your /home directory, then the /home directory should be mapped).

- These particular lines provide examples of what the folder mapping could be, but should be modified based on your environment, preference, and organizational habits.
  - <folder path="/home" name="home"/>
  - <!--folder path="/home/ubuntu" name="ubuntu"/-->

To configure server folder mapping, you must edit the *runtimesettings.xml* file. By default, this file has mapping for home folder (*/home*). You must use one of Ubuntu Linux editing tools like *mcedit* (part of mc), *vi* or *nano*, to edit server mappings.

For example, to use mcedit, start by typing `sudo mc` to open Midnight Commander. Navigate to the */var/GeoMxNGSPipeline* folder and open *runtime-settings.xml* file: Under the *server_folders* node, add folder mappings by adding/changing **folder** elements. Every folder element has 2 attributes:

- **path** – physical path on the server (which can point also to mapped EFS volumes)
- **name** – the name of this mapping.

Remove folder mappings which are incorrect. It is important to keep only valid folder mappings. Otherwise, the GUI will report an error while trying to connect to the server. Press F2 to save your edits. You don't need to restart API server. The changes in `runtime-settings.xml` will be processed automatically.

7. Connect to the newly installed API instance using the GeoMx NGS Pipeline GUI as described in Installing the Pipeline to Run on a Server with GUI on Windows on page 68, or using CLI.

Appendix VI: Connect GeoMx DSP to BaseSpace Workgroup

This option within the **Administration** menu is only visible when accessing the GeoMx DSP Control Center over a remote connection using the Chrome browser. Users can connect their GeoMx DSP to their Illumina BaseSpace workgroup(s) to enable seamless end-to-end workflows. These workflows allow a sequencing job to be sent from the GeoMx DSP directly to the BaseSpace Sequence Hub, eliminating the file processing steps of the conventional workflow.

Requirements:

- Use of Illumina NextSeq 1000/2000 sequencing platform
- Active BaseSpace subscription

To add a connection to a BaseSpace Workgroup:

1. Access the GeoMx DSP over Chrome from a separate computer. Log in as an administrative user.
2. Click on the Administration menu, then Illumina BaseSpace Admin.
3. Click Add Connection to BaseSpace Workgroup.
4. In the pop-up window (Figure 35), create an Account Name for this workgroup connection.
5. From the drop-down menu, select the region of your BaseSpace account (typically the region closest to your geographic location). **DO NOT click Connect to BaseSpace yet.**
6. Open a new Chrome browser tab and navigate to the BaseSpace Sequence Hub. Log in to your account and select the Workgroup to which you wish to connect. **The same workgroup must be used** to log into the NextSeq 1000/2000 instrument and BaseSpace Sequence Hub. **Leave this browser tab open.**
7. Return to the GeoMx DSP browser tab and click **Connect to BaseSpace**. An authentication windows opens. Click Accept to grant permission for the applications to communicate.

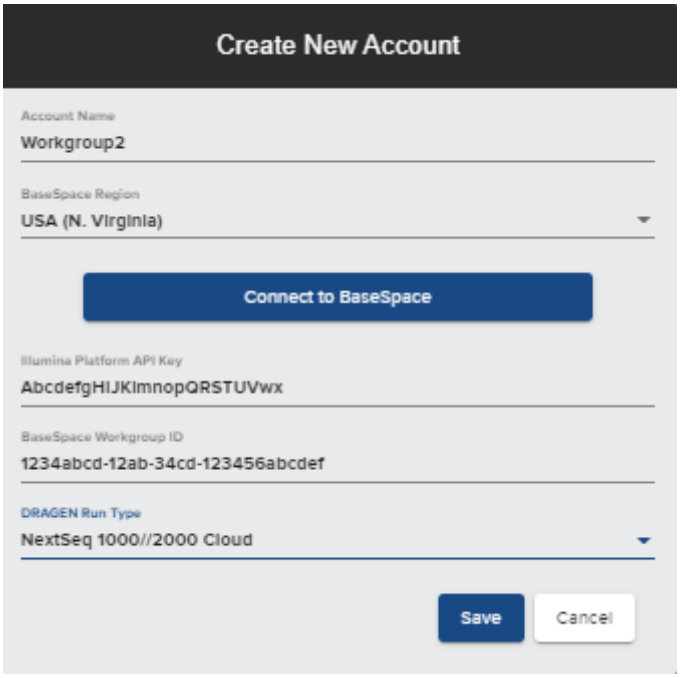


Figure 35: Illumina BaseSpace Admin pop-up

8. Enter the Illumina Platform API Key as follows:

   - In a third browser tab, navigate to https://accounts.login.illumina.com/platform-home/#/apiKey/list (Figure 36). (If your BaseSpace account is part of an Enterprise subscription, navigate to https://[YourEnterpriseDomain].login. illumina.com/platform-home/#/apiKey/list).

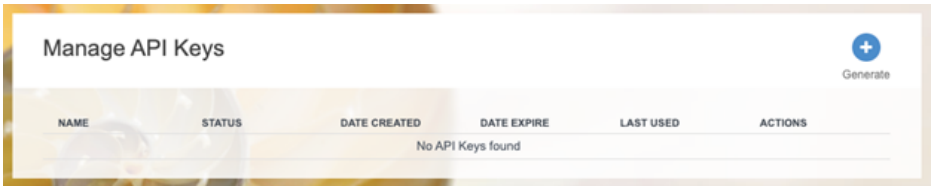


Figure 36: API Key List dialog

   - Click Generate to open the Generate an API Key dialog (Figure 37) .

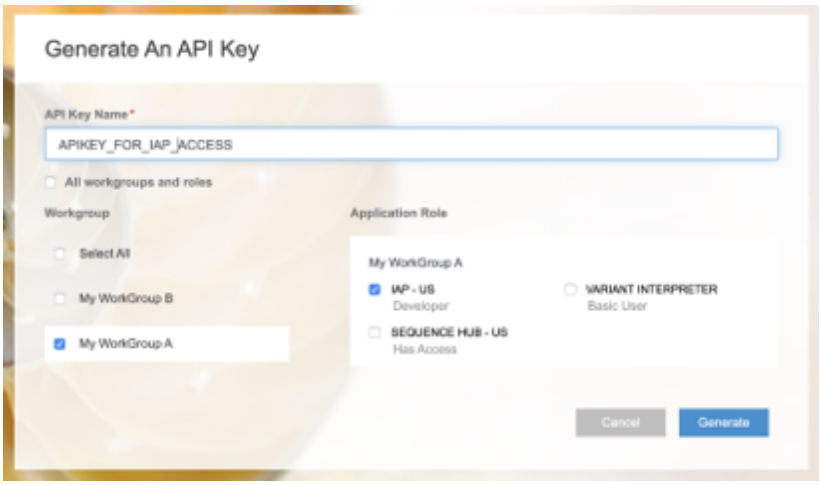


Figure 37: Generate an API Key dialog

   - Enter a name for the key. Un-check the box for "All workgroups and roles" - NanoString recommends to generate a key for a specific workgroup. If your account is under multiple workgroups, choose the one with IAP permissions. Click Generate.
   - A pop-up dialog containing the API key will display. Write down or download the API key.

     A user can only view or download the key at the time of generation. If the key is lost, a user must regenerate it. Keep the key in a secure location and do not share it with others, as such exposure may allow unknown users to access your data. If the key is compromised, a user should regenerate it and configure the GeoMx DSP connection with the new API key.

- Close the API Key Generated dialog, and the newly generated key will appear in the key list. Confirm that the key is set to "Never Expire". Otherwise, the connection will be disrupted by token expiry during operation.

9. Enter the BaseSpace Workgroup ID as follows:
    - Open a fourth Chrome browser tab and navigate to https://api.basespace.illumina.com/v2/users/current/workgroups. (Enterprise users: navigate to https://api.[EnterpriseURL]/v2/users/current/workgroups - replace [EnterpriseURL] with the correct enterprise URL for your account). If you are outside of the United States, refer to the regional API URLs listed at https://developer.basespace.illumina.com/docs/content/documentation/cli/cli-overview#SpecifyAPIserverandAccessToken .
    - A list of workgroups will load. Identify the workgroup to which you wish to connect (**must be the same workgroup as selected in Step 6, above**) and note the ExternalProviderId (Figure 38). Enter this alphanumeric code in the BaseSpace Workgroup ID field of the GeoMx DSP Control Center.

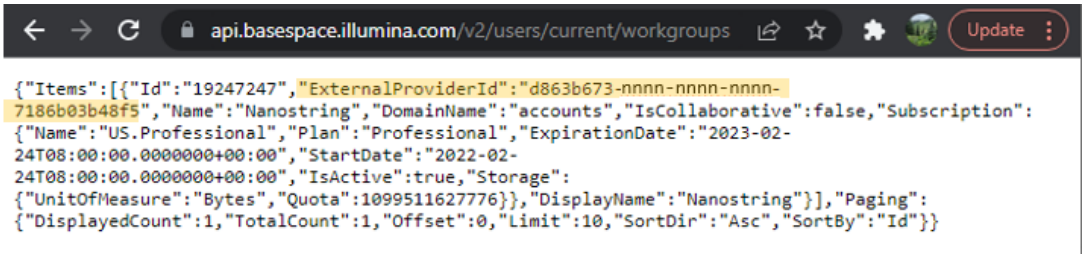


Figure 38: ExternalProviderID

10. Enter the DRAGEN Run Type of your choice (Local or Cloud set-up).
11. Click Save.
12. See the connection listed in the Illumina BaseSpace Administration window of the GeoMx DSP. Click Test to confirm the connection or Edit to make changes if needed.

Troubleshooting

Suggested actions to resolve certain issues are listed below (Table 13). This list is not comprehensive of all possible causes and resolutions. Issues that are rooted in NGS library preparation or pipeline processing, but are not discovered until data analysis, are addressed in the GeoMx DSP Data Analysis User Manual (MAN-10154). Contact Support@nanostring.com for additional support.

Table 13: Troubleshooting NGS readout

Issue	Possible cause	Suggested action
Low yield from library clean-up	Incorrect bead volume used in clean-up	Ensure that the correct volume of AMPure beads was added for a bead-to-sample ratio of 1.2X. Sample volumes should be carefully measured with a pipette.
	Workflow error or low input (e.g. low number of photocleaved oligo inputs due to small-sized ROIs or small number of pooled ROIs)	Ensure PCR best practices on page 26 and thermal cycler parameters. Evaluate experimental conditions that may explain low yield from library prep. Set up a PCR positive control as described in Appendix I: PCR Positive Control on page 58.
	Overamplification in PCR	In many cases it is still worth sequencing the library as long as its concentration can be accurately measured.
Undesired peaks observed in NGS library BioAnalyzer or TapeStation trace (see examples on page 33)	Cross-contamination from other NGS work	Repeat library PCR and AMPure bead clean-up, then evaluate library quality again. Evaluate lab work areas for cross-contamination risk. Follow NGS site readiness guidance on page 20 and PCR best practices on page 26.
	Incorrect PCR cycle settings	Ensure that the protocol is followed as written, with 18 cycles of PCR amplification on page 26.
After clicking "Send to BaseSpace" during Finalize Plate step, there is a Send Run Definition error.	GeoMx DSP is not linked to correct workgroup	Ensure that the steps in Appendix VI: Connect GeoMx DSP to BaseSpace Workgroup on page 72 have been followed correctly. Check that the BaseSpace workgroup ExternalProviderID entered in set-up matches the workgroup where the run is being sent in the Finalize Plate step.

Issue	Possible cause	Suggested action
I want to make adjustments to readout groups	Changes to experiment or sequencing plan	Once a plate is finalized, its readout group assignment can be changed or combined with another finalized readout group (with restrictions; see GeoMx DSP Instrument User Manual (MAN-10152)). Click the plate icon, find the record for the readout group, and click **Move** at the bottom of the Readout Group Information window. The dialog **Move Wells Between Readout Groups** opens. Select the plate barcode to update then click **Choose**. Select the new readout group designation then click **Use Readout Group**. Download the new readout package and use the files to run the GeoMx NGS Pipeline again to process the new data set.
BCL to FASTQ generation yields no FASTQs or low number of reads in each FASTQ	i5 sequence was forward when it should have been reverse complement, or vice versa	• Return to Finalize Readout Group in GeoMx software and change the selection for i5 orientation. • Download a new readout package and re-queue the sequencing job with the new sample sheet. • Run new FASTQ files through the GeoMx NGS Pipeline with the new configuration (.ini) file. Upload new counts to GeoMx.
GeoMx NGS Pipeline software hangs indefinitely (does not finish job)	Insufficient computational resources or space	Ensure software/hardware specifications meet the Appendix V: Installing the GeoMx NGS Pipeline Software on page 66. Ensure the output file location has sufficient space to hold output files.
GeoMx NGS Pipeline software takes a long time to run and creates a huge folder	Pipeline may be set to "Keep interim files"	In the GeoMx NGS Pipeline user interface, un-check the box to keep interim files (described on page 37) . These files are typically needed only in certain troubleshooting situations.

Issue	Possible cause	Suggested action
GeoMx NGS Pipeline software does not recognize FASTQ files, or gives error "System.IndexOutOfRangeException: Index was outside the bounds of the array."	FASTQ file format does not match expected format	The GeoMx NGS Pipeline looks for FASTQ files with a naming structure as follows. Include a lane number even if your flow cell had only 1 lane. DSP-1001250001985-A-A02_S2_L001_R1_001.fastq.gz DSP-1001250001985-A-A02 = Sample ID matching Configuration file, SeqCodeIndices.csv, or Sample ID Translator File. S2 = Sample sheet number L001 = Lane number R1 = Forward or Reverse, read 1 or 2 001 = Should always be 001 .fastq.gz = Indicates a compressed file If issue is not resolved, FASTQs may be corrupted. Re-copy them to the current location.
Processing errors in GeoMx NGS Pipeline	File inputs were manually edited and contain an element that is not allowed	Use a text editor such as Notepad++ to check file inputs like configuration (.ini) file and SampleSheet.csv for extra empty lines, extra quotation marks, or curved quotation marks (only simple vertical quotation marks are allowed).
Processing run failed on GeoMx NGS Pipeline on DRAGEN	Various possible causes	Contact Support@nanostring.com or Illumina Support.
One or more DCC files has size = 1kb and does not contain count data	Pipeline processing error or missing FASTQ input to pipeline	Ensure that FASTQ files for all samples are provided to the GeoMx NGS Pipeline (whether standalone software or on DRAGEN). Re-run the pipeline in case the issue was caused by a temporary interruption in pipeline service.

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