

GeoMx[®] DSP NGS Readout User Manual

Library Preparation & GeoMx NGS Pipeline

NanoString, Inc.
530 Fairview Ave N
Seattle, Washington 98109

www.nanostring.com

T: 206.378.6266

T: 888.358.6266

E: geomxsupport@nanostring.com

Sales Contacts

United States: us.sales@nanostring.com

EMEA: europe.sales@nanostring.com

Asia Pacific & Japan: apac.sales@nanostring.com

Other Regions: info@nanostring.com



EU Authorized Representative

NanoString Technologies
Germany Gmbh
Birketweg 31
80639 Munich
Germany

UK Authorized Representative

NanoString Technologies
Europe Limited
11th Floor Whitefriars
Lewins Mead
Bristol BS1 2NT
United Kingdom

Rights, License, & Trademarks

Use

For research use only. Not for use in diagnostic procedures.

Intellectual Property Rights

This GeoMx[®] Digital Spatial Profiler (DSP) User Manual and its contents are the property of NanoString Technologies, Inc. (“NanoString”), and are intended solely for use by NanoString customers, for the purpose of operating the GeoMx DSP System. The GeoMx DSP System (including both its software and hardware components) and this User Guide and any other documentation provided to you by NanoString in connection therewith, are subject to patents, copyright, trade secret rights and other intellectual property rights owned by, or licensed to, NanoString. No part of the software or hardware may be reproduced, transmitted, transcribed, stored in a retrieval system, or translated into other languages without the prior written consent of NanoString. For a list of patents, see www.nanostring.com/company/patents.

Limited License

Subject to the terms and conditions of the GeoMx DSP System contained in the product quotation, NanoString grants you a limited, non-exclusive, non-transferable, non-sublicensable, research use only license to use the proprietary GeoMx DSP System only in accordance with the manual and other written instructions provided by NanoString. Except as expressly set forth in the terms and conditions, no right or license, whether express, implied or statutory, is granted by NanoString under any intellectual property right owned by, or licensed to, NanoString by virtue of the supply of the proprietary GeoMx DSP System. Without limiting the foregoing, no right or license, whether express, implied or statutory, is granted by NanoString to use the GeoMx DSP System with any third party product not supplied or licensed to you by NanoString or recommended for use by NanoString in a manual or other written instruction provided by NanoString.

Trademarks

NanoString, NanoString Technologies, the NanoString logo, GeoMx, and nCounter are trademarks or registered trademarks of NanoString Technologies, Inc., in the United States and/or other countries. All other trademarks and/or service marks not owned by NanoString that appear in this document are the property of their respective owners.

Copyright

©2022 NanoString Technologies, Inc. All rights reserved.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Table of Contents

GeoMx DSP NGS Readout User Manual	1
Contacts	2
Rights, License, & Trademarks	3
Changes in this revision	7
Conventions	8
GeoMx DSP Workflow	9
User Manuals and Resources	11
Introduction to GeoMx NGS Readout	12
NGS Library Preparation and Sequencing	12
GeoMx NGS Pipeline	14
Two Ways to Run the GeoMx NGS Pipeline	15
File Inputs and Outputs	15
GeoMx NGS Readout Site Readiness	17
GeoMx NGS Readout Equipment, Materials, and Reagents	18
Transferring from the GeoMx DSP run	21
Finalize the GeoMx DSP Collection Plate(s)	21
Download Files for Illumina Processing	21
Prepare the GeoMx DSP Collection Plate for Library Prep	22
Library Preparation	23
PCR setup (2 hours)	23
Pooling and AMPure cleanup (45 minutes)	26
Assessing library quality and quantity	30
Plan Your Sequencing and Pipeline Processing	32
Option 1: Standard Standalone Pipeline	33
1. Set up sequencing run	33
2. Sequence	33
3. Convert BCL to FASTQ files	33
4. Transfer files to a location for GeoMx NGS Pipeline accessibility	34

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

5. Process FASTQ files in the NanoString GeoMx NGS Pipeline	34
6. Upload DCC.zip to GeoMx DSP	37
Option 2. DRAGEN Pipeline on BaseSpace	38
1. Set up sequencing run	38
2. Sequence	38
3. Convert BCL to FASTQ files	38
4. Transfer FASTQ files to BaseSpace	39
5. Process FASTQ files in GeoMx NGS Pipeline on DRAGEN in BaseSpace	39
6. Download DCC.zip from BaseSpace	41
7. Upload DCC.zip to GeoMx	42
Option 3. NextSeq 1000/2000 with DRAGEN Pipeline on BaseSpace	43
1. Set up sequencing run	43
2. Sequence	44
3. Convert BCL to FASTQ files	45
4. Transfer FASTQ files to BaseSpace	45
5. Process FASTQ files in GeoMx NGS Pipeline on DRAGEN in BaseSpace	45
6. Download DCC.zip from BaseSpace	45
7. Upload DCC.zip to GeoMx	46
Option 4. DRAGEN Pipeline with Integrated Sequencing Run Planning on GeoMx DSP	48
1. Set up sequencing run from the GeoMx DSP instrument	49
2. Select location for DRAGEN pipeline processing	50
3. Download readout package	50
4. Sequence	50
5. Download counts	52
6. Upload counts file to GeoMx DSP	53
Appendix I: PCR Positive Control	54
Appendix II: Strategies for Pooling before Sequencing	55
Appendix III: Sequencing Depth	57

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Table of Contents

Appendix IV: Requeue NextSeq 1000/2000 Run with New Sample Sheet using GeoMx NGS Pipeline on DRAGEN in BaseSpace	59
Appendix V: Installing the GeoMx NGS Pipeline Software	62
Appendix VI: Connect GeoMx DSP to BaseSpace Workgroup	68
Troubleshooting	71
Final page	74

Changes in this revision

This **GeoMx DSP NGS Readout User Manual** (MAN-10153-01) replaces the GeoMx-NGS Library Prep User Manual (SEV-00088-05), the GeoMx-NGS Pipeline Software User Manual (SEV-00155-04), and the GeoMx NGS Pipeline on DRAGEN via BaseSpace User Manual (RPP-00535-02).

Other changes in this manual revision include:

- Added instructions to purify the NTC for comparison to pooled PCR library [on page 26](#)
- Added guidance for library preparation from standalone custom RNA-NGS assays [on page 28](#)
- Added examples from Bioanalyzer[®] and TapeStation[®] to section 'Assessing library quality and quantity' [on page 30](#)
- Defined four options for sequencing and pipeline processing configurations [on page 32](#)
- Added instructions to run streamlined workflow from NextSeq 1000/2000 to GeoMx NGS Pipeline on DRAGEN™ on BaseSpace[®] downstream analysis [on page 43](#)
- Added instructions to connect the GeoMx DSP to an Illumina[®] BaseSpace workgroup [on page 68](#) and to run the DRAGEN pipeline with integrated sequencing run planning on GeoMx DSP [on page 48](#)
- Updated Appendices I, II, III, and IV for clarity and to reflect best practices

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Conventions

The following conventions are used throughout this manual 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[®] MAX/FLEX, Pro, or SPRINT to read out GeoMx DSP counts.

GeoMx DSP Workflow

The GeoMx Digital Spatial Profiler (DSP) is a novel platform developed by NanoString. This product relies on antibody or nucleic acid probes 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 quantitated 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 ([see 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 and enter slide/study information. Slides are scanned to capture fluorescent images used to select regions of interest (ROIs). The instrument collects UV-cleaved oligos from the ROIs into the wells of a collection plate.

For NGS readout:

Day 3: Transfer the collected aspirates to a PCR plate and perform **Library Prep** with Seq 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 **NanoString's GeoMx NGS Pipeline** on Illumina DRAGEN[™] accessed via BaseSpace[™] Sequence Hub, or using GeoMx NGS Pipeline standalone software. Upload DCC files on to the GeoMx DSP.

For nCounter readout:

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 MAX/FLEX or Pro Analysis System or SPRINT Profiler.** Upload reporter count conversion (RCC) files to the GeoMx DSP.

- **Day 4 or 5:** Create a **Data Analysis** study in the Data Analysis suite and perform quality-control checks and data analysis, and generate analysis plots.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

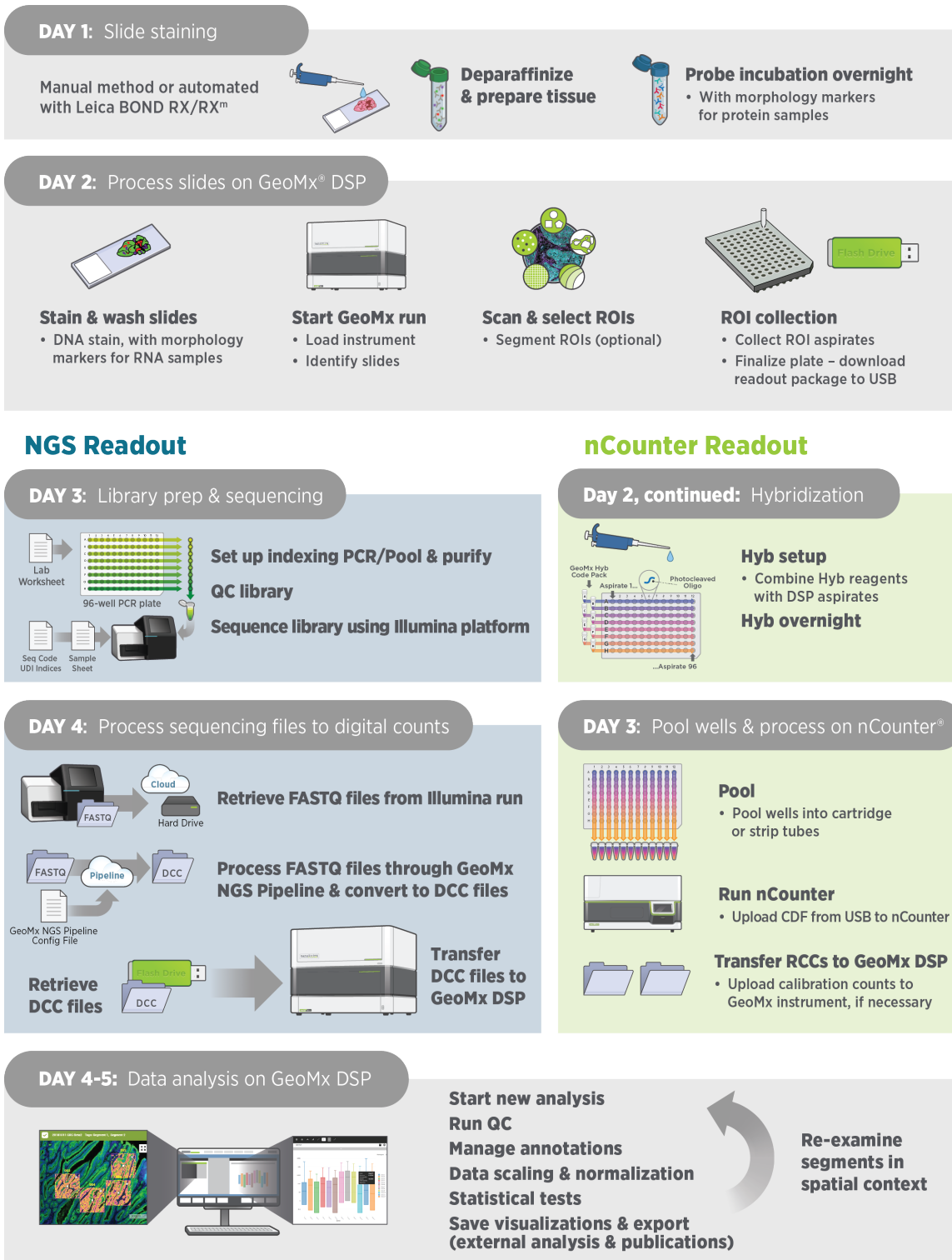


Figure 1: GeoMx DSP workflow summary

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

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	<p style="text-align: center;">For NGS readout: GeoMx DSP NGS Readout User Manual MAN-10153</p>	<p style="text-align: center;">For nCounter readout: GeoMx DSP nCounter Readout User Manual MAN-10089</p>
	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 available in NanoString University.

<p>For NGS readout: For documentation specific to the Illumina platform, see https://support.illumina.com.</p>	<p>For nCounter readout: For documentation specific to the nCounter Pro, MAX/FLEX, and SPRINT instruments, see https://www.nanostring.com/support/support-documentation/ or the NanoString University Document Library at https://university.nanostring.com.</p>
---	--

Introduction to GeoMx NGS Readout

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)** that identifies the biological target ([see Figure 2](#)). Each oligo also includes a **unique molecular identifier (UMI)** sequence, enabling removal of duplicated PCR products 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 primers** in PCR amplification.

During PCR amplification, the GeoMx Seq 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 to Illumina flow cells.

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

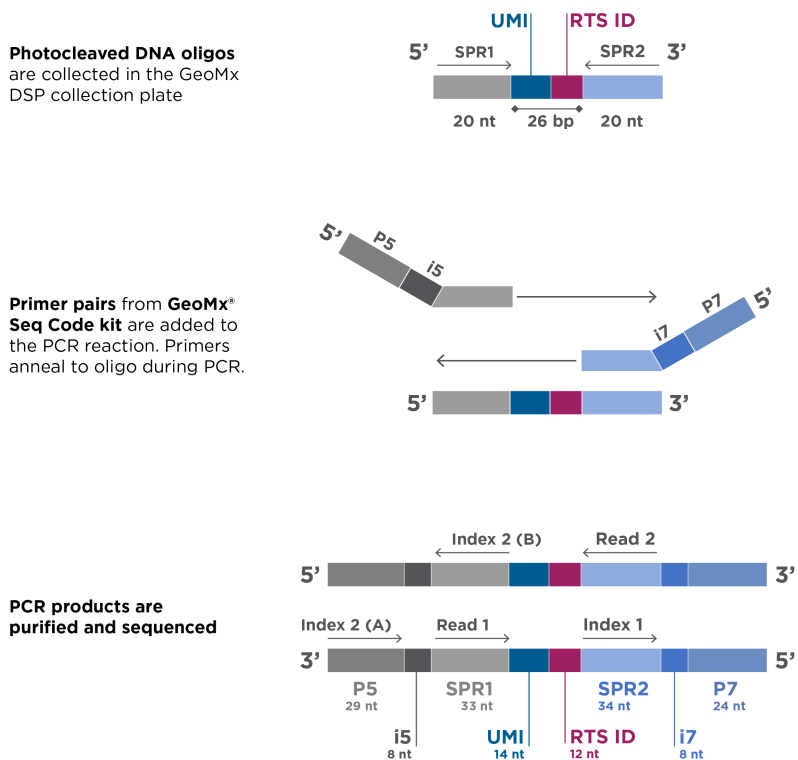


Figure 2: NGS PCR and sequencing diagram

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Introduction to GeoMx NGS Readout

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.

A single molecule in the sequencing library corresponds to one **cluster** on the flow cell. The **paired-end read** strategy recommended by NanoString results in two paired-end reads, forward and reverse, per cluster. This can also be described as **one read pair**.

Table 1. i5 Index Read Direction

i5 Sequence Orientation	Workflow	Illumina Platform
Forward	Forward Strand (Index 2 – Workflow A)	<ul style="list-style-type: none"> • HiSeq 2000/2500 • MiSeq • NovaSeq 6000 with v1.0 reagent kits
Forward	Reverse Complement (Index 2 – Workflow B)	<ul style="list-style-type: none"> • 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)	<ul style="list-style-type: none"> • 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)

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

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 ([see Figure 3](#)) illustrates the steps of the GeoMx NGS Pipeline.

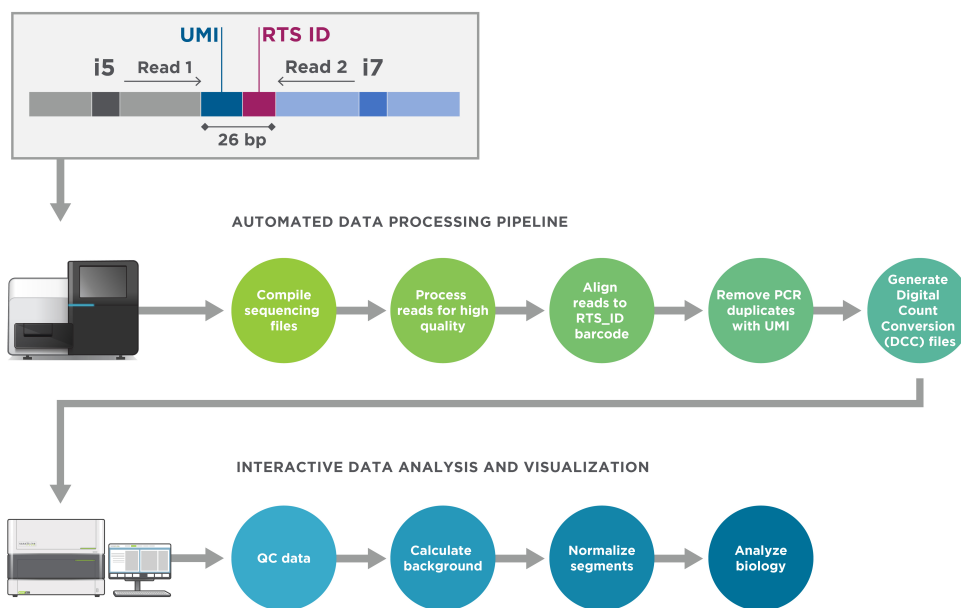


Figure 3: GeoMx NGS Pipeline

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 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 counts to biological target names. Then, PCR duplicates are removed by sorting based on the Unique Molecular Identifier (UMI) 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.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Introduction to GeoMx NGS Readout

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 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).
 - Cloud-based alternative to installing and running the standalone GeoMx NGS Pipeline software.
 - Ideal for users who want to start processing data quickly without IT infrastructure.
 - Requires a subscription to BaseSpace Sequence Hub. Free trial and low-cost subscriptions are available; see <https://support.illumina.com> for more information.

To evaluate the pipeline option that is best for you, refer to [Figure 10 on page 32](#) and the instructions for the different options (starting [on page 33](#)). Additional resources include the [GeoMx DSP IT Specifications and Requirements](#) guidelines, your NanoString Applications Scientist, and technical support from GeoMxSupport@nanosttring.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.

File Inputs and Outputs

File inputs and outputs are illustrated below ([see Figure 4](#)).

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 of the SeqCodeIndices.csv file to create a **SampleSheet.csv** for input to the Illumina sequencer. (NextSeq 1000/2000 users download a SampleSheet.csv and whitelist.txt instead of SeqCodeIndices.csv.)
- **LabWorksheet.txt**, an Excel spreadsheet to refer to in setting up the library.
- **Configuration (.ini) file** containing pipeline processing parameters.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

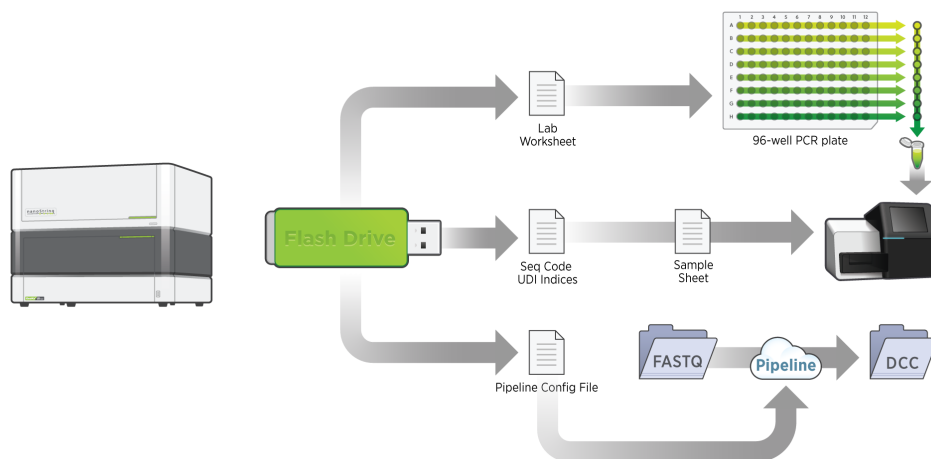


Figure 4: Output files from GeoMx run

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

The **file output from the Illumina NGS** run is the compressed FASTQ.gz file, which contains 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.

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 workflow used, the zipped folder of DCC files is .zip format or output.tar.gz format. The folder is uploaded to the GeoMx DSP instrument for analysis in the Data Analysis suite or export for analysis with your customized bioinformatics tools.

The GeoMx NGS Pipeline also generates a summary.txt file with number of read pairs for each pipeline processing step. The GeoMx NGS Pipeline on DRAGEN via BaseSpace Sequence Hub 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.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

GeoMx NGS Readout Equipment, Materials, and Reagents

The following equipment, materials, and reagents are recommended for this protocol but are **not supplied by NanoString**.

Equipment

Table 2: Equipment not provided by NanoString

Equipment	Manufacturer	Part Numbers
PCR set-up hood (or designated space)	Various	Various
Plate spinner/centrifuge with plate adapter (up to at least 2000 x g)	Various	Various
Qubit™ or similar device for library quantitation	Various, such as Thermo Fisher®	Various, such as Q33238
Vortex	Various	Various
Picofuge	Various	Various
Heated plate sealer and foil seals (alternative to adhesive foils)	Various	Various
Thermal cycler	Various, such as Bio-Rad®	Various, such as 1851197
Capillary electrophoresis device for nucleic acid analysis	Agilent Bioanalyzer® or TapeStation®	G2939BA or G2992AA
Illumina NGS instrument	Illumina	Various

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Equipment, Materials, Reagents**Materials**

Table 3: Materials not provided by NanoString


Materials	Manufacturer	Part Numbers
96-well PCR plates (RNase/DNase free) to match thermal cycler specs	Various	Various
Permeable membranes (dry-down seal)	Sigma®	A9224
96-well PCR plate adhesive foil (alternative to heated plate sealer and foil seals)	Various, such as VWR or Thermo Fisher	60941-076 AB0626
Microcentrifuge tubes (RNase/DNase free)	Various	Various
PCR strip tubes (12-tube or 8-tube strip, RNase/DNase free)	Various	Various
Two* 12-channel P20 multi-channel pipettes	Various	Various
Filter tips (RNase/DNase free)	Various	Various
Cooling block (optional)	Various	Various
Magnetic stand (e.g. DynaMag-2™ Magnet)	Thermo Fisher	12321D
RNase AWAY® or 10% Bleach (RNaseZap® is not a substitute)	Thermo Fisher	7002
DNA LoBind® tubes (for library storage before sequencing)	Various	Various

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

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Reagents

Table 4: Reagents not provided by NanoString

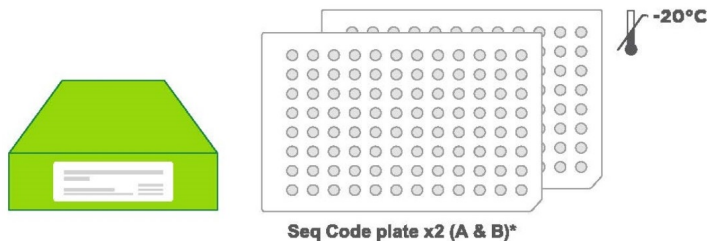
Reagents	Manufacturer	Part Numbers
100% ethanol 	Various	Various
Agencourt® AMPure XP®	Beckman Coulter®	A63880
Elution buffer (Tris-HCl 10 mM with 0.05% Tween-20, pH 8.0) *	Teknova®	T1485
Bioanalyzer DNA High Sensitivity Kit or TapeStation High Sensitivity DNA ScreenTape and reagents	Agilent	5067-4626 or 5067-5584 and 5067-5585
Reagents for Qubit or similar device for NGS library quantitation	Various, such as Thermo Fisher	Various, such as Q32851
PCR grade water	Various	Various

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

The following kits and reagents are available from NanoString.



Master Mix should be stored at -20°C (aliquoting not required).



Seq Code plates are available in packs of A & B, C & D, E & F, and G & H. Store at -20°C. Once thawed, store at 4°C up to 3 months, or freeze again. Seq Code plates can go through 3 freeze-thaw cycles.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

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 15](#) for details on file contents.

- **SeqCodeIndices.csv** (or SampleSheet.csv and whitelist.txt for NextSeq 1000/2000 users)
- **LabWorksheet.txt**
- **Configuration (.ini) file**

NextSeq 1000/2000 users that run the cloud integrated workflow (Option 4 [on page 48](#)) 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.

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

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.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Library Preparation

PCR setup (2 hours)

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

- Decontaminate working surfaces before and after use with 10% bleach (NaOCl), 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 multi-channel pipette.

The instructions here 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 for disinfection.
2. **Program a thermocycler** with a 100°C heated lid ([see 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	
Anneal	65°C	60 sec	18 X
Extend	68°C	30 sec	
Final extension	68°C	5 min	1 X
Hold	4°C	∞	1 X

3. **Keep 5X PCR Master Mix on ice** or cooling block during setup.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

4. Refer to the Lab Worksheet (see [Figure 5](#)) to choose the correct letter of **GeoMx Seq 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, 5X PCR Master Mix, and DSP collection plate to 1000 x g.

	A	B	C	D
1	Experiment Summary			
2	Readout group name	BigTest		
3	Date	11/7/2019 0:15		
4	Analyte	RNA		
5	Readout mode	NGS		
6	Number of Collection		4	
7	Number Of AOIs		288	
8	Library Prep Protocol Version			
9				
10	Library Prep Summary			
11	Library Prep Plate	Collection Plate	Primer Plate	
12	BigTest-B	1012348731122	GeoMx Seq Code B	
13	BigTest-D	1012238721122	GeoMx Seq Code D	
14	BigTest-E	1011221121122	GeoMx Seq Code E	
15	BigTest-H	1011223321222	GeoMx Seq Code H	
16				

Figure 5: Lab Worksheet

6. **Set up the PCR reactions in a new 96-well PCR plate** as in [Table 7](#) and [Figure 6](#). Ensure that the **primer plate**, **DSP collection plate** and **PCR plate** are all in the correct orientation (well A1 at upper left). Use of a multichannel pipette is recommended. Ensure that each component is added to the bottom of the well.
 - a. Aliquot **2 µL of 5X PCR Master Mix** to each well of the new PCR plate.
 - b. Transfer **4 µL of primer** from each well of the Seq Code Primer Plate to the corresponding well of the PCR plate. Use of a multichannel pipette is encouraged. Change pipette tips for every row/column.
 - c. Transfer **4 µL of DSP aspirate including the NTC** from each well of the DSP collection plate to the corresponding well of the PCR plate. Change pipette tips for every row/column.

Table 7: PCR reaction

Aliquot from 5X PCR Master Mix tube to PCR plate	Transfer from Seq Code plate to PCR plate	Transfer from DSP collection plate to PCR plate
2 µL 5X PCR Master Mix	4 µL Primer Mix	4 µL DSP aspirate

The final volume in each well of the PCR plate is 10 µL.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

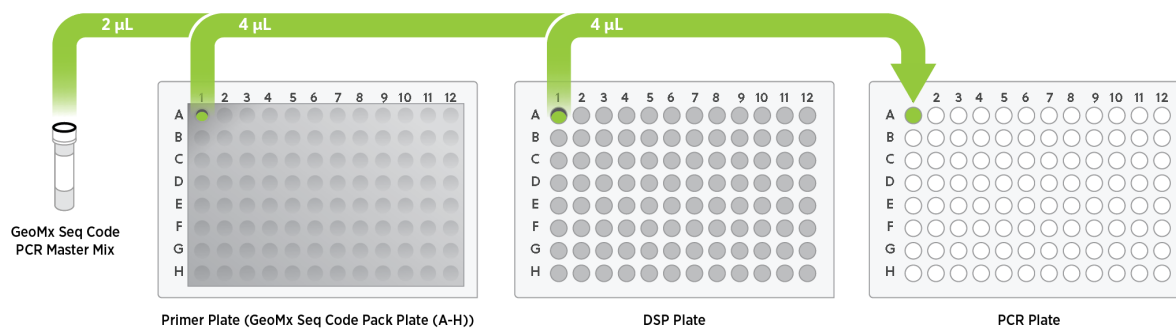
Library Preparation

Figure 6: PCR plate setup

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 ([see Table 6](#)).

After the PCR run is complete, the PCR plate may be stored at 4°C overnight. For longer storage, store at -20°C.

Instructions to set up a PCR positive control reaction are included in [Appendix I: PCR Positive Control on page 54](#). 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 29](#).

1. Once PCR is complete, **pulse centrifuge PCR plate** to 1000 x g.
2. Pool PCR products as follows ([see Figure 7](#)).

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 μ L 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 **NTC PCR product** (approximately 6 μ L) into its own 1.5 mL tube.
- c. **If you set up a PCR Positive Control**, pipette the 10 μ L PCR Positive Control to its own (i.e. a third) 1.5 mL tube.

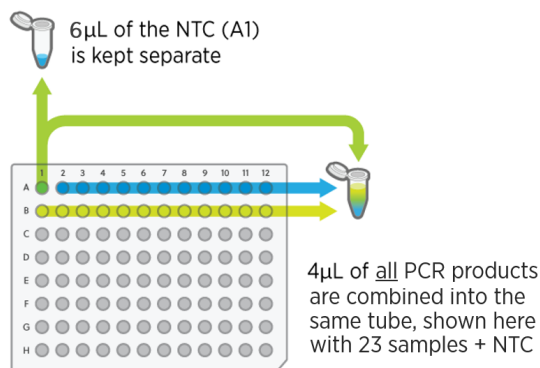


Figure 7: Pooling PCR reactions

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.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Library Preparation

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**, incubating 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 μ L Elution Buffer. Mix well** by pipetting.
17. **Pellet beads on a magnetic stand for 5 minutes** or until the solution is clear.
18. **Extract 50 μ L of supernatant** to a new tube. **Leave 2–4 μ L** of supernatant in tube, if necessary, to avoid disturbing beads. This supernatant contains the sequencing library.
19. **Add 60 μ L of Ampure XP beads** to the **50 μ L 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.
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.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Library Preparation

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. 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.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Library Preparation

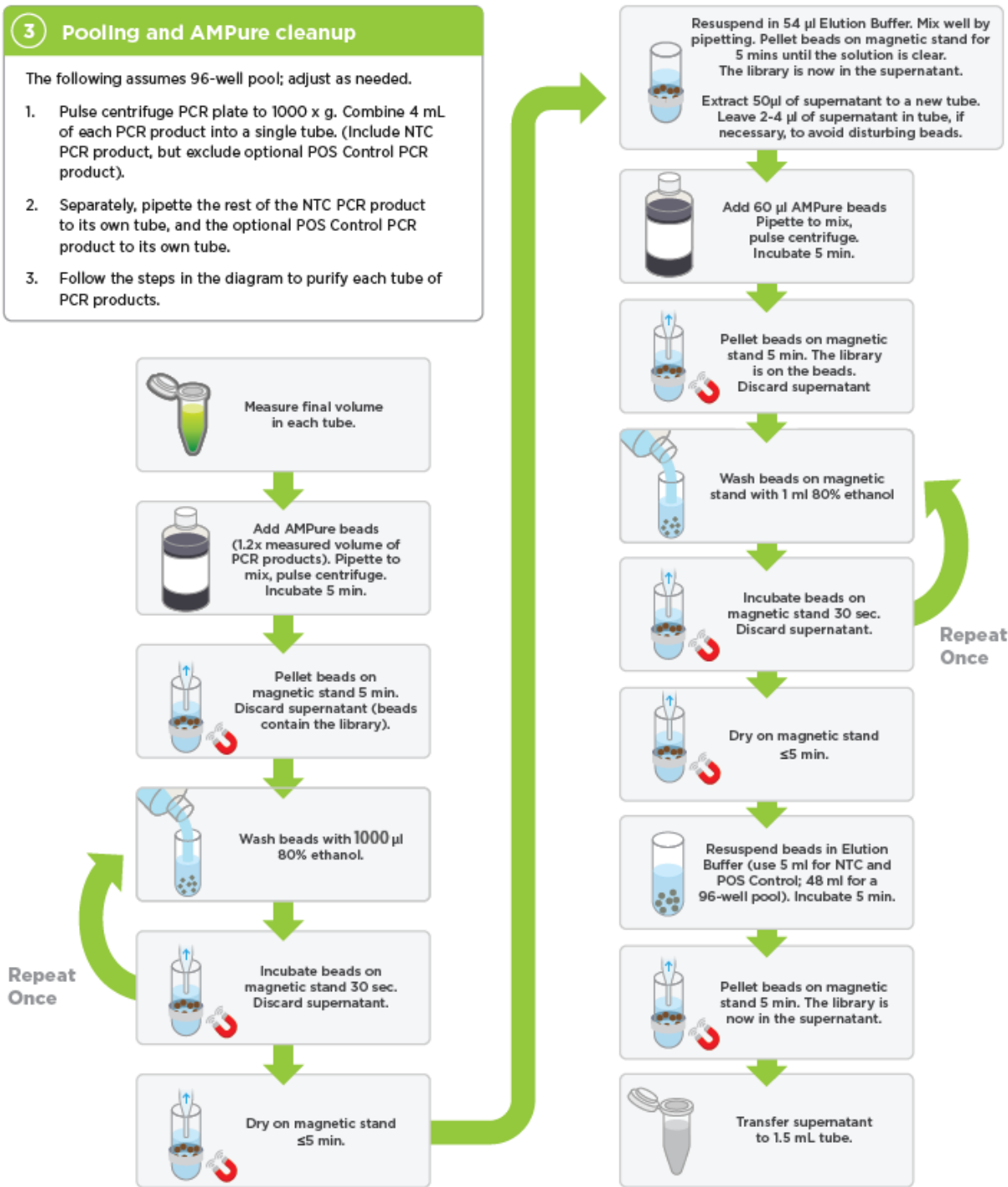


Figure 8: AMPure purification

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Assessing library quality and quantity

1. Combine 2 μL of library and 14 μL of Elution Buffer to create a 1:7 dilution. For a standalone Custom RNA-NGS Panel of low plex, prepare a 1:4 library dilution.
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 fragment analysis. 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 ([see Figure 9](#)):

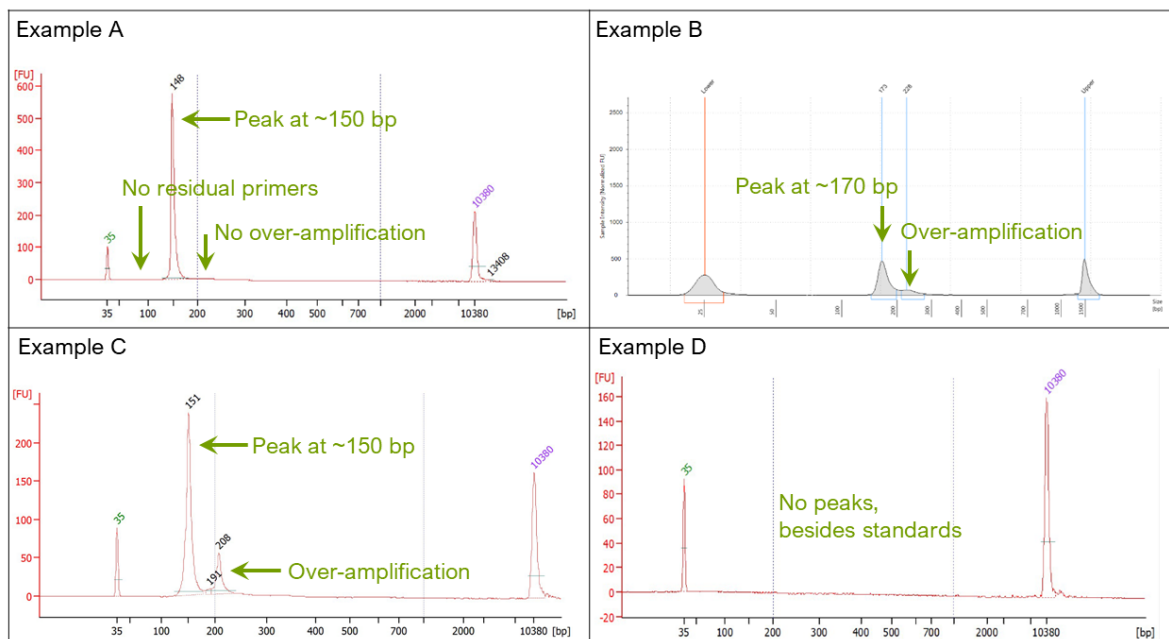


Figure 9: Library QC examples

- 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. Using the Bioanalyzer, the library will appear as ~150 bp. Using the TapeStation, the library will appear as ~170 bp.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Library Preparation

Verify that the concentration of the ~150 bp amplicon peak of either the undiluted library or the 1:7 dilution falls within the Bioanalyzer assay's quantitative range (5 – 500 pg/ μ L).

4. Assess quantity of library stock using Qubit or qPCR.
5. If combining more than one collection plate (Sample Pool) into one sequencing run (one Sequencing Pool), refer to [Appendix II: Strategies for Pooling before Sequencing on page 55](#).
6. Refer to Illumina recommendations for final loading concentration of the library, based on the sequencer and flow cell in use for your project. Some examples are listed in [Table 9](#). These values may be adjusted based on empirical testing. **Include 1-5% PhiX spike-in (by volume)**. Refer to [Appendix III: Sequencing Depth on page 57](#) for guidance in planning the sequencing depth.

Table 9: Suggested loading concentration per Illumina platform

Illumina platform	Flow cell	Final 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 (not Xp loading)

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Plan Your Sequencing and Pipeline Processing

Use the diagram in [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.

Plan your Sequencing and Pipeline Processing

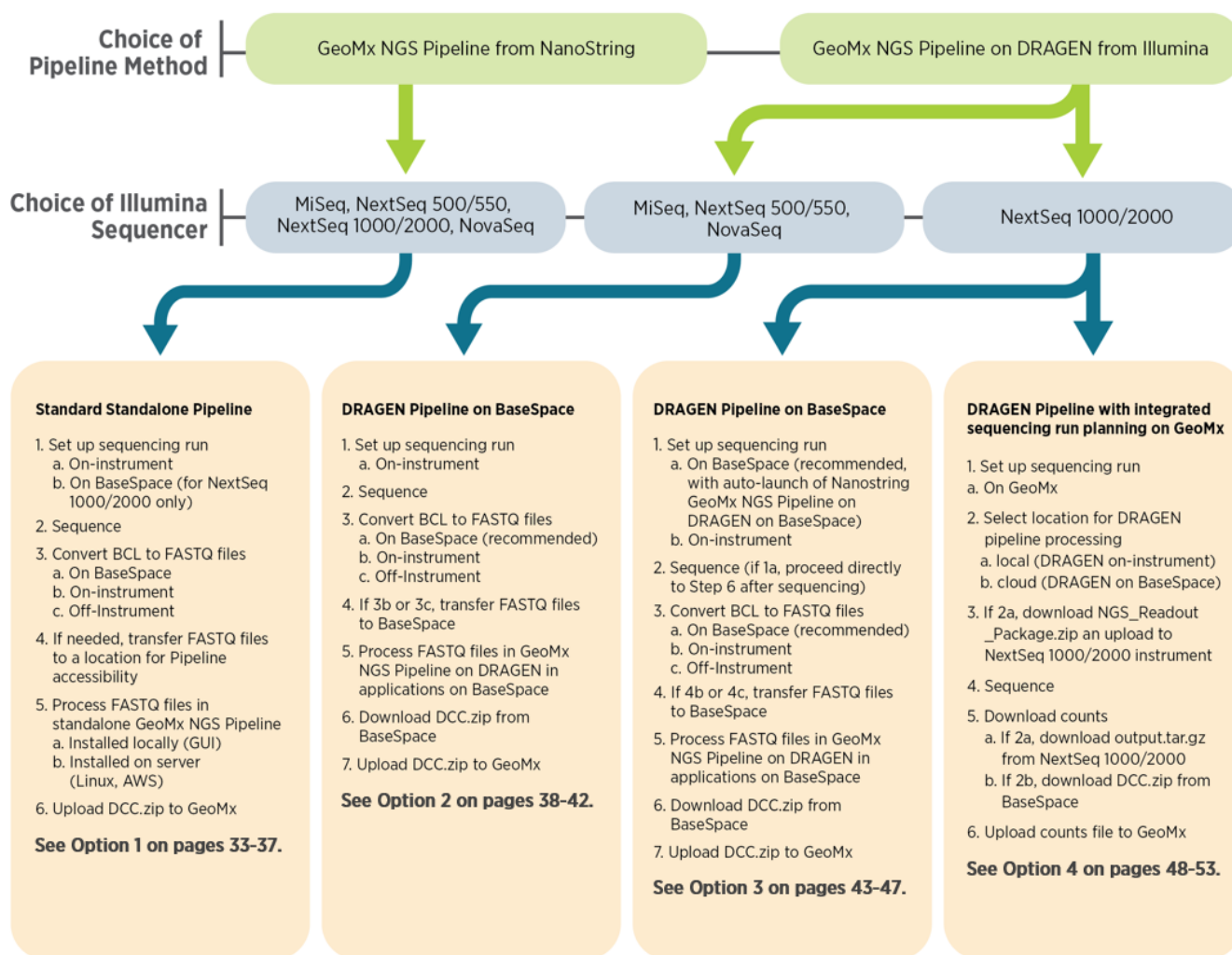


Figure 10: Plan your Sequencing and Pipeline Processing diagram

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Option 1. NanoString GeoMx NGS Pipeline

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 62](#).

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. (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, you may 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
- Index adapter kit: NanoString GeoMx Seq Code
- Index reads: 2 indexes
- Index mismatch: 1 (default)
- Read type: Paired-end
- Read length: Read 1, 27 bp; Read 2, 27bp
- Index 1 (i7) = 8 bp; Index 2 (i5) = 8 bp
- Generate FASTQ files (*may not be an option, depending on your platform*)

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.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Option 1. NanoString GeoMx NGS Pipeline

NextSeq 1000/2000 DRAGEN 3.8 and above no longer uses standard FASTQ names with lanes. To ensure compatibility with the GeoMx NGS Pipeline, please use DRAGEN 3.7.4 or below for BCL to FASTQ conversion.

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.

5. Process FASTQ files in the NanoString GeoMx NGS Pipeline**Running the pipeline using Graphical User Interface (GUI) on Windows or Mac**

1. Open the **GeoMx NGS Pipeline software**.
2. Select **Run locally** or **Run remotely**, depending on how you plan to run the pipeline. Review [Installation options on page 62](#) 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 available resources. To run to process 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** ([see Figure 11](#)).
4. **Browse** to your **Input directory** (the folder housing your Illumina FASTQ files in format fastq.gz).

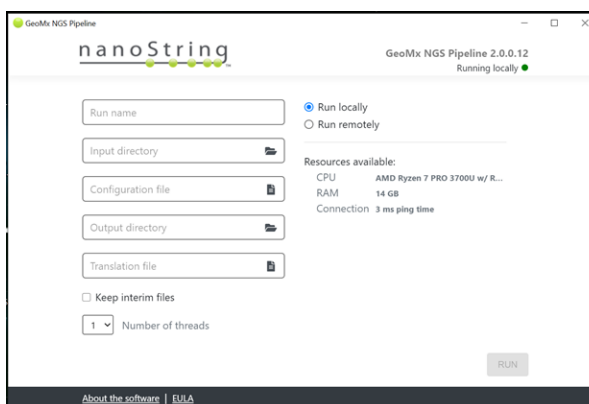


Figure 11: GeoMx NGS Pipeline run setup window

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Option 1. NanoString GeoMx NGS Pipeline

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 GeoMxSupport@nanosttring.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, 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 ([see 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 may proceed and the pipeline will generate “empty” DCC files (no counts for any probes) for the ROIs/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.

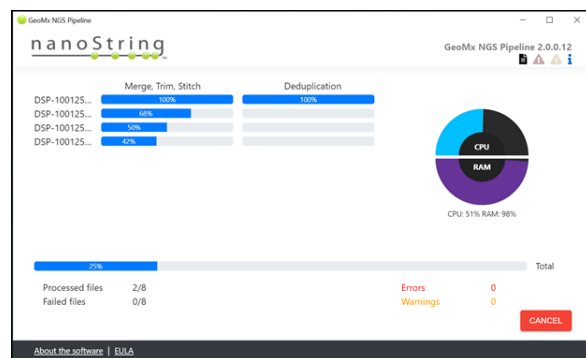


Figure 12: GeoMx NGS Pipeline run monitoring window

12. When the process is complete, the status bar will read 100%. Click **Done**.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Option 1. NanoString GeoMx NGS Pipeline

- Open the output folder and locate the zipped **DCC files** subfolder. Proceed to [6. Upload DCC.zip to GeoMx DSP on page 37.](#)



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

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.

- 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.

- Log in to the server. If you are processing on a remote server, you need to run the command from the server.
- 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.
- Copy configuration (.ini) and FASTQ files to server.
- 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 already have installed **GeoMxNGSPipelineAPI** on this server and you rerun the installation, you need to check the ownership and permissions of the `/var/tmp/.net/` subfolder. To do this, navigate 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]
```

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Option 1. NanoString GeoMx NGS Pipeline

- 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 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 are now re-uploading 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).

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

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. Confirm the sequencing details in the Sample Sheet. If corrections are needed, you may 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
- Index adapter kit: NanoString GeoMx Seq Code
- Index reads: 2 indexes
- Index mismatch: 1 (default)
- Read type: Paired-end
- Read length: Read 1, 27 bp; Read 2, 27bp
- Index 1 (i7) = 8 bp; Index 2 (i5) = 8 bp
- Generate FASTQ files (*may not be an option, depending on your platform*)

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.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Option 2. GeoMx NGS Pipeline on DRAGEN**4. Transfer FASTQ files to BaseSpace**

Skip this step if your FASTQ files were generated in BaseSpace. Otherwise, transfer FASTQ files using the BaseSpace Web Uploader (<https://help.basespace.illumina.com/articles/tutorials/upload-data-using-web-uploader/>) or command line interface (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 NGS Pipeline. 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 ([see Figure 13](#)).

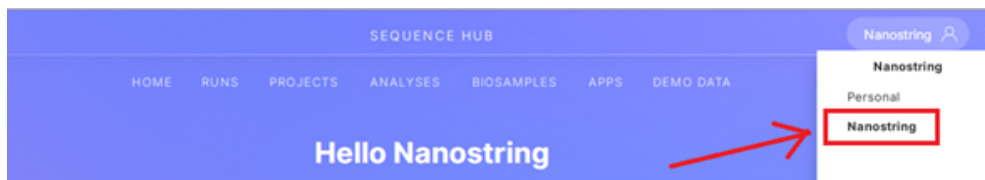


Figure 13: Selecting workgroup in BaseSpace

2. Select Apps then search for GeoMx and select GeoMx NGS Pipeline ([see Figure 14](#)).

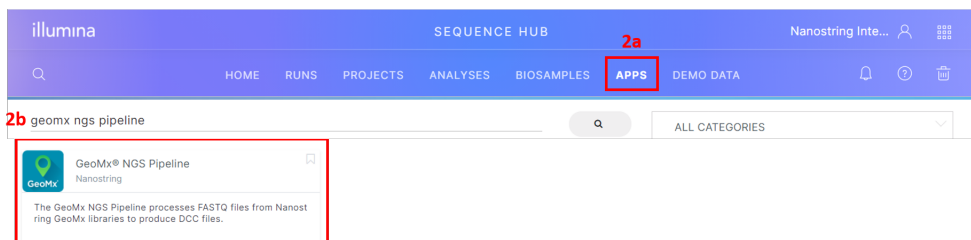


Figure 14: Find GeoMx NGS Pipeline application

3. Launch application ([see Figure 15](#)).

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

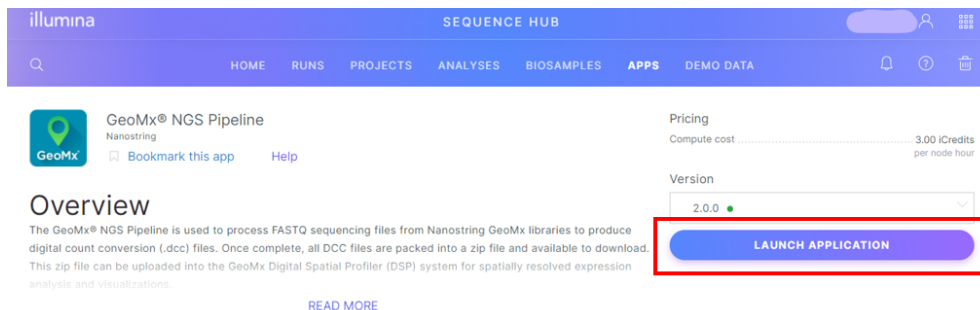


Figure 15: Launch application

4. Choose the **Start From: FASTQ files** option (see 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

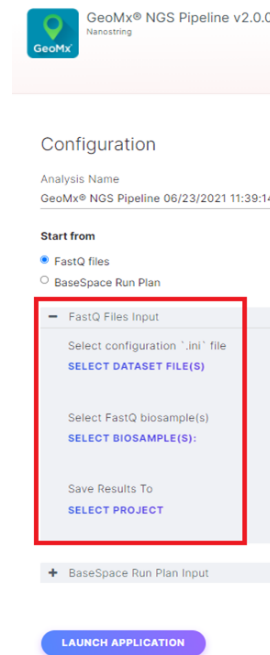


Figure 16: Choose file inputs

Option 2. GeoMx NGS Pipeline on DRAGEN

6. Download DCC.zip from BaseSpace

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

ANALYSIS NAME	APPLICATION	PROJECT	UPDATED	SIZE	COMMENTS	DELIVERY	STATUS
GeoMx NGS Pipeline [BETA] 10/20/2020 7:50...	GeoMx NGS Pipeli...	Margaret_dryrunte...	2020-10-20	105 MB		None	Complete
Instrument Uploader for ZZ244-ns2k-2	Instrument Upload...	ZZ244-ns2k-2_197...	2020-10-17	244 MB		None	Complete
GeoMx NGS Pipeline [TEST] 10/16/2020 10:13...	GeoMx NGS Pipeli...	ZZ243-miseq1	2020-10-16	24 MB		None	Complete
ZZ243_config	Imported v1.0.0	ZZ243-miseq1	2020-10-16	2 MB		None	Complete

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** (see [Figure 18](#)). (Despite its name, the folder "DCC_Files" is not the zipped DCC output for download).

NAME	CREATED	TYPE	PATH
< Back			
DCC_Files		Folder	DCC_Files
Dragen_Files		Folder	Dragen_Files
GeoMx_NGS_Pipeline_TEST_10_16_20...	2020-10-16 11:58	zip	
summary.txt	2020-10-16 11:58	txt	

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.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

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 are now re-uploading 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 38](#)).

1. Log into BaseSpace at <https://BaseSpace.illumina.com/dashboard>.
2. Select your preferred workgroup ([see Figure 19](#)).

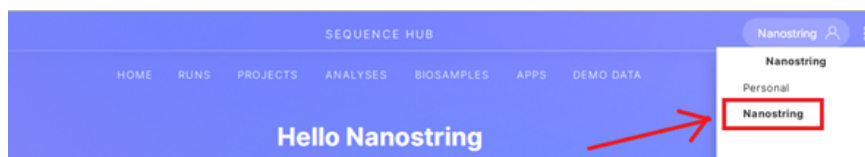


Figure 19: Select workgroup

3. Create a run by selecting **Runs** then **Instrument Run Setup** ([see Figure 20](#)).

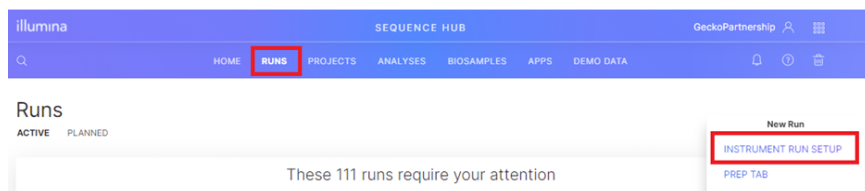


Figure 20: Instrument run setup

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: NanoString GeoMx NGS Pipeline – 2.0.0

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Option 3. NextSeq 1000/2000 + DRAGEN Pipeline

- Library prep kit: NanoString GeoMx Seq Code
 - Index adapter kit: NanoString GeoMx Seq Code
 - Index reads: 2 indexes
 - Read type: Paired-end
 - Read length: Read 1, 27 bp; Read 2, 27bp
 - Index 1 (i7) = 8 bp; Index 2 (i5) = 8 bp
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 ([see Figure 21](#)).

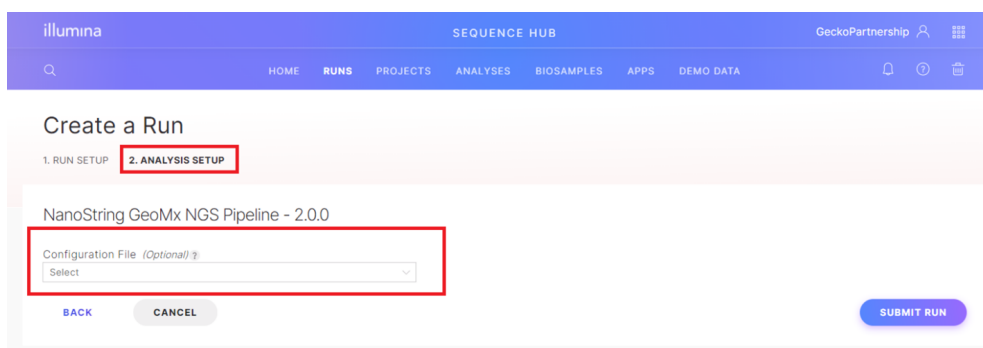


Figure 21: Upload configuration (.ini) file

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 38](#)).

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 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**.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Option 3. NextSeq 1000/2000 + DRAGEN Pipeline

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 38](#).

NextSeq 1000/2000 DRAGEN BCL Convert 3.8 and above no longer uses Illumina naming convention for FASTQ files and omits lane numbers. To ensure compatibility with the GeoMx NGS Pipeline, please use DRAGEN BCL Convert 3.7.4 or below.

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 39](#).

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 39](#).

6. Download DCC.zip from BaseSpace

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

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

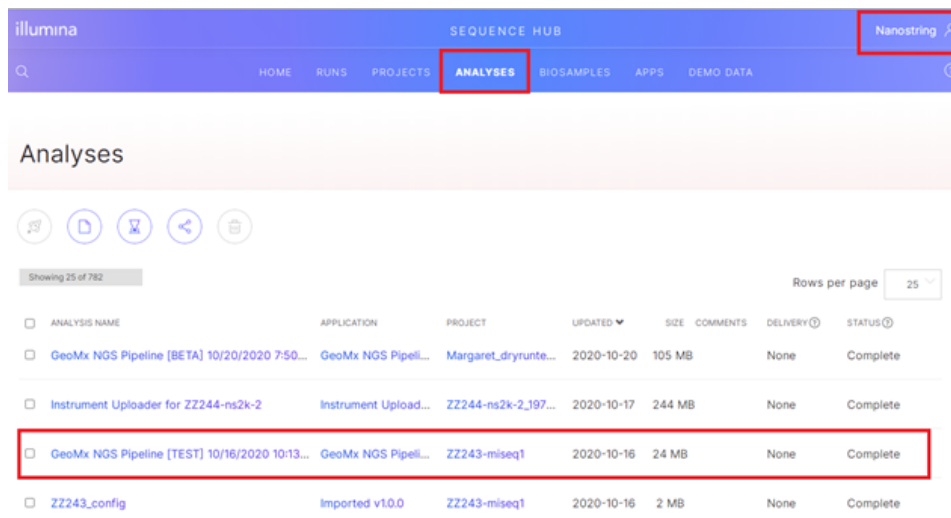


Figure 22: Analyses in BaseSpace

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

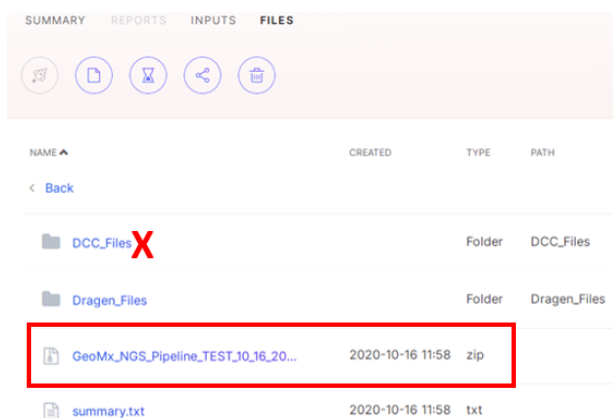


Figure 23: DCC.zip file output from GeoMx NGS Pipeline



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

- In the GeoMx DSP Control Center, hover over the Data Collection button and select **Upload Counts**.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Option 3. NextSeq 1000/2000 + DRAGEN Pipeline

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 are now re-uploading 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)). Users have the choice of the **local** or **cloud** integrated workflows:

- 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.
- 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 68](#).
 - On the NextSeq 1000/2000, go to **Settings** and select **Online Run Setup** and **Proactive, Run Monitoring, and Storage**. Save settings.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Option 4. NextSeq 1000/2000 Integrated Run Plan**1. Set up sequencing run from the GeoMx DSP instrument**

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

Readout Group Information

Search by:

Readout Group: 20211221_CWW Counting Device Model:

Counting Platform: Illumina Read Strategy: Single Paired

Read Length 1:

Read Length 2:

i5 Sequence Orientation: Forward Reverse

Illumina BaseSpace Account:

Readout Group Information by plate:

Plate Barcode	Plate Rows	Date Collected	Wells	GeoMx Seq Code
1001250003340	A - G	12/22/2021	81	A

Figure 24: Finalize Readout Group window

Option 4. NextSeq 1000/2000 Integrated Run Plan

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 ([see Figure 24](#)), click **Send to BaseSpace**. Proceed to Step 4 Sequence.

3. Download readout package

From the **Finalize Readout Group** window ([see 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 BaseSpace 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).

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Option 4. NextSeq 1000/2000 Integrated Run Plan**• Cloud integrated workflow:**

1. Log into BaseSpace and select 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.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Option 4. NextSeq 1000/2000 Integrated Run Plan**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.

• **Cloud integrated workflow:**

1. When the run is complete, log into BaseSpace, navigate to the workgroup, and select the **Analyses** tab (see [Figure 25](#)).
2. Click on the analysis of interest (see [Figure 25](#)).

The screenshot shows the BaseSpace interface. The top navigation bar includes 'HOME', 'RUNS', 'PROJECTS', 'ANALYSES', 'BIOSAMPLES', 'APPS', and 'DEMO DATA'. The 'ANALYSES' tab is selected and highlighted. Below the navigation bar, there are several icons for file management. A table titled 'Analyses' displays a list of analyses. The table has columns for 'ANALYSIS NAME', 'APPLICATION', 'PROJECT', 'UPDATED', 'SIZE', 'COMMENTS', 'DELIVERY', and 'STATUS'. The row for 'GeoMx NGS Pipeline [TEST] 10/16/2020 10:13...' is highlighted with a red box.

ANALYSIS NAME	APPLICATION	PROJECT	UPDATED	SIZE	COMMENTS	DELIVERY	STATUS
GeoMx NGS Pipeline [BETA] 10/20/2020 7:50...	GeoMx NGS Pipeli...	Margaret_dryrunte...	2020-10-20	105 MB		None	Complete
Instrument Uploader for ZZ244-ns2k-2	Instrument Upload...	ZZ244-ns2k-2_197...	2020-10-17	244 MB		None	Complete
GeoMx NGS Pipeline [TEST] 10/16/2020 10:13...	GeoMx NGS Pipeli...	ZZ243-miseq1	2020-10-16	24 MB		None	Complete
ZZ243_config	Imported v1.0.0	ZZ243-miseq1	2020-10-16	2 MB		None	Complete

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** (see [Figure 26](#)). (Despite its name, the folder "DCC_Files" is not the zipped DCC output for download).

Option 4. NextSeq 1000/2000 Integrated Run Plan

NAME	CREATED	TYPE	PATH
< Back			
DCC_Files		Folder	DCC_Files
Dragen_Files		Folder	Dragen_Files
GeoMx_NGS_Pipeline_TEST_10_16_20...	2020-10-16 11:58	zip	
summary.txt	2020-10-16 11:58	txt	

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 are now re-uploading 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).

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

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 a dilution series of the RNA-NGS or Protein-NGS probe pool as directed below. Prepare the positive control in the slide preparation area to avoid cross-contamination.

- Dilute probe pool through a dilution series:
 - Dilution 1: Take 5 μL of stock probe pool and add to 495 μL of Elution Buffer
 - Dilution 2. Take 5 μL of Dilution 1 and add to 495 μL of Elution Buffer
 - Dilution 3. Take 5 μL of Dilution 2 and add to 495 μL of Elution Buffer
 - Dilution 4. Take 2.5 μL of Dilution 3 and add to 497.5 μL of Elution Buffer
- Use 4 μL of Dilution 4 as the PCR template (instead of DSP aspirate) in a PCR positive control reaction.
- Take the PCR product (10 μL) and combine with 12 μL AMPure beads in the library cleanup workflow, alongside 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.
- We recommend all samples from a single collection plate be equivolume-pooled into a single Sample Pool, irrespective of segment size (see [Figure 7 on page 26](#)).
- 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 in Column H 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 loading concentration for the Illumina sequencer and flow cell in use for the project. Include 1-5% PhiX spike-in.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

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%	
	<i>Sum = total area of Sequencing Pool = 10,000,000 μm^2</i>	<i>Sum = 100%</i>	

- If you need additional support to plan your library pooling strategy and sequencing, please contact GeoMxSupport@nanosttring.com or your Field Applications Scientist or Product Applications Scientist before your sequencing run.

Appendix III: Sequencing Depth**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.
- Recommended sequencing depths (number of sequenced read pairs) for different GeoMx applications are listed in [Table 11](#).

Table 11: Read pairs recommended for different GeoMx NGS panels

Type	Assay	Sequencing Depth Factor	Calculation
RNA	Cancer Transcriptome Atlas (CTA)	30	Number of sequenced read pairs = total collection area (μm^2) x Sequencing Depth Factor
RNA	Whole Transcriptome Atlas (WTA)	100	Number of sequenced read pairs = total collection area (μm^2) x Sequencing Depth Factor
RNA	Standalone Custom RNA-NGS Panel with number of targets equal to n	0.05	Number of sequenced read pairs = total collection area (μm^2) x Sequencing Depth Factor x n
Protein	Protein-NGS Panel with number of targets equal to n	2	Number of sequenced read pairs = total collection area (μm^2) x Sequencing Depth Factor x n

- 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.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Appendix III: 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.

Appendix IV: Requeue NextSeq 1000/2000 run**Appendix IV: Requeue NextSeq 1000/2000 Run with New Sample Sheet using GeoMx NGS Pipeline on DRAGEN in BaseSpace**

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 (see [Figure 27](#)).

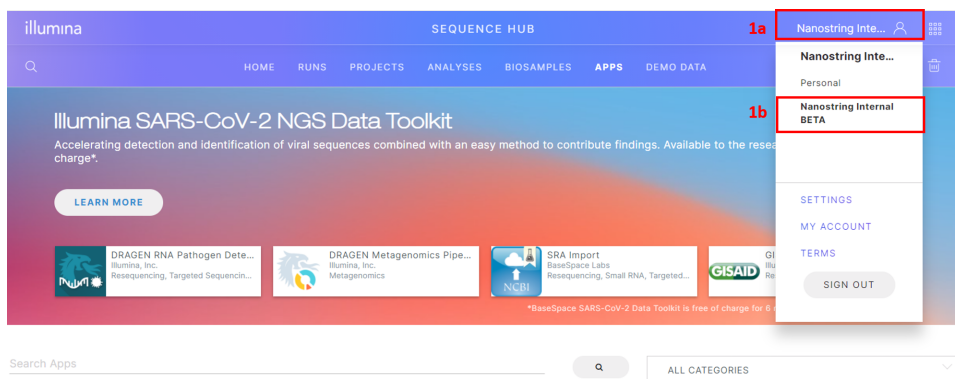


Figure 27: Select workgroup

2. Select “Apps”, search for “GeoMx”, and select “GeoMx NGS Pipeline” (see [Figure 28](#)).

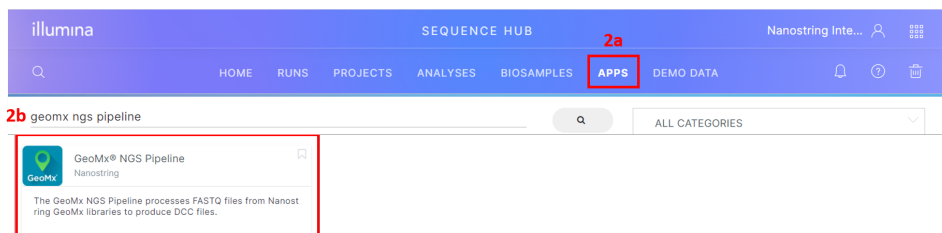


Figure 28: Locate GeoMx NGS Pipeline app

3. Launch application (see [Figure 29](#)).

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

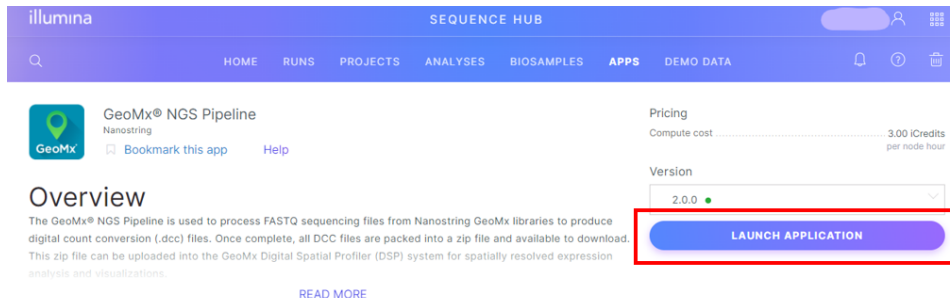


Figure 29: Launch GeoMx NGS Pipeline application

4. For **Start From**, choose **BaseSpace Run Plan**, and enter only the input files associated with BaseSpace Run Plan (see Figure 30).
5. Under **Select BaseSpace Run**, choose the run to requeue, and select an override sample sheet and/or an override configuration file.

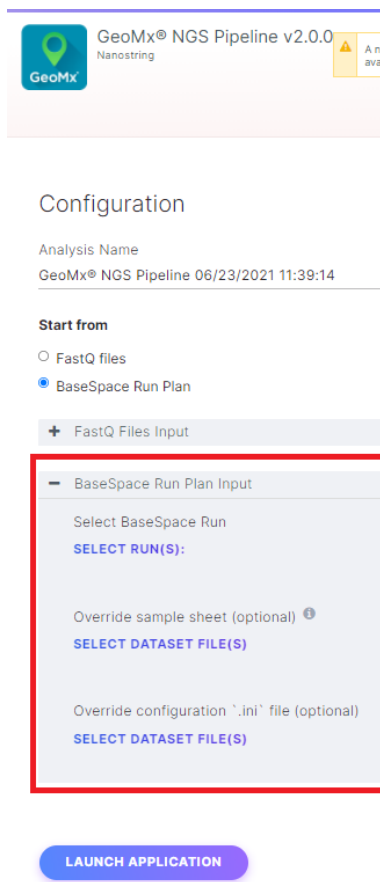


Figure 30: Start From option

6. Click **Launch Application**.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Appendix IV: Requeue NextSeq 1000/2000 run

7. Check run output by logging into BaseSpace, navigating to Workgroup, and selecting the **Analyses** tab (see [Figure 31](#)).

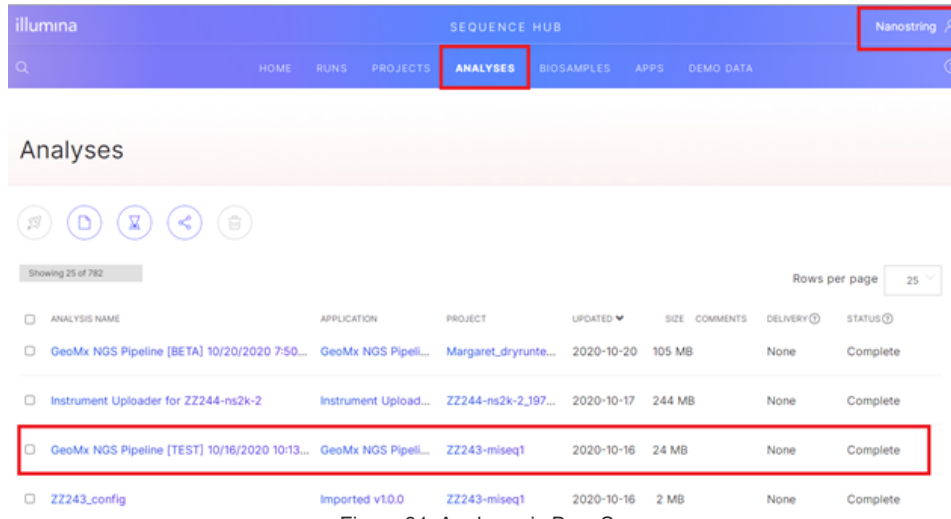


Figure 31: Analyses in BaseSpace

8. Within **Analyses**, open folder **GeoMx_NGS_Pipeline**. Download the DCC.zip output with the **name:GeoMx_NGS_Pipeline_...** and **file type: zip** (see [Figure 32](#)). (Despite its name, the folder "DCC_Files" is not the zipped DCC output for download).

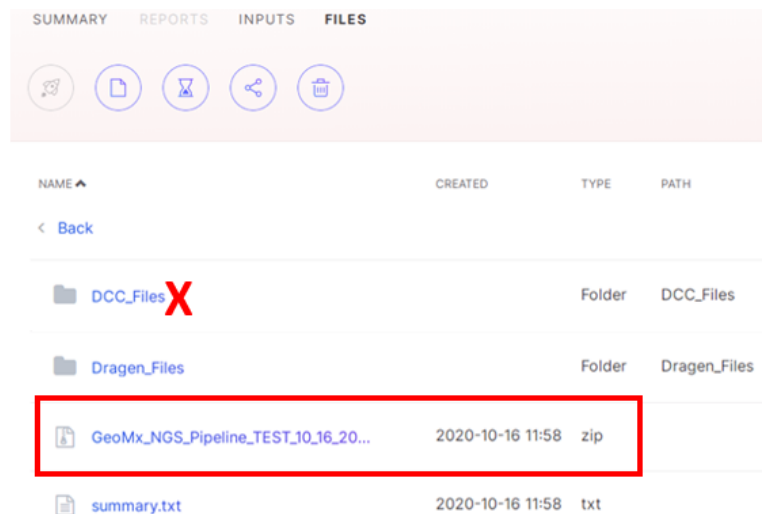


Figure 32: DCC.zip file output from GeoMx NGS Pipeline



IMPORTANT: Check DCC file sizes and summary.txt to ensure files were processed as expected.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Appendix V: Installing the GeoMx NGS Pipeline Software

This section does not apply to users of the GeoMx NGS Pipeline on DRAGEN via Illumina BaseSpace Sequence Hub.

Installation options

The GeoMx NGS Pipeline software can be run on a **Linux** or **Amazon Web Services® (AWS)** server, or locally (only for very small studies). To interact with the GeoMx NGS Pipeline Software, users have two options: a **graphical user interface (GUI)** or a **command line interface (CLI)**.

[Table 12](#) lists possible installation combinations:

Table 12: GeoMx NGS Pipeline software installation combinations

Operating System	User Interface	Pipeline location
Windows®	GUI	Local
		Server (Linux/AWS)
MacOS®	GUI	Local
		Server (Linux/AWS)
Linux®	CLI	Server (Linux/AWS)

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 [Installing on Linux or AWS server on page 65](#).
- 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.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Appendix V: Installing Pipeline Software**Features of Windows/MacOS GUI connected to Linux server**

- Connecting to a Linux server using the Windows or MacOS 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. Users also need the GeoMx NGS Pipeline installed on their computers.

Features of CLI control of remote 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 or if you have a fast CPU and a lot of RAM, you can use the Windows, Mac 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.

System requirements

Local and remote runs	Interface	Pipeline location	OS	CPU	Memory (GB)
Windows	GUI	Local or server (Linux/AWS)	Windows 10	Intel® Core™ i5-4750 3.20 GHz	16
MacOS	GUI	Local or server (Linux/AWS)	MacOS Catalina® V.10.15.5	Intel Core i5 2.60 GHz	16
Linux	CLI	Server (Linux/AWS)	Linux Ubuntu® 18.04	AMD Phenom 8650 2.30 MHz	16
AWS instance type: AWS t2.xlarge	CLI	Server (AWS)	AWS Ubuntu	vCPU 4	16

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

If running locally (small experiments):

The specifications below reflect the needs of a pipeline run and may be impacted by other programs running on the same machine. These resources are adequate for runs containing up to 96 segments/ROIs (with up to ~50 million reads per segment/ROI).

For MacOS:

- Macbook Pro® with a CPU at 1.4 GHz processor or better
- 16 GB RAM

For Windows:

- Intel Core i5-8350U @ 1.70GHZ 1.90 GHz
- 16 GB RAM
- 64-bit Operating System, x64-based processor

If running GeoMx NGS Pipeline on a Linux server or AWS instance (larger experiments):

The GeoMx NGS Pipeline input files should be available on the server. The specifications below reflect needs of a pipeline run and may be impacted by other programs running on the same server. These resources are adequate for runs containing segments/ROIs exceeding ~50 million reads per segment/ROI.

Linux server:

- OS: Ubuntu 18 and up
- 16 GB RAM
- Adequate storage for data files via EBS, EFS or attached NAS. NanoString recommends at least 2 GB RAM per thread and at least 1 GB of available free memory.

Installing the pipeline

Installing Locally on Windows/Mac/Linux

1. **Download** the GeoMx NGS Pipeline Software installation file from <https://nanosttring.com/products/geomx-digital-spatial-profiler/software-updates/>.
2. Right-click on the installation file and select **Extract Here**.
3. Double-click on the resulting **installer application**.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Appendix V: Installing Pipeline Software

- 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.
 - If you plan on running the Pipeline on your local computer, check the box **GeoMxNGSPipeline Local Server**.
4. Once the GeoMx NGS Pipeline software has been installed, **open the application**.
 5. (Optional) If you plan on using a **remote server** for processing, you will first need to **install the GeoMx NGS Pipeline on your server**. See next section.

- Once installed, add the server in the UI by clicking **new server**, entering the Public IPv4 address of the server (four integers separated by periods followed by **:5000**), then clicking **Add** (see [Figure 33](#)).

- Enter **API server address**, including port (insert your server name in lieu of the red text): `http:// <remote server address>:5000`

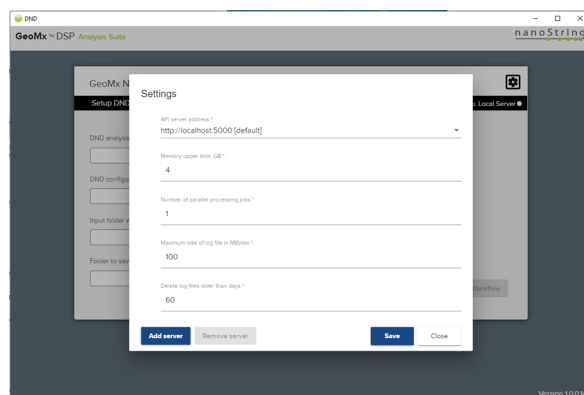


Figure 33: Adding a server

- 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).
- 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 and MacOS 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 GeoMxSupport@nanosttring.com for support.

Installing 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.nanosttring.com/geomx-ngs-pipeline-software-on-aws>.

Installation comes as zip archive which contains the following files:

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Appendix V: Installing Pipeline Software

- GeoMxNGSPipeline_Linux_2.0.0.15.sh (or similar) – this is the installation script
- GeoMxNGSPipeline.tgz – this is the installation package file

During the installation you will need to 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** (Ubuntu) distributive as well as **sudo** user privileges on that server.

1. Use secure copy protocol (SCP) to **copy installation files** on the server. You can use WinSCP or similar software. **Unzip the installation archive** and use SCP (WinSCP) to **copy** *GeoMxNGSPipeline_Linux_2.0.0.15.sh* (or similar) 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_2.0.0.15.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_2.0.0.15.sh`. This will add execute **x** permission and the permission set will look like: `rw-rw-r-x`.
4. **Run the installation script:** `sudo ./GeoMxNGSPipeline_Linux_2.0.0.15.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.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Appendix V: Installing Pipeline Software



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 (optional). This step is required only 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 you connect to.
 - 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 need to edit the `runtimeSettings.xml` file. By default, this file has mapping for home folder (`/home`). You will need to 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.

Also 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. Hit F2 to save your edits. You don't need to restart API server. The changes in `runtime-settings.xml` will be processed automatically.

7. Finally, connect to newly installed API instance using GeoMx NGS Pipeline GUI.

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 ([see Figure 34](#)), 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 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

The screenshot shows a 'Create New Account' pop-up window. It has a dark header with the title 'Create New Account'. Below the header, there are several input fields and a button. The first field is 'Account Name' with the value 'Workgroup2'. The second field is 'BaseSpace Region' with a dropdown menu showing 'USA (N. Virginia)'. Below these fields is a large blue button labeled 'Connect to BaseSpace'. The next field is 'Illumina Platform API Key' with the value 'AbcdefgHIJKlmnopQRSTUVWXYZ'. The fourth field is 'BaseSpace Workgroup ID' with the value '1234abcd-12ab-34cd-123456abcdef'. The fifth field is 'DRAGEN Run Type' with a dropdown menu showing 'NextSeq 1000//2000 Cloud'. At the bottom right of the form are two buttons: 'Save' and 'Cancel'.

Figure 34: Illumina BaseSpace Admin pop-up

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Appendix VI: Connect GeoMx to BaseSpace

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.
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> (see **Figure 35**). (If your BaseSpace account is part of an Enterprise subscription, navigate to [https://\[YourEnterpriseDomain\].login.illumina.com/platform-home/#/apiKey/list](https://[YourEnterpriseDomain].login.illumina.com/platform-home/#/apiKey/list)).

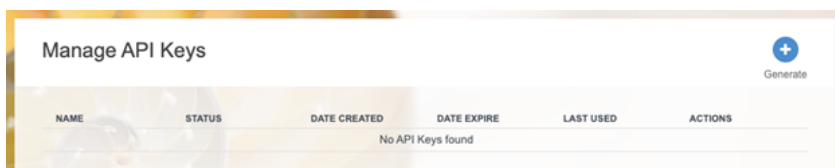


Figure 35: API Key List dialog

- Click Generate to open the Generate an API Key dialog (see **Figure 36**).

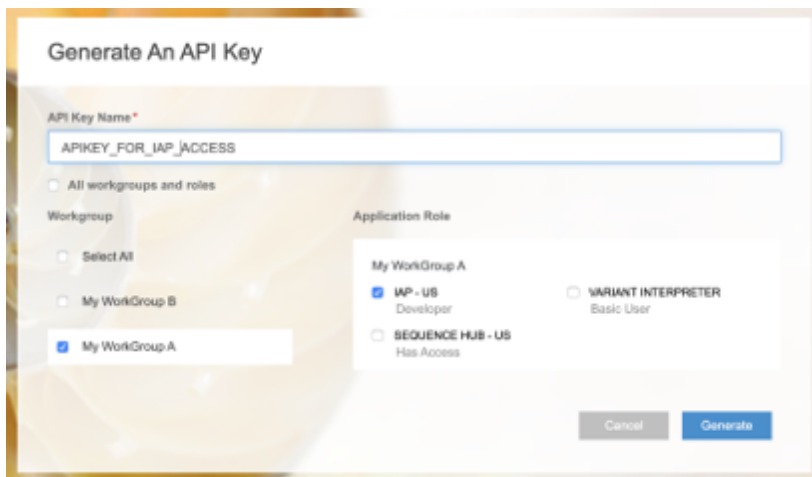


Figure 36: Generate an API Key dialog

- 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.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

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](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 (**see Figure 37**). Enter this alphanumeric code in the BaseSpace Workgroup ID field of the GeoMx DSP Control Center.

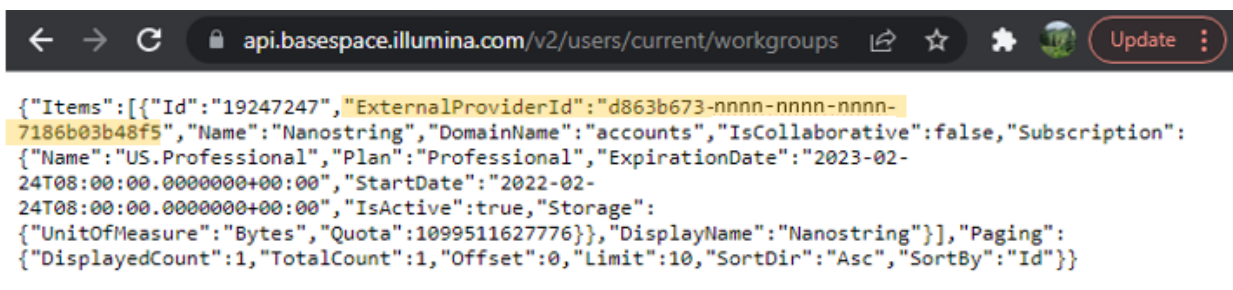


Figure 37: ExternalProviderID

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.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Troubleshooting

Suggested actions to resolve certain issues are listed below ([see Table 13](#)). This list is not comprehensive of all possible causes and potential 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 GeoMxSupport@nanosttring.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 23 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 54 .
Undesired peaks observed in NGS library BioAnalyzer or TapeStation trace (see examples on page 30)	Overamplification in PCR	In many cases it is still worth sequencing the library as long as its concentration can be accurately measured.
	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 17 and PCR best practices on page 23 .
	Incorrect PCR cycle settings	Ensure that the protocol is followed as written, with 18 cycles of PCR amplification on page 23 .

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Troubleshooting

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. 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.
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 68 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.
BCL to FASTQ generation yields no FASTQs or low number of reads in each FASTQ	i5 sequence was inadvertently read forward when it should have been reverse complement, or vice versa	<ul style="list-style-type: none"> • 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 System requirements on page 63 . Ensure the output file location has sufficient space to hold output files.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

Issue	Possible cause	Suggested action
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 34). These files are typically needed only in certain troubleshooting situations.
GeoMx NGS Pipeline software does not recognize FASTQ files	FASTQ file format does not match expected format	<p>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.</p> <p><u>DSP-1001250001985-A-A02_S2_L001_R1_001.fastq.gz</u></p> <p>DSP-1001250001985-A-A02 = Sample ID matching Configuration file, SeqCodeIndices.csv, or Sample ID Translator File.</p> <p>S2 = Sample sheet number</p> <p>L001 = Lane number</p> <p>R1 = forward or reverse, read 1 or 2</p> <p>001 = Should always be 001</p> <p>.fastq.gz = Indicates a compressed file</p>
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 GeoMxSupport@nanosttring.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.

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.



NanoString Technologies, Inc.
530 Fairview Ave North
Seattle, Washington 98109 USA
www.nanostring.com

CONTACT US
info@nanostring.com
Tel: +1 888 358 6266
Fax: +1 206 378 6288

SALES CONTACTS
United States: us.sales@nanostring.com
EMEA: europe.sales@nanostring.com
Asia Pacific & Japan: apac.sales@nanostring.com
Other regions: info@nanostring.com

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.