GeoMx® DSP
nCounter® Readout
User Manual

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nanoString®

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Table of Contents

Changes in this revision

- Edited text, figures, and structure for clarity
- Updated items and links in Equipment, Materials, and Reagents lists **on page 13** and **25**
- Updated text to include nCounter® Pro Analysis System as an option for readout platform
- Added a section on Troubleshooting **on page 39**

Conventions

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

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

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

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

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

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

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

For nCounter readout: Content in green boxes denotes steps or information specific to nCounter readout of GeoMx DSP. Follow these instructions if using nCounter® 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.

GeoMx DSP Workflow

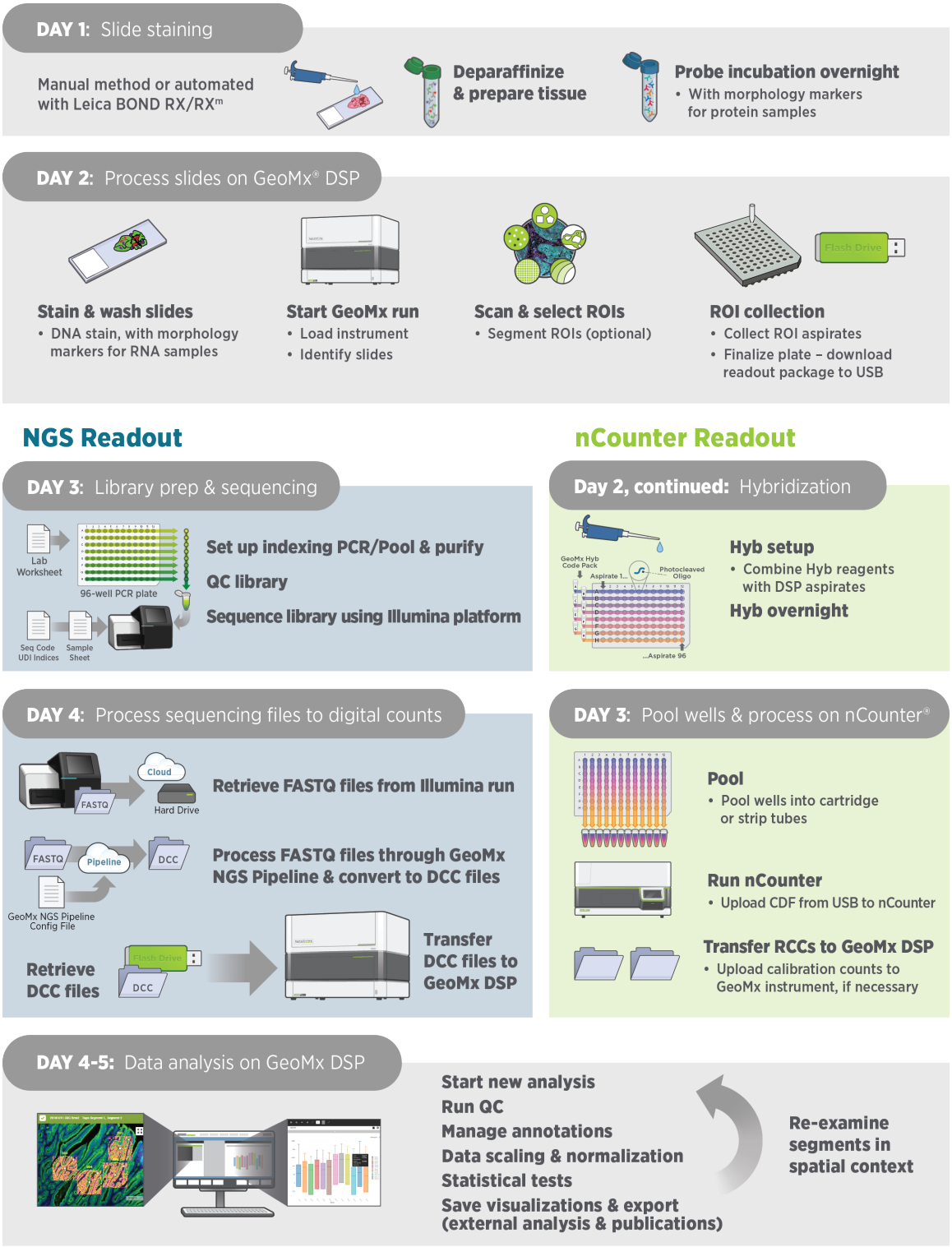

Figure 1: GeoMx DSP workflow summary

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User Manuals and Resources

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

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

User manuals and other documents can be found online in the NanoString University Document Library at https://university.nanostring.com.

Instrument and workflow training courses are available in NanoString University.

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

Introduction to nCounter Readout

GeoMx DSP assays with nCounter readout use nCounter molecular barcoding to quantify gene and protein expression in spatial context. Aspirates containing UV-cleaved oligos that correspond to a specific target are collected by the GeoMx DSP instrument. The collection plate is removed from the instrument and prepared for counting on an nCounter platform **(see Figure 2)**. In addition, the plate is finalized in the GeoMx DSP software generating a **lab worksheet** and **cartridge definition file (CDF)**, which are needed for sample preparation and readout. For more information about finalizing the plate, see the GeoMx DSP Instrument User Manual (MAN-10152).

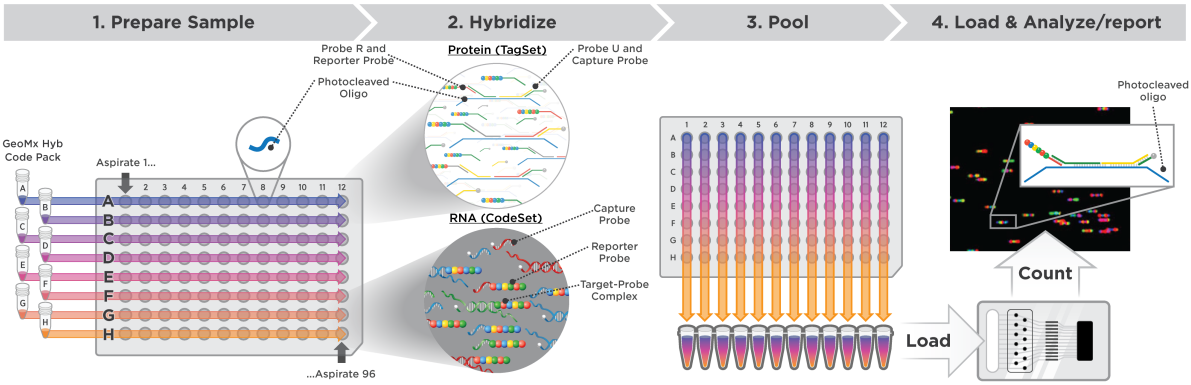


Figure 2: GeoMx DSP nCounter readout overview

To prepare the samples for counting on the nCounter platform, the aspirates are dried down and then rehydrated in the DSP collection plate. Samples are transferred to a new plate for hybridization and combined with **GeoMx Hyb Code Pack** reagents. The hybridization reaction **(see Figure 3)** takes place overnight and products are then pooled by column into a strip tube.

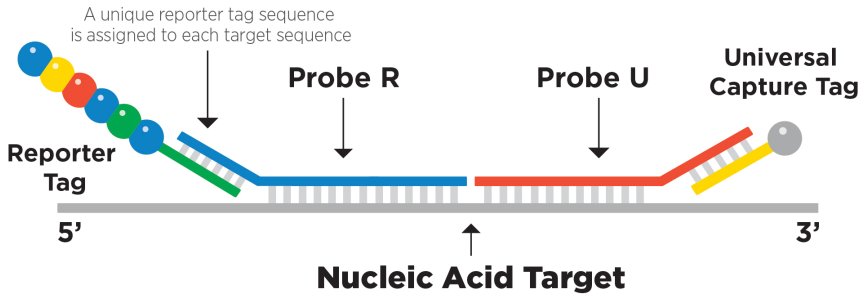


Figure 3: Depiction of probe interactions in GeoMx DSP protein assays with nCounter readout. GeoMx DSP RNA assays with nCounter readout use slightly different chemistry, with direct binding between the reporter tag, the nucleic acid target, and the capture tag or probe (ICP).

The pooled samples are loaded on the nCounter Analysis System or SPRINT Profiler for counting of the oligos collected by the GeoMx DSP. When counting is complete, the nCounter system generates **reporter code count (RCC)** files. The user uploads the RCCs onto the GeoMx DSP system to integrate oligo counts with spatial data, and then proceeds to data analysis.

To complete nCounter readout, the correct corresponding Hyb Code calibration data must be uploaded to the GeoMx DSP (see instructions in **Appendix III: GeoMx Hyb Code Calibration on page 38**). Calibration data will be stored on the instrument and associated with any counts corresponding to that Hyb Code Pack lot.

GeoMx Hyb Code reagents enable multiplexing

GeoMx Hyb Code reagents are formulated with positive and negative controls and allow for multiplexing of up to 96 samples on a single nCounter cartridge. Each GeoMx Hyb Code reagent tube (A–H) is sufficient for 12 reactions.

- **The row of the plate that DSP aspirates are collected into must match the Hyb Code letter used in the nCounter readout portion of the workflow.** For example, aspirates collected into row A must be hybridized with Hyb Code A, row B with Hyb Code B, etc. Therefore, consider the Hyb Code reagents you have on hand when planning your GeoMx DSP experiment.
- Use the **lab worksheet** generated after finalizing the collection plate to **determine the required GeoMx Hyb Code reagents** for your nCounter readout. For example, running 24 samples that are in rows A and B requires two Hyb Codes (A and B). Running 60 samples that are in rows D-H requires five Hyb Codes (D-H). Use only the Hyb Codes listed in the lab worksheet.
- **DO NOT use the same GeoMx Hyb Code reagent** twice in the same nCounter readout run. For example, do not use two GeoMx Hyb Code A tubes in one experiment, as it will be impossible to de–multiplex the data. For the same reason, DO NOT pool and count hybridizations from two full-plate experiments using the same Hyb Codes.
- Hybridization of GeoMx Hyb Codes to their targets is performed overnight. **The hybridization plate wells must be completely sealed** with compatible foil seals to avoid evaporation. NanoString recommends a heat sealer to effectively seal the hybridization plate and avoid loss of sample.

Protein Assays nCounter Readout

Equipment, Materials, and Reagents

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

Table 1: Equipment for protein nCounter readout not provided by NanoString

Equipment	Manufacturer	Part No.
Heated plate sealer (with compatible heat-sealing foil seals)*	Various, e.g. Thermo Fisher®	Various, e.g. AB-1443A
Thermal cycler (NOTE: Ensure a compatible fit with the 96-well PCR plates (see Materials))	Various, e.g. Bio-Rad®	Various, e.g. 1851197
Picofuge	Various	Various
Vortex	Various	Various
Plate spinner/centrifuge (up to at least 2000 x *g*)	Various	Various

*NanoString recommends a heated plate sealer for this protocol. Adhesive foil seals (e.g. Thermo Fisher AB0626) may work, but have not been validated by NanoString. Test plate sealing method before overnight hybridization.

Table 2: Materials for protein nCounter readout not provided by NanoString

Materials	Manufacturer	Part No.
Pipettes for 0.1–1,000 µL	Various	Various
12-channel P20 multi-channel pipetter	Various	Various
Filter tips (DNase/RNase free)	Various	Various
Microcentrifuge tubes (DNase/RNase free)	Various	Various
Permeable membranes (included in Training Kit)	Sigma-Aldrich®	A9224
96-well PCR plates (compatible with thermal cycler, plate sealer, and heat-sealing foils (see Equipment))	Various	Various, e.g. E951020346 to match thermal cycler linked above
Heat-sealing foil seals (compatible with plate sealer)	Fisher Scientific®	AB-0559
RNase AWAY® or 10% Bleach (RNaseZap® is not a substitute)	Thermo Fisher	7003PK
USB drive v3.0, 64 GB or higher (able to be NTFS formatted)	SanDisk® (or comparable)	SDCZ800-128G-G46
PCR strip tubes (12-tube or 8-tube strip, DNase/RNase free) NOTE: nCounter readout on MAX/FLEX/Pro requires the strip tubes from NanoString's Master Kit.	Various	Various

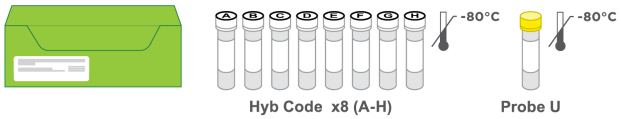
Table 3: Reagents for protein nCounter readout not provided by NanoString (RT = room temperature)

Reagents	Source	Storage
Nuclease-free or DEPC-treated water	Various	RT
(Optional) TE-Tween (10 mM Tris pH8, 1 mM EDTA, 0.1% Tween-20)	Various	RT

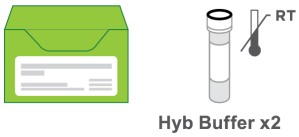
NanoString Reagents

The following reagents are **supplied by NanoString.** Contact your NanoString Sales Representative to use our reagent planning tools to calculate required quantities.

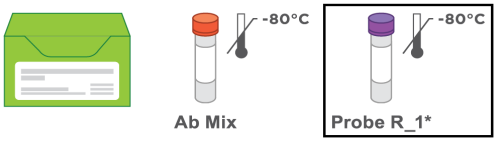
GeoMx Hyb Code Pack for Protein



GeoMx Hybridization Buffer



Probe R from GeoMx Protein Core and optional Module kits for nCounter readout - various available



> **IMPORTANT:** Not all nCounter protein modules are compatible with one another. Each module is assigned a Probe R number. Do not combine two modules with a common Probe R number in the same experimental run, or the data will not be interpretable. Instead, use Substitute Probe R (available from NanoString). See nCounter protein modules' Probe R designations in **Appendix I: Substitute Probe R Guidance on page 36**.

In addition, certain equipment, materials, and reagents are required to run the nCounter Analysis System (MAXFLEX/Pro) or SPRINT Profiler (see platform-specific user manuals at https://nanostring.com/support/support-documentation/ or https://university.nanostring.com/page/document-library).

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1 Transferring Files from the GeoMx DSP

Finalize the GeoMx DSP Collection Plate

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

During the plate finalization step, enter the **GeoMx Hyb Code Pack** lot number (see Figure 4) to be used in downstream nCounter processing; select **Update**. If you do not know the lot number, you can skip this field and enter it when you upload nCounter data.

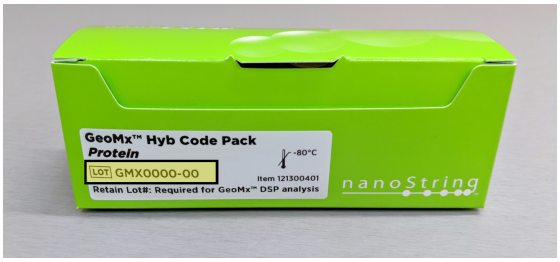


Figure 4: Hyb Code Pack lot number highlighted in yellow

IMPORTANT: The row letter into which DSP aspirates were collected must match the Hyb Code letter used in the nCounter readout portion of the workflow. Aspirates collected into row A must be hybridized with Hyb Code A, row B with Hyb Code B, etc. Ensure you have the correct reagents on hand before beginning the nCounter readout protocol.

Download Files for nCounter Readout

After finalizing the plate, download the following files from the GeoMx DSP **Finalize Plate window** (see Figure 5):

- Under "Definition File", **Download** the Cartridge Definition File (CDF) containing plate map information of the DSP collection plate. Do not edit the contents of the CDF and ensure it is in a folder in the root drive of the USB titled **CDFData**.
- Under "Library Prep Instructions", **Download** the **lab worksheet** to use as a reference during setup of the hybridization reactions.

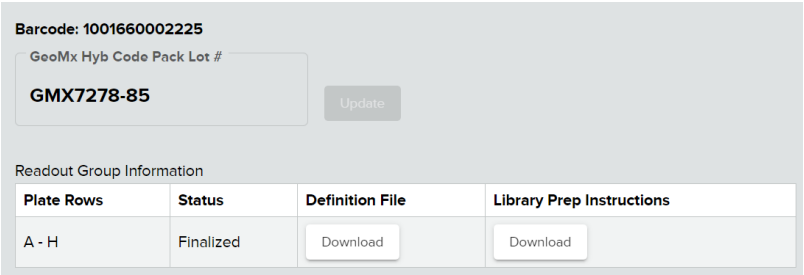


Figure 5: Finalize Plate window

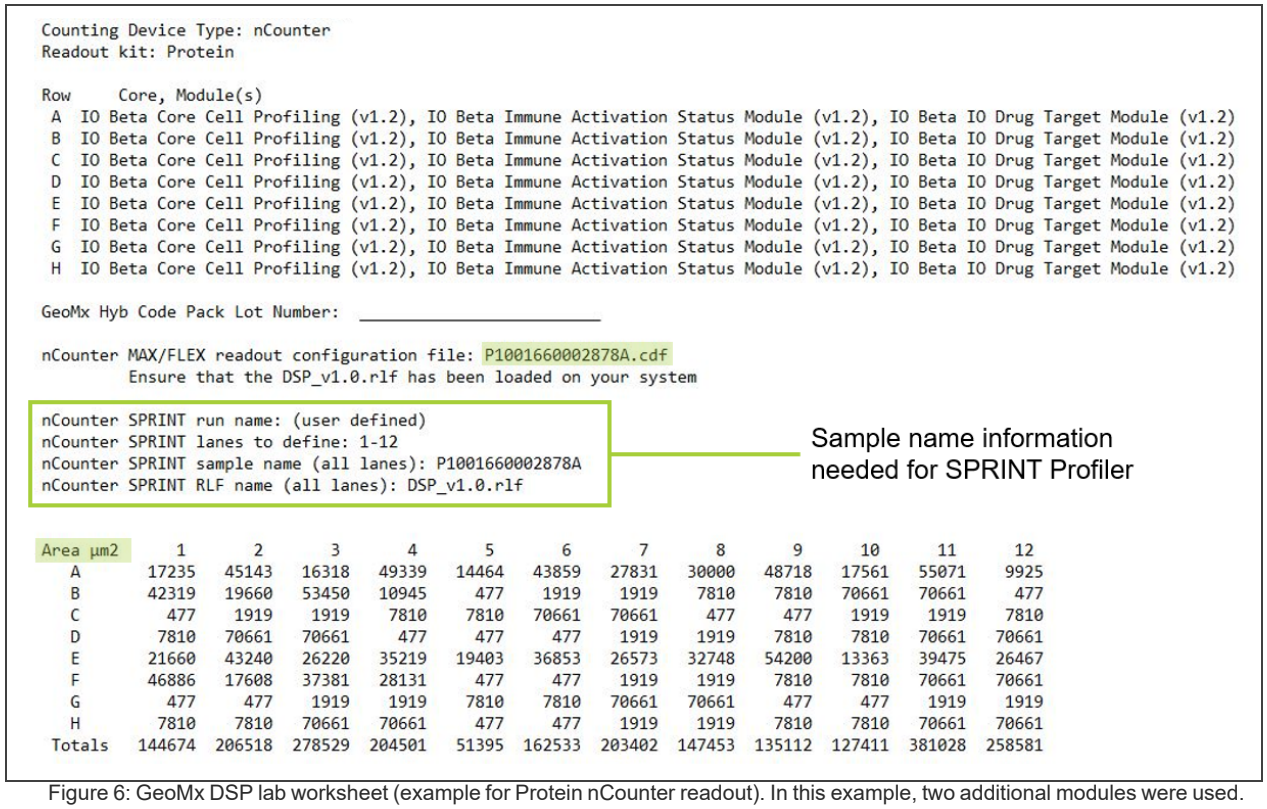
Transfer Run Information to an nCounter System

- If you are using a MAX/FLEX/Pro instrument, upload the **CDF** from a USB drive to the Digital Analyzer starting from the home screen, or while scanning is paused. When initiating the scan, the uploaded CDF will be available in the [**load existing**] option. Do not edit the contents of the CDF and ensure it is in a folder in the root drive of a USB titled **CDFData**.
- If you are using a SPRINT Profiler, manually transfer information from the **lab worksheet** to a New Run using the SPRINT Control Center web interface (in order to control sample names). Once saved to the Run Queue, this Run will be available for selection on the Profiler.

  NOTE: Sample name entered in the SPRINT Control Center must match the sample name listed on the lab worksheet. Sample name is the same for all lanes.

For more information on setting up nCounter runs, see the nCounter instrument user manuals at https://nanostring.com/support/support-documentation/ or https://university.nanostring.com/page/document-library.

The lab worksheet indicates the core and any module kits used, the rows in which aspirates were collected, the total area collected per well and per column, the CDF name, and information needed to set up a SPRINT run **(see Figure 6)**.



Counting Device Type: nCounter
Readout kit: Protein

Row	Core, Module(s)
A	IO Beta Core Cell Profiling (v1.2), IO Beta Immune Activation Status Module (v1.2), IO Beta IO Drug Target Module (v1.2)
B	IO Beta Core Cell Profiling (v1.2), IO Beta Immune Activation Status Module (v1.2), IO Beta IO Drug Target Module (v1.2)
C	IO Beta Core Cell Profiling (v1.2), IO Beta Immune Activation Status Module (v1.2), IO Beta IO Drug Target Module (v1.2)
D	IO Beta Core Cell Profiling (v1.2), IO Beta Immune Activation Status Module (v1.2), IO Beta IO Drug Target Module (v1.2)
E	IO Beta Core Cell Profiling (v1.2), IO Beta Immune Activation Status Module (v1.2), IO Beta IO Drug Target Module (v1.2)
F	IO Beta Core Cell Profiling (v1.2), IO Beta Immune Activation Status Module (v1.2), IO Beta IO Drug Target Module (v1.2)
G	IO Beta Core Cell Profiling (v1.2), IO Beta Immune Activation Status Module (v1.2), IO Beta IO Drug Target Module (v1.2)
H	IO Beta Core Cell Profiling (v1.2), IO Beta Immune Activation Status Module (v1.2), IO Beta IO Drug Target Module (v1.2)

GeoMx Hyb Code Pack Lot Number: _____

nCounter MAX/FLEX readout configuration file: P1001660002878A.cdf
Ensure that the DSP_v1.0.rlf has been loaded on your system

nCounter SPRINT run name: (user defined)
nCounter SPRINT lanes to define: 1-12
nCounter SPRINT sample name (all lanes): P1001660002878A
nCounter SPRINT RLF name (all lanes): DSP_v1.0.rlf

Sample name information needed for SPRINT Profiler

Area µm2	1	2	3	4	5	6	7	8	9	10	11	12
A	17235	45143	16318	49339	14464	43859	27831	30000	48718	17561	55071	9925
B	42319	19660	53450	10945	477	1919	1919	7810	7810	70661	70661	477
C	477	1919	1919	7810	7810	70661	70661	477	477	1919	1919	7810
D	7810	70661	70661	477	477	477	1919	1919	7810	7810	70661	70661
E	21660	43240	26220	35219	19403	36853	26573	32748	54200	13363	39475	26467
F	46886	17608	37381	28131	477	477	1919	1919	7810	7810	70661	70661
G	477	477	1919	1919	7810	7810	70661	70661	477	477	1919	1919
H	7810	7810	70661	70661	477	477	1919	1919	7810	7810	70661	70661
Totals	144674	206518	278529	204501	51395	162533	203402	147453	135112	127411	381028	258581

Figure 6: GeoMx DSP lab worksheet (example for Protein nCounter readout). In this example, two additional modules were used.

2 Prepare the GeoMx DSP Collection Plate for nCounter Readout

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°C.

   > **IMPORTANT:** Deviating from the safe storage guidelines may result in reductions in data quality.

   When ready to process the 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 thermal cycler or heat block at 65°C for 1 hour. The lid of the thermal cycler needs to be in the open position to allow evaporation. 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.
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, return the plate to the thermal cycler and dry down until all liquid is evaporated.
6. **Rehydrate the samples** with **7 µL nuclease-free water**. Pipette up and down 5 times to mix, then allow the collected targets to solubilize for **10 minutes** at room temperature. Use an adhesive plate seal to keep the sample from re-evaporating.
7. **Pulse centrifuge the plate** to 1000 x *g* to ensure all liquid is collected at the bottom.

3 Create Probe R and Probe U Working Pools

Perform steps at room temperature unless otherwise noted. Refer to the Introduction to nCounter Readout on page 11 for information about the role of Probe U, Probe R, and Hyb Code in the hybridization reaction.

> **IMPORTANT: The row letter into which DSP aspirates were collected must match the Hyb Code letter used in the nCounter readout portion of the workflow.** Aspirates collected into row A must be hybridized with Hyb Code A, row B with Hyb Code B, etc.

1. Thaw the **Probe U Master Stock** and the **Probe R Master Stocks** appropriate for the protein core and modules used in the slide preparation protocol. To prepare for a subsequent step, thaw the required **GeoMx Hyb Codes** noted on the lab worksheet. Record the lot number of the GeoMx Hyb Code Pack (see Figure 7).

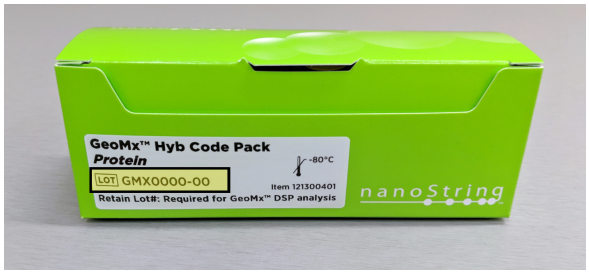


Figure 7: Hyb Code Pack lot number

2. Make the **Probe R** and **Probe U Working Pools**:
   - Refer to Appendix II: Preparing Probe R Master Stock for Custom Barcoded Antibodies from Abcam on page 37 if using a custom barcoded antibody from Abcam.
   - After thawing the **Probe R** and **U Master Stocks**, vortex and spin down before diluting them in Working Pools.
   - The **number of rows** finalized on the DSP collection plate determines the **volumes of the Working Pools** and the **number of GeoMx Hyb Codes** used.
   - Make the **Probe R Working Pool** in a new tube according to Table 4. Refer to the appropriate row of the table to prepare enough Probe R Working Pool for the number of Hyb Codes required for your hybridization. If different combinations of core and modules were used, make separate Probe R Working Pools for each combination.

Table 4: Probe R working pool dilutions

# of Hyb Codes	Core Probe R	Module1 Probe R	Module2 Probe R	Other Modules	Nuclease-free Water	Total Volume
1	2 µL	2 µL	2 µL	...	___ µL	16.5 µL
2-3	4 µL	4 µL	4 µL	...	___ µL	33 µL
4-6	6 µL	6 µL	6 µL	...	___ µL	49.5 µL
7-8	8 µL	8 µL	8 µL	...	___ µL	66 µL

- Make the **Probe U Working Pool** in a new tube according to Table 5. Refer to the appropriate row of the table to prepare enough Probe U Working Pool for the number of Hyb Codes required for your hybridization.

Table 5: Probe U working pool dilutions

# of Hyb Codes	Probe U Master Stock	Nuclease-free Water	Total Volume
1	2 µL	14.5 µL	16.5 µL
2-3	4 µL	29 µL	33 µL
4-6	6 µL	43.5 µL	49.5 µL
7-8	8 µL	58 µL	66 µL

4 Create Probe/Buffer Mix

Create the Probe/Buffer Mix following Figure 8 and Table 6.

1. Pipette **80 µL of Hybridization Buffer per GeoMx Hyb Code** to be used into a new tube (referenced as Probe/Buffer Mix tube).

2. Add **8 µL Probe R Working Pool per GeoMx Hyb Code** to be used into the Probe/Buffer Mix tube.

   For example,
   1 Hyb Code → add 8 µLWorking Pool
   4 Hyb Codes → add 32 µLWorking Pool
   .

3. Add **8 µL Probe U Working Pool per GeoMx Hyb Code** to be used to the Probe/Buffer Mix tube.

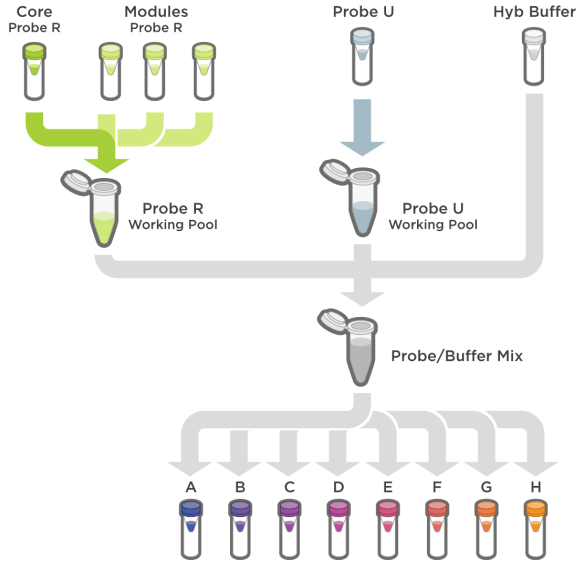


Figure 8: Probe/buffer workflow

Table 6: Probe/Buffer mix

# of Hyb Codes	Probe R Working Pool	Probe U Working Pool	Hybridization Buffer
	(*n* x 8 µL)	(*n* x 8 µL)	(*n* x 80 µL)
n = ___	___ µL	___ µL	___ µL

4. Flick to mix and spin down in a picofuge.

5 Create GeoMx Hyb Code Master Mixes

> **IMPORTANT: The row letter into which DSP aspirates were collected must match the Hyb Code letter used in the nCounter readout portion of the workflow.** Aspirates collected into row A must be hybridized with Hyb Code A, row B with Hyb Code B, etc.

1. After the **GeoMx Hyb Code** tubes have thawed completely, flick to mix and spin down in a picofuge.
2. Add **84 µL of Probe/Buffer Mix** containing Probe R, Probe U and Hybridization Buffer to each tube of **GeoMx Hyb Code** to be used (see Figure 8).

   If preparing a plate with different protein modules and different Probe R designations across different rows, refer to the lab worksheet to confirm the correct Probe R ends up in the correct Hyb Code tube.

3. Mix by flicking the tubes, NOT vortexing. Spin briefly in picofuge.

   These are the **GeoMx Hyb Code Master Mixes**. There should be one GeoMx Hyb Code Master Mix for each row of the plate to be hybridized.

6 Set Up Hybridization

1. Set up for the hybridization reaction:
   - The hybridization plate (a new 96-well plate) must be sealable with a tight foil seal that does not allow evaporation in an **overnight** incubation at **67°C. Test your hybridization reaction set-up for evaporation before running experimental samples.**
   - Confirm that the plate sits completely in the thermal cycler that will be used.
   - Set the thermal cycler to **67°C** and set the heated lid to **72°C** to prevent condensation on the plate seal. If the thermal cycler is programmable, it can be set to ramp down to 4°C indefinitely after the16-24 hr hybridization.

     > **IMPORTANT:** When using a new plate sealer; consider testing the apparatus with spare plates until optimal conditions (resulting in sealed foil without melted plastic) have been identified. Use 160ºC for 1.5 seconds as a default starting point.

2. Pipette **8 µL** of each **GeoMx Hyb Code Master Mix** into each well of the appropriate row of the hybridization plate, matching GeoMx Hyb Code letter A–H to the respective plate row letter (see Figure 9).

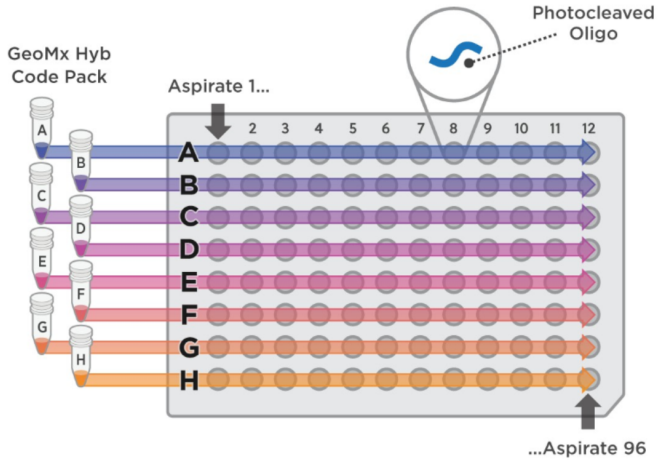


Figure 9: GeoMx Hyb Code and aspirate set-up

3. Transfer **7 µL** of DSP aspirate from the DSP collection plate to the corresponding well in the hybridization plate (e.g. A1 to A1).
4. Mix by gently pipetting each **15 µL** hybridization volume up and down 5 times.
5. Seal the plate carefully using a heated plate sealer.
6. Quick spin the hybridization plate, spinning just long enough to reach 2,000 *x g*.
7. Incubate the plate at 67°C for 16–24 hours in a thermal cycler with a heated lid at 72°C.

7 Pool the Hybridized Samples

1. Remove the plate from the thermal cycler.
2. If the plate is not already at 4°C, cool the plate on ice for 5 minutes.
3. Quick spin the plate, spinning just long enough to reach 2,000 x *g*.
4. Plan the pooling strategy:
   - Locate the **total illuminated segment area** per column listed on the downloaded lab worksheet.
   - Based on this value and referring to Table 7, determine the **volume** of hybridization product to pool for each column. This value may be different for each column.

Table 7: Protein hybridization volumes for pooling

Total illuminated segment area for entire column (μm^2)	MAX/FLEX/Pro volume per well to pool	SPRINT volume per well to pool
≤ 47,000	15.0 µL	15.0 µL†
≤ 63,000	13.5 µL	9.0 µL†
≤ 140,000	6.8 µL	4.5 µL†
≤ 280,000	3.0 µL	2.0 µL
≤ 420,000	2.0 µL	1.3 µL*
≤ 560,000	1.5 µL	1.0 µL*
≤ 770,000	1.2 µL*	0.8 µL*
≤ 1,540,000	0.6 µL*	0.4 µL*
≤ 2,310,000	0.4 µL*	0.3 µL*
≤ 3,100,000	0.3 µL*	0.2 µL*

* Make serial dilutions in **TE-Tween** (10 mM Tris pH 8, 1 mM EDTA, 0.1% Tween-20) for smaller volumes. For example, instead of pipetting 0.4 µL of hybridization product from its well into the pool, make a 1:10 dilution of the hybridization product by mixing 2 µL hybridization product with 18 µL TE-Tween, then pipetting 4 µL of diluted hybridization product into the pool.

† Since the maximum loading volume for a SPRINT cartridge well is **35 µL**, users may need to adjust any pools greater than 35 µL.

Certain tissue types may have extremely high levels of some proteins (e.g., smooth muscle actin in muscle tissue, or HER2 in HER2+ breast cancer tissue), which may lead to saturation even after following the pooling guidelines. If your tissue type falls into this category, please contact geomxsupport@nanostring.com to discuss attenuation strategies.

5. Pool the products by column into a 12-well strip tube (see Figure 10). When using the MAX/FLEX/Pro system, use NanoString- supplied strip tubes and ensure they are oriented correctly (notch after position 1 and 8).
6. Mix the final pool by gently pipetting up and down 5 times.
7. Cap the strip tube and briefly spin down.

   Reseal the hybridization plate and freeze any remaining unpooled hybridization products at -80°C.

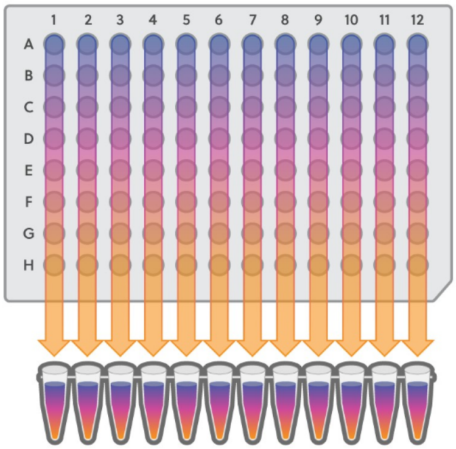


Figure 10: GeoMx Hyb Code and aspirate pooling

If necessary, pooled hybridization products may be stored in the strip tube at -80°C until running on the nCounter instrument.

8 Load the nCounter

1. Load the pooled samples on the MAX/FLEX/Pro Prep Station or SPRINT Profiler, as indicated in the platform-specific user manual. Find the manuals at https://nanostring.com/support/support-documentation/ or https://university.nanostring.com/ page/document-library.
   - For the MAX/FLEX/Pro Prep Station, load the strip tube containing the pools. Select **High Sensitivity** mode.
   - For the SPRINT, load **30-35 µL** from each tube into the corresponding lane on a SPRINT cartridge. If the pool has <30 µL, add nuclease-free water to bring the volume to 30 µL before loading.
2. Transfer the run information from the GeoMx DSP system to the nCounter system, if not already done. See **Transfer Run Information to an nCounter System on page 16**.

9 Run nCounter

Begin the run on the nCounter instrument following platform-specific user manuals (see links, above).

10 Transfer nCounter counts to GeoMx DSP system

After the nCounter run is complete, copy your zipped RCC files to a USB drive and transfer them to the GeoMx DSP.

1. In the GeoMx DSP Control Center, click on **Data Collection** then **Upload Counts/Cal Files**. The **Upload Count Data and Cal Files** window opens (see Figure 11).
2. Click **Choose File** and navigate to the **zipped** counts folder (RCC.zip).

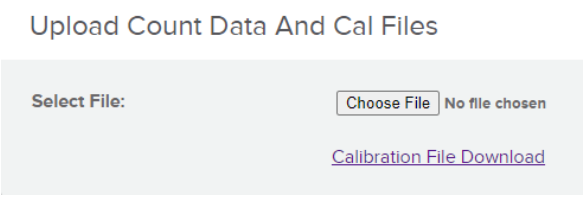


Figure 11: Upload Count Data and Cal Files window

3. A notification will appear under the Notifications Bell indicating that counts were uploaded successfully. This may take a few moments.

   If you encounter an error in uploading counts, check these points:

   - Make sure there is not a folder within the RCC.zip folder.
   - Make sure the correct Hyb Code Lot number is associated with the experiment. Check by clicking on the plate icon and entering the plate barcode.
   - Make sure the correct CDF was used for the nCounter run.
   - Make sure SampleID in the CDF matches SampleID and CartridgeID in the RCC files.
   - nCounter data require a **calibration file** for each new lot of Hyb Code. See the instructions in Appendix III: GeoMx Hyb Code Calibration on page 38 to upload lot-specific calibration file data.

> **IMPORTANT:** If you previously uploaded counts and then re-upload 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.

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

RNA Assays nCounter Readout

Equipment, Materials, and Reagents

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

Table 8: Equipment for RNA nCounter readout not provided by NanoString

Equipment	Manufacturer	Part No.
Heated plate sealer (with compatible heat-sealing foil seals)*	Various, e.g.Thermo Fisher®	Various, e.g. AB1443A
Thermal cycler (NOTE: Ensure a compatible fit with the 96-well PCR plates (see Materials))	Various, e.g.Bio-Rad®	Various, e.g. 1851197
Picofuge	Various	Various
Vortex	Various	Various
Plate spinner/centrifuge (up to at least 2000 x *g*)	Various	Various

*NanoString recommends a heated plate sealer for this protocol. Adhesive foil seals (e.g. Thermo Fisher AB0626) may work, but have not been validated by NanoString. Test plate sealing method before overnight hybridization.

Table 9: Materials for RNA nCounter readout not provided by NanoString

Materials	Manufacturer	Part No.
Pipettes for 0.1–1,000 µL	Various	Various
12-channel P20 multi-channel pipetter	Various	Various
Filter tips (DNase/RNase free)	Various	Various
Microcentrifuge tubes (DNase/RNase free)	Various	Various
Permeable membranes (included in Training Kit)	Sigma	A9224
96-well PCR plates (compatible with thermal cycler, plate sealer, and heat-sealing foils (see Equipment))	Various	Various, e.g. E951020346 to match thermal cycler linked above
Heat-sealing foil seals (compatible with plate sealer)	Fisher Scientific®	AB-0559
RNase AWAY® or 10% Bleach (RNaseZap® is not a substitute)	Thermo Fisher	7003PK
USB drive v3.0, 64 GB or higher (able to be NTFS formatted)	SanDisk® (or comparable)	SDCZ800-128G-G46
PCR strip tubes (12-tube or 8-tube strip, DNase/RNase free) NOTE: nCounter readout on MAX/FLEX/Pro requires the strip tubes from NanoString's Master Kit.	Various	Various

Table 10: Reagents for RNA nCounter readout not provided by NanoString. RT = room temperature.

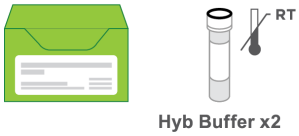
Reagents	Source	Storage
Nuclease-free or DEPC-treated water	Various	RT

NanoString Reagents

The following kits and reagents are **supplied by NanoString.** Contact your NanoString Sales Representative to use our reagent planning tools to calculate required quantities.

GeoMx Hyb Code Pack for RNA

GeoMx Hybridization Buffer



In addition, certain equipment, materials, and reagents are required to run the nCounter Analysis System (MAXFLEX/Pro) or SPRINT Profiler (see platform-specific user manuals at https://nanostring.com/support/support-documentation/ or https://university.nanostring.com/page/document-library).

1 Transferring Files from the GeoMx DSP

Finalize the GeoMx DSP Collection Plate

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

During the plate finalization step, enter the **GeoMx Hyb Code Pack** lot number (see Figure 12) to be used in downstream nCounter processing; select **Update**. If you do not know the lot number, you can skip this field and enter it when you upload nCounter data.

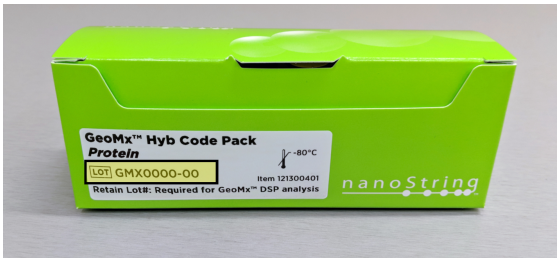


Figure 12: Hyb Code Pack lot number highlighted in yellow

IMPORTANT: The row letter into which DSP aspirates were collected must match the Hyb Code letter used in the nCounter readout portion of the workflow. Aspirates collected into row A must be hybridized with Hyb Code A, row B with Hyb Code B, etc. Ensure you have the correct reagents on hand before beginning the nCounter readout protocol.

Download Files for nCounter Readout

After finalizing the plate, download the following files from the GeoMx DSP **Finalize Plate window** (see Figure 13):

- Under "Definition File", **Download** the Cartridge Definition File (CDF) containing plate map information of the DSP collection plate. Do not edit the contents of the CDF and ensure it is in a folder in the root drive of the USB titled **CDFData**.
- Under "Library Prep Instructions", **Download** the **lab worksheet** to use as a reference during setup of the hybridization reactions.

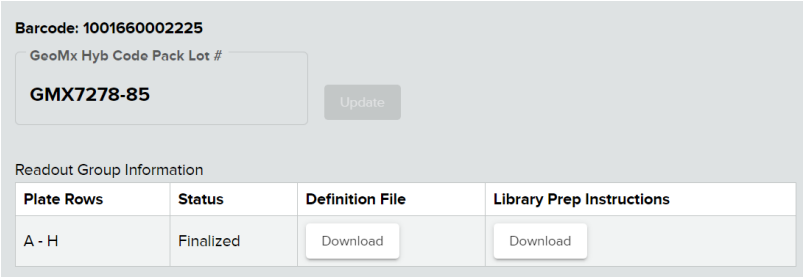


Figure 13: Finalize Plate window

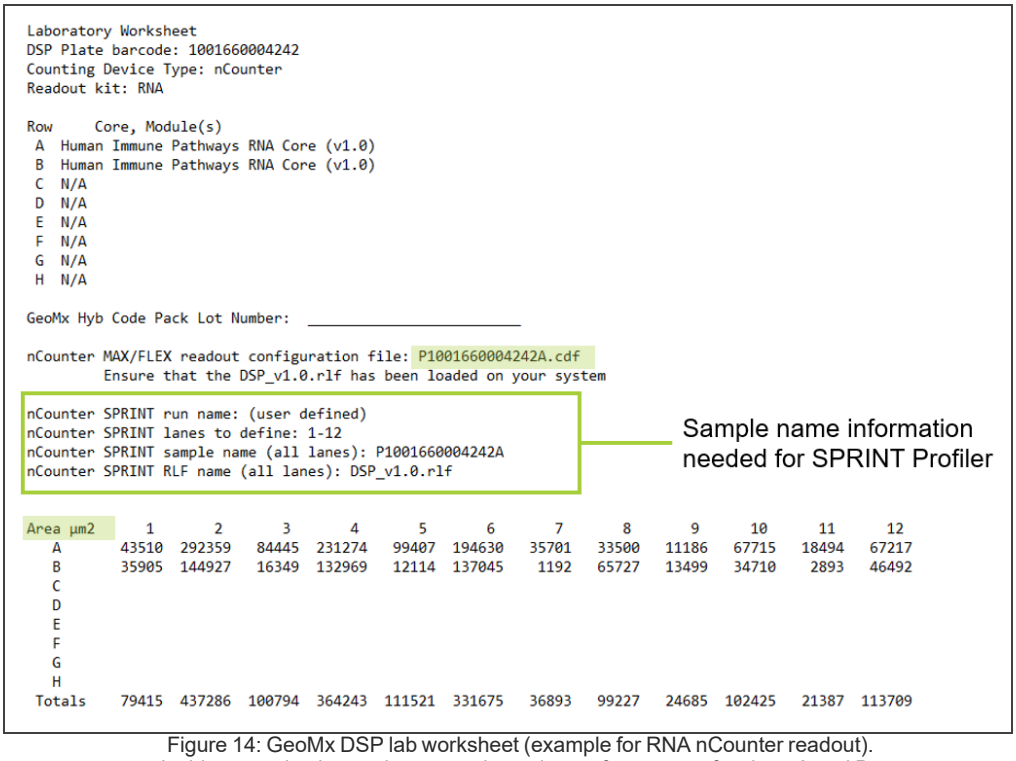
Transfer Run Information to an nCounter System

- If you are using a MAX/FLEX/Pro instrument, upload the **CDF** from the USB drive to the Digital Analyzer starting from the home screen, or while scanning is paused. When initiating the scan, the uploaded CDF will be available in the [**load existing**] option. Do not edit the contents of the CDF and ensure it is in a folder in the root drive of the USB titled **CDFData**.
- If you are using a SPRINT Profiler, manually transfer information from the **lab worksheet** to a New Run using the SPRINT Control Center web interface (in order to control sample names). Once saved to the Run Queue, this Run will be available for selection on the Profiler.

  NOTE: Sample name entered in the SPRINT Control Center must match the sample name listed on the lab worksheet. Sample name is the same for all lanes.

For more information on setting up nCounter runs, see the nCounter instruments user manuals at https://nanostring.com/support/support-documentation/ or https://university.nanostring.com/page/document-library .

The lab worksheet indicates the RNA assay used, the rows in which aspirates were collected, the total area collected per well and per column, CDF name, and information to set up a SPRINT run **(see Figure 14)**.



Laboratory Worksheet
DSP Plate barcode: 1001660004242
Counting Device Type: nCounter
Readout kit: RNA

Row	Core, Module(s)
A	Human Immune Pathways RNA Core (v1.0)
B	Human Immune Pathways RNA Core (v1.0)
C	N/A
D	N/A
E	N/A
F	N/A
G	N/A
H	N/A

GeoMx Hyb Code Pack Lot Number: _____

nCounter MAX/FLEX readout configuration file: P1001660004242A.cdf
Ensure that the DSP_v1.0.rlf has been loaded on your system

nCounter SPRINT run name: (user defined)
nCounter SPRINT lanes to define: 1-12
nCounter SPRINT sample name (all lanes): P1001660004242A
nCounter SPRINT RLF name (all lanes): DSP_v1.0.rlf

Sample name information needed for SPRINT Profiler

Area µm2	1	2	3	4	5	6	7	8	9	10	11	12
A	43510	292359	84445	231274	99407	194630	35701	33500	11186	67715	18494	67217
B	35905	144927	16349	132969	12114	137045	1192	65727	13499	34710	2893	46492
C												
D												
E												
F												
G												
H												
Totals	79415	437286	100794	364243	111521	331675	36893	99227	24685	102425	21387	113709

Figure 14: GeoMx DSP lab worksheet (example for RNA nCounter readout).
In this example, the readout group is made up of two rows of a plate, A and B.

2 Prepare the GeoMx DSP Collection Plate for nCounter Readout

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°C.

   > **IMPORTANT:** Deviating from the safe storage guidelines may result in reductions in data quality.

   When ready to process the 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 thermal cycler or heat block at 65°C for 1 hour. The lid of the thermal cycler needs to be in the open position to allow evaporation. 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.
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, return the plate to the thermal cycler and dry down until all liquid is evaporated.
6. **Rehydrate the samples** with **7 µL nuclease-free water**. Pipette up and down 5 times to mix, then allow the collected targets to solubilize for **10 minutes** at room temperature. Use an adhesive plate seal to keep the sample from re-evaporating.
7. **Pulse centrifuge the plate** to 1000 x *g* to ensure all liquid has been collected at the bottom.

3 Create the In Situ Capture Probe (ICP) Working Pool

Perform steps at room temperature unless otherwise noted. Refer to the Introduction to nCounter Readout on page 11 for information about the role of ICP and Hyb Code in the hybridization reaction.

IMPORTANT: Set up the hybridization reaction in a workspace separate from RNA probe mix preparation to avoid contamination.

IMPORTANT: The row letter into which DSP aspirates were collected must match the Hyb Code letter used in the nCounter readout portion of the workflow. Aspirates collected into row A must be hybridized with Hyb Code A, row B with Hyb Code B, etc.

If processing less than a full collection plate, NanoString recommends aliquoting reagent ICP and freezing unused aliquots at -80°C.

1. Thaw the reagent **ICP**. To prepare for a subsequent step, thaw the required **GeoMx Hyb Codes** noted on the lab worksheet. Record the lot number of the GeoMx Hyb Code Pack (see Figure 15).
2. Make the **ICP Working Pool** following Table 11, according to the number of Hyb Codes to be hybridized:

Figure 15: Hyb Code Pack lot number

Table 11: ICP working pool dilutions

# of Hyb Codes	ICP Master Stock	Nuclease-free Water	Total Volume
1	4 µL	29 µL	33 µL
2–3	8 µL	58 µL	66 µL
4–6	14 µL	102 µL	116 µL
7–8	20 µL	145 µL	165 µL

4 Create ICP/Buffer Mix

Create ICP/Buffer Mix following Figure 16 and Table 12.

1. Pipette **80 µL** of **Hybridization Buffer per GeoMx Hyb Code** to be used into a new tube.
2. Add **16 µL** of **ICP Working Pool per GeoMx Hyb Code** to be used into the tube of Hybridization Buffer to create the ICP/Buffer Mix.

   For example,
   1 Hyb Code → add 16 µL of Working Pool
   4 Hyb Codes → add 64 µL of Working Pool

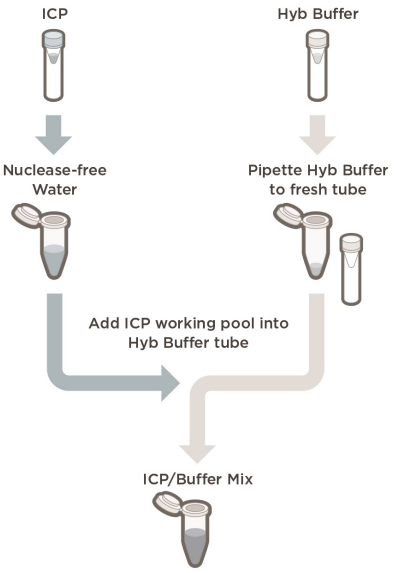


Figure 16: ICP/Buffer mix workflow: ICP is diluted in water to make the ICP Working Pool, which is then combined with Hyb Buffer to make the ICP/Buffer Mix.

Table 12: ICP/Buffer mix

# of Hyb Codes	ICP Working Pool	Hybridization Buffer
	(n x 16 µL)	(n x 80 µL)
n = ___	___ µL	___ µL

3. Flick to mix and spin down in a picofuge.

5 Create GeoMx Hyb Code Master Mixes

> **IMPORTANT: The row letter into which DSP aspirates were collected must match the Hyb Code letter used in the nCounter readout portion of the workflow.** Aspirates collected into row A must be hybridized with Hyb Code A, row B with Hyb Code B, etc.

1. After the **GeoMx Hyb Code** tubes have thawed completely, flick to mix and spin down in a picofuge.
2. Add **84 µL** of **ICP/Buffer Mix** to each tube of **GeoMx Hyb Code** to be used **(see Figure 17)**.
3. Mix by flicking the tubes, NOT vortexing. Spin briefly in picofuge.

   These are the **GeoMx Hyb Code Master Mixes**. There should be one GeoMx Hyb Code Master Mix for each row of the plate to be hybridized.

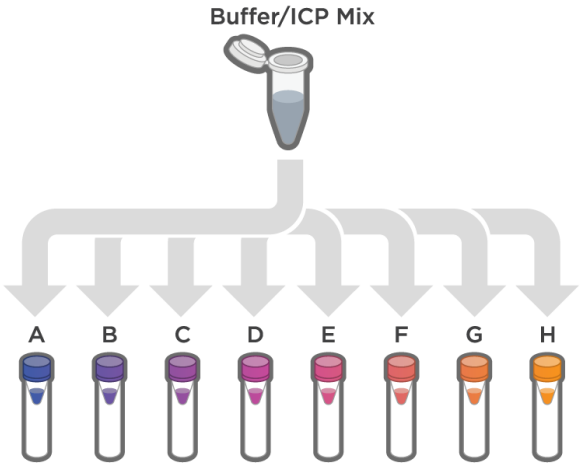


Figure 17: Distributing ICP/Buffer Mix into GeoMx Hyb Code tubes

6 Set Up Hybridization

1. Set up for the hybridization reaction:
   - The hybridization plate (a new 96-well plate) must be sealable with a tight foil seal that does not allow evaporation in an **overnight** incubation at **65°C. Test your hybridization reaction set-up for evaporation before running experimental samples.**
   - Confirm that the plate sits completely in the thermal cycler that will be used.
   - Set the thermal cycler to **65°C** and set the heated lid to **70°C** to prevent condensation on the plate seal. If the thermal cycler is programmable, it can be set to ramp down to 4°C indefinitely after the16-24 hr hybridization.

> **IMPORTANT:** When using a new plate sealer; consider testing the apparatus with spare plates until optimal conditions (resulting in sealed foil without melted plastic) have been identified. Use 160ºC for 1.5 seconds as a default starting point.

2. Pipette **8 µL** of each **GeoMx Hyb Code Master Mix** into each of the 12 wells of the appropriate row of the hybridization plate, matching GeoMx Hyb Code A–H to the respective plate row **(see Figure 18)**.
3. Transfer **7 µL of DSP aspirate** from the DSP collection plate to the corresponding well in the hybridization plate (e.g. A1 to A1).
4. Mix by gently pipetting each **15 µL** hybridization reaction up and down 5 times.

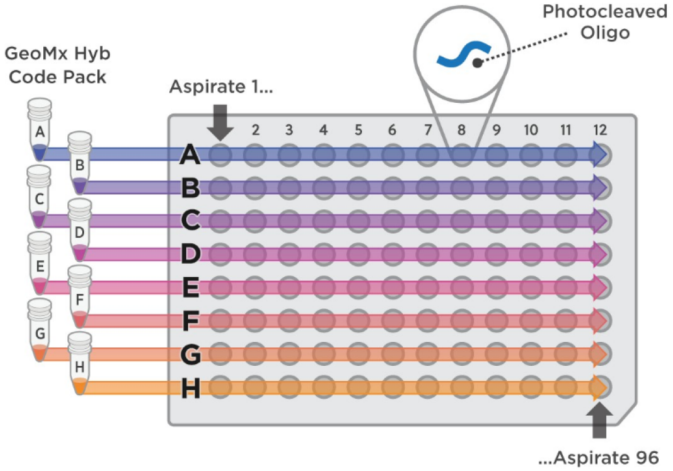


Figure 18: Setting up the hybridization plate

5. Seal the plate carefully using a heated plate sealer.
6. Quick spin the hybridization plate, spinning just long enough to reach 2,000 x *g*.
7. Incubate the plate at 65°C for 16–24 hours in a thermal cycler with a heated lid set at 70°C.

7 Pool the Hybridized Samples

1. Remove the plate from the thermal cycler.
2. If the plate is not already at 4°C, cool the plate on ice for 5 minutes.
3. Quick spin the plate, spinning just long enough to reach 2,000 x *g*.
4. Pool the products by column into a strip tube **(see Figure 19)**. Pool the full volume of each well (15 µL). When using the MAX/FLEX/Pro system, use NanoString-supplied strip tubes and ensure they are oriented correctly (notch after position 1 and 8).

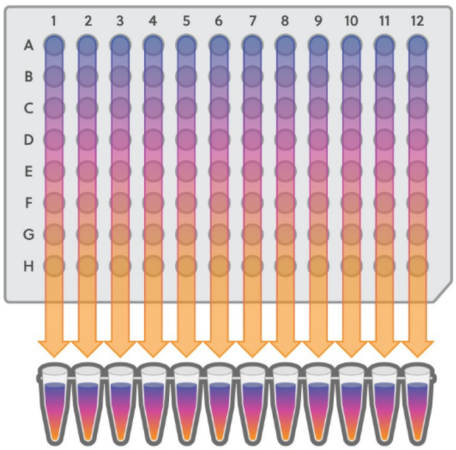


Figure 19: GeoMx Hyb Code and aspirate pooling

5. Mix the final pool by gently pipetting up and down 5 times.
6. Cap the strip tube and briefly spin down.

   If necessary, pooled hybridization products may be stored in the strip tube at -80°C until running on the nCounter instrument.

8 Load the nCounter

1. Load the pooled samples on the MAX/FLEX/Pro Prep Station or SPRINT Profiler, as indicated in the platform-specific user manual. Find the manuals at https://nanostring.com/support/support-documentation/ or https://university.nanostring.com/ page/document-library.
   - For the MAX/FLEX/Pro Prep Station,load the strip tube containing the pools. Select **High Sensitivity** mode.
   - For the SPRINT, load **30-35 µL** from each tube into the corresponding lane on a SPRINT cartridge. If the pool has <30 µL, add nuclease-free water to bring the volume to 30 µL before loading.
2. Transfer the run information from the GeoMx DSP system to the nCounter system, if not already done. See **Transfer Run Information to an nCounter System on page 28**.

9 Run nCounter

Begin the run on the nCounter instrument following platform-specific user manuals (see links, above).

10 Transfer nCounter counts to GeoMx DSP system

After the nCounter run is complete, copy your zipped RCC files to a USB drive and transfer them to the GeoMx DSP.

1. In the GeoMx DSP Control Center, click on **Data Collection** then **Upload Counts/Cal Files**. The **Upload Count Data and Cal Files** window opens (see Figure 20).
2. Click **Choose File** and navigate to the **zipped** counts folder (RCC.zip).

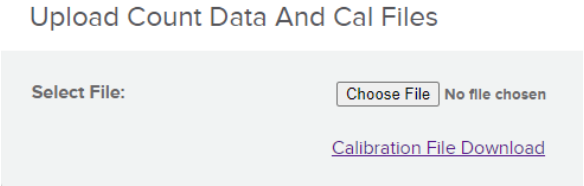


Figure 20: Upload Count Data and Cal Files window

3. A notification will appear under the Notifications Bell indicating that counts were uploaded successfully. This may take a few moments.

   If you encounter an error in uploading counts, check these points:

   - Make sure there is not a folder within the RCC.zip folder.
   - Make sure the correct Hyb Code Lot number is associated with the experiment. Check by clicking on the plate icon and entering the plate barcode.
   - Make sure the correct CDF was used for the nCounter run.
   - Make sure SampleID in the CDF matches SampleID and CartridgeID in the RCC files.
   - nCounter data require a **calibration file** for each new lot of Hyb Code. See the instructions in Appendix III: GeoMx Hyb Code Calibration on page 38 to upload lot-specific calibration file data.

> **IMPORTANT:** If you previously uploaded counts and then re-upload 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.

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

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

Appendix I: Substitute Probe R Guidance

A Core protein panel can be run with up to 6 Modules at once. Core and Modules must all fall within the same group (e.g., Human IO, Mouse IO, or Human Neuroscience).

Each panel is assigned a Probe R number for nCounter readout (see Table 13). **Do not combine two modules with a common Probe R number in the same experimental run, or the data will not be interpretable.** Substitute Probe Rs are available from NanoString to allow the combination of modules that share a Probe R number, such as MAPK Signaling and Immune Cell Typing.

IO Core and Modules

Table 13: Protein panels and their corresponding Probe R number

Panel	Probe R number	Substitute Probe Rs available
Immune Cell Profiling Core	IO R_1	
IO Drug Target	IO R_2	
Immune Activation Status	IO R_3	
Immune Cell Typing	IO R_4	
MAPK Signaling	IO R_4	IO R_2 IO R_3 IO R_5 IO R_6 IO R_7
Pan-Tumor	IO R_5	
Cell Death	IO R_6	
PI3K/AKT Signaling	IO R_7	
Custom	IO R_8 IO R_9	

Neuroscience Core and Modules

As of May 2022, all Human Neuroscience modules are compatible with one another, and all Mouse Neuroscience modules are compatible with one another, without Substitute Probe R.

Appendix II: Preparing Probe R Master Stock for Custom Barcoded Antibodies from Abcam

Each GeoMx protein panel is assigned a Probe R number for nCounter readout. When purchasing individual custom barcoded GeoMx antibodies from Abcam, the corresponding Probe R is provided for each antibody. These individual Probe Rs must be combined to make the Probe R Master Stock required in the protein assay nCounter readout protocol.

1. **Thaw the individual Probe R tubes** (up to 5) corresponding to the custom barcoded antibodies from Abcam used in slide preparation.
2. **Pipette 2 µL** of each Abcam Probe R together into a single, new tube.
3. **Add nuclease-free water** to bring the total volume up to **10 µL**.
4. Add the appropriate volume of Probe R Master Stock for the number of Hyb codes in your assay, as indicated in the Probe R working pool dilutions table on page 18.

Appendix III: GeoMx Hyb Code Calibration

GeoMx Hyb Code reagents require lot-specific calibration. NanoString generates new calibration data for each Hyb Code lot and posts the data files on the NanoString website. The appropriate calibration files must be downloaded from the website and uploaded to the GeoMx DSP system to complete the nCounter readout.

Downloading Calibration Files from Website

1. Visit www.nanostring.com/dspcalibfiles.
2. Select the file that matches the lot of GeoMx Hyb Code in use (lot number is printed on the front of the Hyb Code box, shown in **Figure 4 on page 15**. If you do not see the lot number you need, please contact bioinformatics@nanostring.com for assistance).
3. Download the zipped file to a USB drive or other location accessible from your GeoMx DSP system.

Uploading Calibration Files to GeoMx DSP

1. In the **DSP Control Center**, hover over the **Data Collection** button and select **Upload Counts/Cal Files**. The Upload Count Data and Cal Files window opens **(see Figure 21)**.
2. In the **Upload Count Data** window, select **Choose File,** then browse to the location of the saved zipped calibration files and select **Open.** Alternatively, select **Calibration File Download** to access the calibration files website from the GeoMx DSP Control Center.

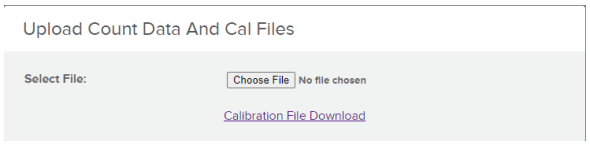


Figure 21: Upload Count Data And Cal Files

3. A notification will appear under the Notifications Bell indicating that calibration file was uploaded successfully.

Troubleshooting

Suggested actions to resolve certain issues are listed below. For additional support, contact GeoMxSupport@nanostring.com.

Issue	Possible Cause	Suggested Actions
I associated the wrong Hyb Code lot number with the plate	User error	Click on the plate icon (or where it says "No plate") to open the Plate Information window. Enter the plate barcode for which you need to adjust the Hyb Code information. Edit the Hyb Code lot number associated with the plate. You may need to re-upload the count data (RCC files) to begin data analysis.
I don't have the right Hyb Code reagents on hand	Various	Hyb Code letter must match the collection plate row letter. Order Hyb Code reagents to match the collected aspirates' row(s).
I mixed Hyb Code with the wrong rows of the collection	User error	Hyb Code letter must match the collection plate row letter. The best resolution may be to re-scan the slide and perform another collection from different ROIs.
Overnight hybridization evaporated	Plate was not sealed properly	Samples that evaporated are unfortunately lost. The experiment would need to be repeated. NanoString recommends using a heated plate sealer and foil seals to minimize the risk of evaporation.
Error when uploading counts (RCCs) to GeoMx		Make sure there is not a folder within the RCC.zip folder. Make sure the correct Hyb Code Lot number is associated with the experiment. Check by clicking on the plate icon. Make sure the correct CDF was used for the nCounter run. Make sure SampleID in the CDF matches SampleID and CartridgeID in the RCC files. nCounter data require a **calibration file** for each new lot of Hyb Code. See the instructions in **Appendix III: GeoMx Hyb Code Calibration on page 38** to upload lot-specific calibration file data.
Problem with nCounter MAX/FLEX/Pro or SPRINT Profiler		Refer to platform-specific user documentation at https://nanostring.com/support/support-documentation/ or https://university.nanostring.com/page/document-library .

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