GeoMx® DSP
Manual Slide Preparation
User Manual

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nanoString®

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Changes in this Revision

This **GeoMx DSP Manual Slide Preparation User Manual** (MAN-10150) covers protein and RNA sample preparation using manual (non-automated) methods, for assays with NGS or nCounter readout. The GeoMx DSP Automated Slide Preparation User Manual (MAN-10151) covers sample preparation using semi- and fully-automated methods on the BOND RX/RX^{m} Fully Automated IHC/ISH Stainer from Leica Biosystems®.

Changes in this manual revision include:

- Added instructions to use the new Immuno-oncology Proteome Atlas (IPA) with Pro Code indices on page 22. Please refer to the following page for a summary of key information and workflow changes for this assay.
- Updated Protein slide unmounting and restaining procedure, which does not require a new hydrophobic barrier, on page 25
- Clarified that adhesive slide labels should not overlap the scan area or slide gasket, throughout the manual
- Updated typeface for improved readability

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

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

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

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

Conventions

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

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

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

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

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

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

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

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

GeoMx DSP Workflow

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

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

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

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

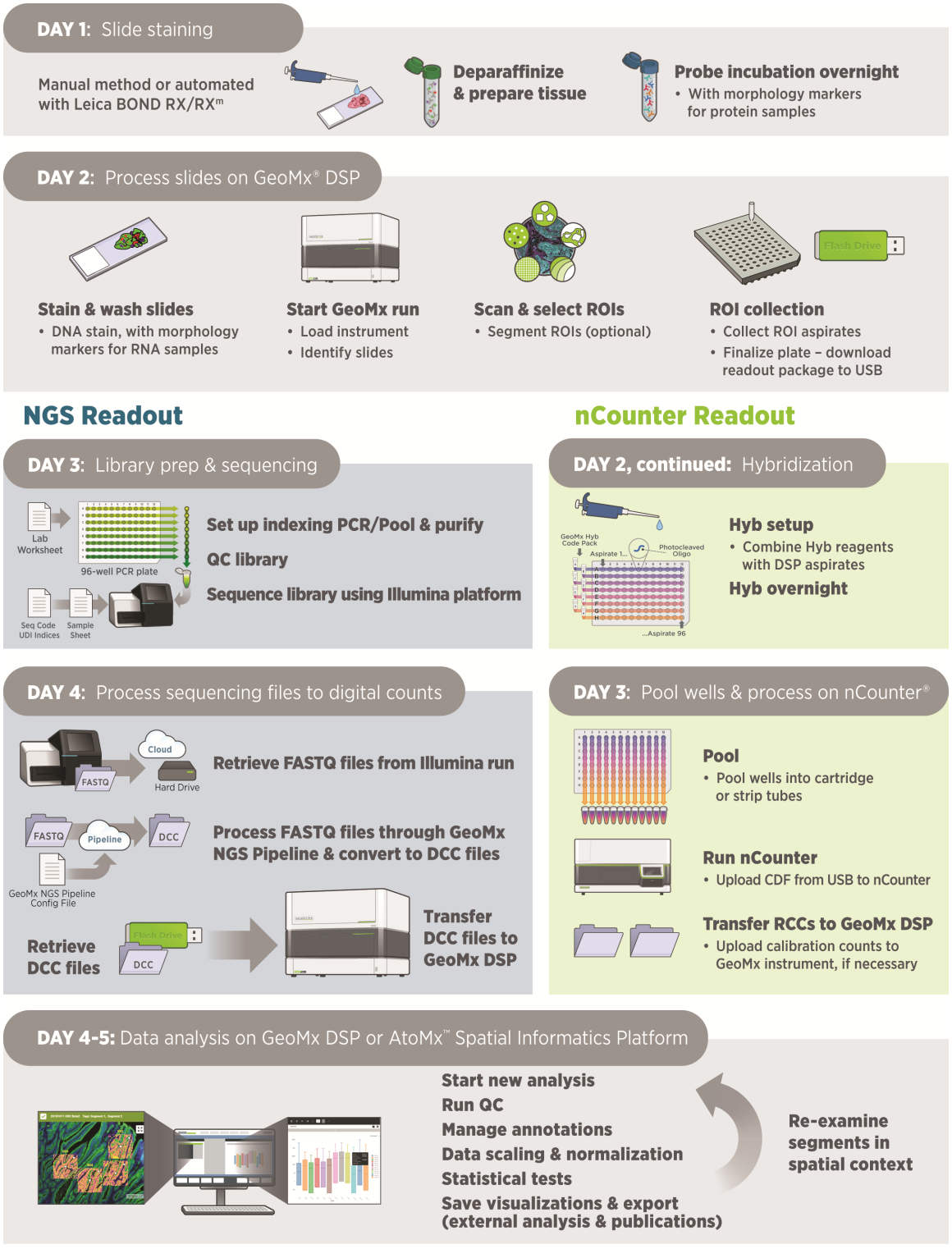


Figure 1: GeoMx DSP workflow summary

User Manuals and Resources

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

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

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

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

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

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

Slide Prep Introduction

Slide preparation is the first step of the GeoMx DSP workflow. Tissue sections are processed for staining, followed by the addition of morphology reagents and biological probes (Figure 2).

- **Morphology reagents** are antibody-fluorophore complexes that bind to specific targets on the tissue. Tissue structure and cell components most important to your analysis are illuminated using the fluorescence imaging on the DSP system. Alternative labeling techniques are described in the Appendices.
- **Biological probes** are either antibodies or *in situ* hybridization (ISH) probes that bind to protein or RNA targets, respectively. Each probe is coupled to a photocleavable oligonucleotide. These oligos, when exposed to the GeoMx DSP instrument's UV light, will be released into solution and aspirated into a collection plate well for downstream processing.

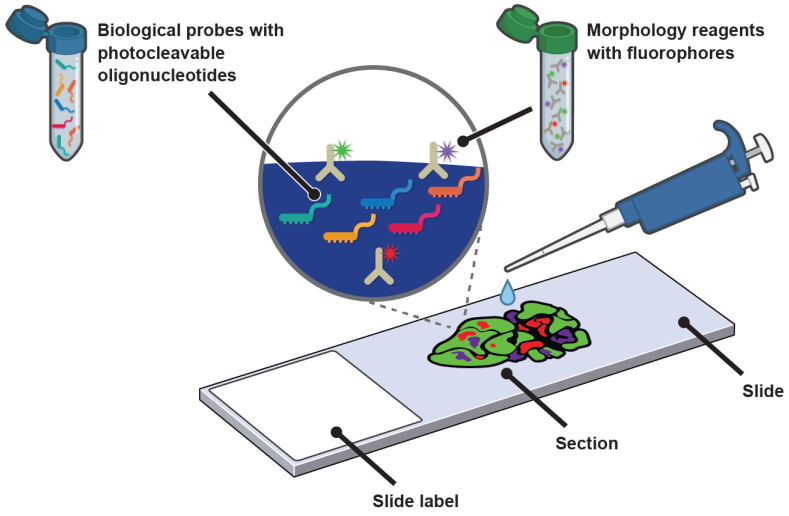


Figure 2: RNA slide preparation with ISH probes

Slide Prep Equipment, Materials, and Reagents

Required equipment, materials, and reagents are listed at the start of each slide preparation process in the manual. Individual **Equipment, Materials, and Reagents** lists for every application of the GeoMx DSP are available in the NanoString University Document Library (https://university.nanostring.com).

Protein Slide Preparation Protocol (FFPE)

1 Equipment, Materials, and Reagents

The following tables list equipment, materials, and reagents that are required for this protocol but are **not supplied by NanoString**.

Table 1: Equipment for protein slide prep not supplied by NanoString.

Equipment	Source, Catalog No.
Baking oven	Quincy Lab, Inc., various GC models (or comparable)
TintoRetriever pressure cooker (rated for 110V; requires transformer to operate on 220V)*	Bio SB, BSB 7008 or BSB 7015
Picofuge	Various
Vortex	Various

*The TintoRetriever pressure cooker is validated for this protocol. These alternatives have been recommended by GeoMx DSP users, but have not been validated by NanoString: Cuisinart CPC-600N1 (discontinued by manufacturer but available from some vendors); Tefal CY505E 6 Liter; AmazonBasics multipurpose pressure cooker 5.5L.

Table 2: Materials for protein slide prep not supplied by NanoString.

Materials	Source, Catalog No.
Pipettes for 5–1,000 µL	Various
Filter tips (DNase/RNase free)	Various
Microcentrifuge tubes (DNase/RNase free)	Various
RNase AWAY® or 10% Bleach (RNaseZap® is not a substitute)	Thermo Fisher, 7003PK
Superfrost Plus microscope slides or Leica BOND Plus microscope slides (more adhesive; recommended for tissues prone to detaching from slides)	Fisher Scientific, 12-550-15 or Leica Biosystems, S21.2113.A
Slide staining jars (Coplin jars) (qty 12; recommend at least 1 of plastic for use in pressure cooker) and slide holder inserts	VWR, 25608-904, 25608-868 (or comparable)
Humidity chamber	Simport, M920-2 (select black lid) (or comparable)
Hydrophobic barrier pen	Vector Labs, H-4000 (or comparable)
Razor blades	Various
Coverslips	Various
Heat/cold protectant handling glove	Various
Kimwipes™ (or comparable)	Various
USB drive v3.0, 64 GB or higher (able to be NTFS formatted)	SanDisk, SDCZ800-128G-G46 ; AmazonBasics LS21USB128G1; or comparable

Table 3: Reagents for protein slide prep not supplied by NanoString. RT = room temperature

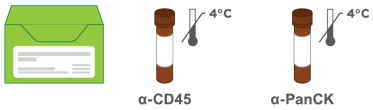
Reagents	Source, Catalog No.	Storage
DEPC-treated water	Thermo Fisher, AM9922 (or comparable) NOTE: As an alternative to commercial DEPC-treated water, prepare your own following standard protocols.	RT
10X tris-buffered saline (TBS)	Cell Signaling Technologies, 12498S	RT
10X TBS with Tween®20 (TBS-T)	Cell Signaling Technologies, 9997S (1% Tween20 in concentrate, for 0.1% in 1X formulation)	RT
4 or 16% paraformaldehyde (PFA) 	Thermo Scientific, 4% concentration, FB002, R37814 or 16% concentration (must be diluted to 4% with PBS), 28906, 28908 (or comparable)	4°C (or manufacturer instructions)
1X phosphate buffered saline pH 7.4 (PBS) (used only to dilute 16% PFA)	Thermo Fisher, 10010031KU (or comparable)	RT
Fluoromount-G mounting media	SouthernBiotech, 0100-01	RT
CitriSolv **or** Xylene or D-Limonene ((R)-(+)-Limonene) 	Fisher Scientific, 04-355-121 Sigma Aldrich, 183164-100ML or 183164-500ML (or comparable)	RT
100% ethanol (EtOH): ACS grade or better 	Various	RT
10X citrate buffer pH 6	Sigma Aldrich, C9999-100ML or C9999-1000ML (or comparable)	4°C

NanoString Reagents

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

GeoMx Protein Slide Prep Kit

GeoMx Morphology Kit — Human or Mouse Protein compatible (various available)

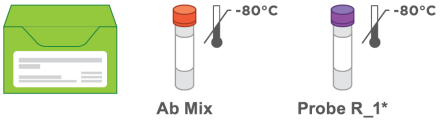


GeoMx Nuclear Stain Morphology Kit

For NGS readout:
GeoMx Protein Cores and Modules for NGS readout (various available, including custom options)

NOTE: Immuno-oncology Proteome Atlas (IPA) uses Pro Code indices. Please pay attention to Pro Code-specific instructions throughout GeoMx DSP user manuals.

For nCounter readout:
GeoMx Protein Cores and Modules for nCounter readout (various available, including custom options)



*Each Protein Core and Module for nCounter readout includes a Probe R reagent, from Probe R_1 to Probe R_9. **DO NOT combine two modules with a common Probe R_number in the same experiment run**. Substitute Probe R reagents are available for assays with overlapping Probe R_numbers. Refer to Appendix IV: Substitute Probe R Guidance on page 54 for more information and to plan Probe R usage in your experiment.

Morphology marker antibodies are stored at 4°C and aliquoting is not required. Each tube of Protein Core/Module (Ab mix) for nCounter readout contains sufficient reagent for 12 slides (112 µL). If using the entire Ab mix in one week, store at 4°C. If not, **aliquot the Ab mix** into 4-slide aliquots (37 µL + 37 µL + 38 µL) and freeze unused aliquots at -80°C. Do not exceed more than 2 freeze/thaw cycles and do not freeze diluted antibody.

Prepare reagents

Prepare your reagents using the dilution instructions (Table 4). The actual volume of reagents used in the protocol will vary – the volumes to prepare in Table 4 are suggestions.

Table 4: Reagent prep for protein slide preparation.

Reagent	Dilution	Storage
1X citrate buffer pH 6	To prepare 250 mL of 1X citrate buffer, add 25 mL 10X citrate buffer to 225 mL DEPC-treated water. **Must** be prepared on the day of slide prep. Do not prepare ahead of time.	4°C
95% ethanol (EtOH)	To prepare 500 mL of 95% ethanol, add 25 mL of DEPC-treated water to 475 mL of 100% ethanol.	RT
4% paraformaldehyde (PFA)	NOTE: Use *only* for post-fixation step. If starting with 16% stock, prepare 1 mL of 4% PFA by adding 250 µL 16% PFA to 750 µL 1X PBS, aliquot to 250 µL each, and store.	4°C (or manufacturer instructions)
1X TBS with 0.1% Tween20 (TBS-T)	To prepare 1 L, dilute 100 mL of 10X TBS-T in 900 mL DEPC-treated water. Total volume needed for slide prep depends on volume of staining jars to be used.	RT
1X TBS	To prepare 5 mL 1X TBS, dilute 500 µL of 10X TBS in 4.5 mL DEPC-treated water.	RT

2 Prepare tissue samples

Appendix I: Selecting and Sectioning FFPE Samples on page 45 covers FFPE block selection and sectioning in detail. Review it prior to beginning the Protein Slide Preparation protocol.

GeoMx has been validated for sample blocks up to 3 years old prepared from tissues with a cold ischemic time of less than 1 hour using 10% NBF or similar fixative. For best results, do not use FFPE blocks more than 10 years old. Assay performance will be influenced by tissue block age and treatment conditions such as cold/warm ischemic time, fixative, and storage.

Slide Preparation

1. Tissue sections should be **5 µm** thick and mounted on **Superfrost Plus or BOND Plus slides.** Tissue sections must be placed in the **Scan Area** (shown in green in Figure 3) in the center of the slide and be no larger than 35.3 mm long by 14.1 mm wide. Tissue should not overlap the slide gasket (shown in blue) or the tip calibration area (shown in red). If mounting multiple sections per slide, ensure that tissues are at least 2–3 mm apart and still fit within the Scan Area. Adhesive slide labels should not overlap Scan Area or slide gasket.

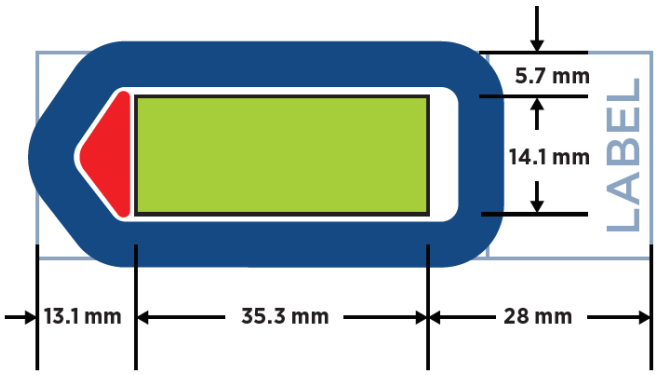


Figure 3: Slide dimensions. Measure from label edge of slide as reference point.

If sections are too large and/or placed off-center, continue with slide preparation as usual. Just before loading the slide in the instrument slide tray, scrape off the parts of the tissue exceeding the scan area, making sure the slide gasket and tip calibration area are free of tissue. Scraping off tissue before slide preparation could generate tissue folds that may result in staining or binding artifacts.

IMPORTANT: The GeoMx DSP instrument will only image the area inside the Scan Area. **Tissue outside of the Scan Area will not be imaged and may cause problems in tissue detection by the optical system.**

2. **Bake slides with mounted sections** in a 60°C drying oven for 30 minutes to 3 hours prior to deparaffinization. Stand slides vertically during baking to allow excess paraffin to flow off. Longer baking times may be necessary for some tissues to sufficiently adhere to the slide (e.g., overnight at 37°C followed by 2-4 hr at 60°C); this should be empirically tested.

3 Deparaffinize and rehydrate FFPE tissue sections (45 minutes)

Needed for this step: **Staining jars, Citrisolv** (or acceptable substitute)**, 100% EtOH, 95% EtOH,** and **DEPC-treated water**. See the Equipment, Materials, and Reagents lists on page 13 for more details.

1. Place slides in a rack and perform the washes in staining jars as illustrated below. Ensure you have sufficient buffer volume to cover the slides. Dip slides up and down gently several times when placing in and before removing from staining jars.

2. During the above washes, **prepare the pressure cooker** for the next step by adding water to the correct level per the manufacturer's instructions (for the TintoRetriever, above 4 cups) and preheating to 80°C. **Do not preheat the Citrate Buffer.**

⚠ **WARNING:** Dispose of CitriSolv or its substitute in accordance with your lab's safety procedures.

4 Perform antigen retrieval (1 hour)

Needed for this step: **staining jars**, **preheated pressure cooker, 1X Citrate Buffer** and **1X TBS-T**. See the Equipment, Materials, and Reagents lists on page 13 and Prepare Reagents steps on page 16.

A plastic staining jar is recommended for this step to avoid the risk of glass breaking. If using a glass jar, be aware of the risk of glass breaking during incubation or upon removal of the jar to room temperature.

1. **Place FFPE slides in a staining jar containing 1X freshly prepared Citrate Buffer pH 6 at room temperature**. Place a lid on the staining jar to prevent evaporation. To prevent pressurization, DO NOT tighten or seal the jar lid.

2. Place the staining jar containing the slides and lid **into the preheated pressure cooker**.
3. Secure the pressure cooker lid and run on **high pressure and high temperature for 15 minutes.**

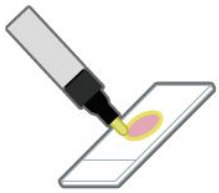
4. When the timer reaches zero, **carefully release the pressure** and **transfer** the staining jar with slides to a lab bench (room temperature), **remove** the staining jar lid, and **let stand** for at least 25 minutes (maximum one hour).
5. **Wash** the slides **in 1X TBS-T** in a staining jar **for 5 minutes**.

5 Blocking (1 hour)

Needed for this step: **hydrophobic pen, humidity chamber,** and **Buffer W**. See the Equipment, Materials, and Reagents lists on page 13 for more details.

IMPORTANT: When creating a hydrophobic barrier around the tissue on a slide, prepare slides one at a time, leaving the others in the TBS-T solution. **DO NOT** allow the tissue sections to dry out during slide preparation.

1. **Prep the humidity chamber** by lining with Kimwipes and adding enough water to cover the bottom of the chamber. If your chamber is light-permeable, minimize light exposure (e.g., wrap the lid in aluminum foil).
2. **Remove one slide from the 1X TBS-T**, tap on an absorbent, clean surface such as a paper towel, then use an absorbent wipe to carefully **remove excess buffer** from the slide, without touching the tissue.
3. **Make a closed hydrophobic barrier** around each tissue section with a hydrophobic pen. Ensure that a complete barrier is made while minimizing the area within the barrier. Keep in mind that the barrier will be removed in a later step, so leave enough room to remove the barrier without damaging the tissue.



4. **Place the slide in the humidity chamber** in a horizontal position and add enough Buffer W to completely fill the hydrophobic barrier (up to **200 µL** per slide, depending on the size of the tissue).
5. Repeat steps 2-4 for any additional slides.

6. **Incubate slides with Buffer W for 1 hour** at room temperature in the closed humidity chamber.
7. **Thaw detection probe antibody (Ab) mix** (cores and modules) on ice. Keep tube protected from light.

6 Primary antibody incubation (overnight)

Needed for this step: **prepared humidity chamber, thawed detection antibody mix** (core and optional modules)**, morphology markers,** and **Buffer W**. See the Equipment, Materials, and Reagents lists on page 13 for more details.

IMPORTANT: Probe mixes should be assembled in an area separate from nCounter work, NGS library prep, or other GeoMx workflows. GeoMx detection reagents can cross- contaminate probe mixes and give misleading or incorrect results. Areas should be cleaned thoroughly with RNase AWAY after probe mix formulation. Alternatively, probe mixes can be made in PCR workstations that are decontaminated with UV light. Change gloves after handling probe mixes to avoid cross-contamination.

Due to the high sensitivity of this assay, it is recommended that you change pipette filter tips for every step, change gloves frequently, and use fresh wipes to remove excess liquids.

For nCounter readout: Not all protein modules are compatible with one another. Some combinations require Substitute Probe R for nCounter readout. Plan your modules by referring to Appendix IV: Substitute Probe R Guidance on page 54.

1. **Mix** the detection Ab mix by flicking, then spin down. **Do not vortex.**

   Morphology marker antibodies are stored at 4°C and aliquoting is not required. Each tube of Protein Core/ Module (Ab mix) for nCounter readout contains sufficient reagent for 12 slides (112 µL). If using the entire Ab mix in one week, store at 4°C. If not, **aliquot the Ab mix** into 4-slide aliquots (37 µL + 37 µL + 38 µL) and freeze unused aliquots at -80°C. Do not exceed more than 2 freeze/thaw cycles and do not freeze diluted antibody.

2. **Make a working Ab solution** by diluting detection antibodies and morphology markers into Buffer W (Table 5). Adjust to reflect the number of core, module, and morphology reagents, and the number of slides to be prepared (**up to a total volume of 210 µL per slide**).

Table 5: Working antibody mix equation for protein slide prep for NGS or nCounter assays (*n* = number of slides).

Panel	Core Mix	Module 1	Each addl. Module*	Morph Marker1**	Morph Marker2	Addl Markers	Buffer W***	Final Volume
Immuno-oncology Proteome Atlas	8.4 µL x *n* (IPA tube 1 of 2)	52.5 µL x *n* (IPA tube 2 of 2)	8.4 µL x *n* (i.e., custom)	5.3 µL x *n*	5.3 µL x *n*	5.3 µL x *n* per marker	(up to 210 µL) x *n*	210 µL x *n*
All other panels (NGS or nCounter readout)	8.4 µL x *n*	8.4 µL x *n*	8.4 µL x *n* per module	5.3 µL x *n*	5.3 µL x *n*	5.3 µL x *n* per marker	(up to 210 µL) x *n*	210 µL x *n*

* If adding a custom-barcoded detection antibody, follow instructions in Appendix V: Adding Custom Barcoded Antibodies on page 55.

** If using non-NanoString morphology markers, optimal concentration in the working antibody mix must be determined by user testing.

*** If using a different number of detection or morphology reagents, Buffer W amount must be adjusted to bring total volume up to **210 µL/slide.**

3. **Remove slide from humidity chamber and remove excess liquid** by tapping the slide on a clean, absorbent surface, such as a paper towel, then using an absorbent wipe to carefully remove excess buffer from the slide, without touching the tissue.
4. **Place the slide back into the humidity chamber in a horizontal position. Cover the tissue with 200 µL of the diluted antibody solution**. Make sure the entire tissue is covered and no bubbles are present.

IMPORTANT: From this point on, minimize the slides' exposure to light to preserve the integrity of the photocleavable barcodes.

5. **Transfer the humidity chamber** to a 4°C refrigerator and **incubate overnight**. Ensure the humidity chamber stays level to avoid losing antibody solution.

7 Postfix (70 minutes)

Needed for this step: **staining jars, 1X TBS-T,** and **4% PFA**. See the Equipment, Materials, and Reagents lists on page 13 and Prepare Reagents steps on page 16.

> **IMPORTANT:** Everything that comes into contact with the antibody solution, such as containers for TBS-T, must be dedicated to this protocol and thoroughly cleaned with RNase AWAY, as probes may contaminate subsequent runs. Use separate staining jars for different probe mixes.

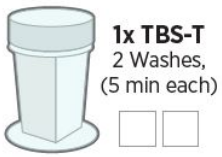
1. **Removing one slide at a time from the humidity chamber, carefully tap off the antibody solution from each slide** on a clean, absorbent surface, such as a paper towel.

2. Wash the slides in **3 washes of 1X TBS-T for 10 minutes each**.

> **IMPORTANT:** Washes are critical for best quality data. Do not shorten or skip washes.

3. **Removing one slide at a time, carefully tap off each slide** on a clean, absorbent surface to remove excess wash solution.
4. Ensure that the hydrophobic barrier is still intact or draw a fresh barrier over the old one using the hydrophobic pen.
5. Cover the sample with up to **200 µL 4% PFA** and **incubate for 30 minutes** in the humidity chamber at room temperature.

6. (Optional) Remove SYTO 13 nuclear stain from -20°C and allow it to warm to room temperature for use in a subsequent step.
7. Carefully tap each slide on clean, absorbent surface to remove excess 4% PFA. Wash slides in **two washes of 1X TBS-T for 5 minutes** each.



8 Nuclei staining (20 minutes)

Needed for this step: **humidity chamber, staining jars, razor blade, SYTO 13 nuclear stain, 1X TBS, and 1X TBS-T**. See the Equipment, Materials, and Reagents lists on page 13 and Prepare Reagents steps on page 16.

> **IMPORTANT:** Before using the humidity chamber in the following steps, clean it with RNase AWAY. Prepare the humidity chamber by lining with Kimwipes and adding just enough water to cover the bottom of the chamber.

1. Allow SYTO 13 to warm to room temperature.
2. Once thawed, **vortex then picofuge SYTO 13** for at least **1 minute** to bring the solution and insoluble particles to the bottom of the vial. When pipetting SYTO 13, pipette from the top of the vial to avoid insoluble particles.
3. **Dilute SYTO 13 1:10 in 1X TBS** (to final concentration of 500 nM). Prepare a sufficient volume per slide to completely cover tissue (~200 µL per slide). Mix by pipetting up and down. Close SYTO 13 stock vial tightly and store at -20°C.
4. **Remove one slide at a time from the 1X TBS-T,** remove excess liquid by tapping on a clean, absorbent surface, then place slides in humidity chamber in a horizontal position and **cover the tissue with diluted SYTO 13**.

5. **Stain for 15 minutes** at room temperature in the humidity chamber.
6. **Wash** slides by dipping into a staining jar with **1X TBS-T**.
7. **Transfer** to another staining jar with fresh **1X TBS-T**.
8. Working with one slide at a time, and dipping back into 1X TBS-T to avoid drying out, carefully **scrape off the hydrophobic pen** with a razor blade. Be sure to remove all of the wax without damaging or removing any of the tissue.

9. **Keep stained slides in 1X TBS-T** protected from light until loading on the GeoMx DSP (see the GeoMx DSP Instrument User Manual (MAN-10152)). For storage longer than 1 day, see next page. **DO NOT let slides dry out.**

Safe storage guidelines for protein slides

- **Storage for up to 1 day:** submerge in 1X TBS-T and store at 4°C, protected from light to maintain the integrity of the photocleavable barcodes.
- **Storage for 1 day to 3 months:**
    1. **Rinse slide** to be mounted with TBS-T or PBS-T. Touch the slide edge to a paper towel to remove excess liquid. Place slide on a flat surface.
    2. Using a pipette tip (200 µL tip works well), **add one drop (~50 µL) of Fluoromount-G** to the slide; add more as necessary to ensure the slide does not dry out and tissue is adequately covered.
    3. **Mount coverslip** by aligning one edge of the coverslip then slowly lowering from one side to the other. Remove excess mounting medium.
    4. **Allow slide to dry** at room temperature overnight, protected from light (e.g. in a bench drawer).
    5. Store slide at 4°C, protected from light, for up to 3 months.

Slide unmounting procedure

1. Submerge mounted slide in 1X TBS-T or PBS-T until coverslip is loose or has fallen off. With gentle agitation, the coverslip typically falls off within 15 minutes.
2. If coverslip has fallen off, slide is ready for use. If coverslip is still attached but loose, gently remove it using tweezers.
3. Wash slide with fresh 1X TBS-T for 5 minutes to ensure removal of mounting media.
4. SYTO 13 signal may have faded during storage. If it is necessary to re-stain with SYTO 13, repeat steps 1-9 on the previous page. It is typically not necessary to redraw the hyrophobic barrier.

Stripping and re-probing procedure for protein slides

GeoMx Protein assay slides are reusable and can be restained with a different commercial or custom panel following this procedure. This procedure requires a UV light box or transilluminator capable of emitting 302/312 nm UV light (example) and 1X TBS-T.

1. Place slide flat on the surface of a UV transilluminator.
2. Apply enough **1X TBS-T** to completely cover the tissue (50–200 µL depending on the size of the tissue).
3. **Expose to UV light** for **3 minutes** to cleave tags from bound antibodies.
4. Carefully tap slide on a clean, absorbent surface (e.g. paper towel) to remove liquid and avoid oligo contamination.
5. **Wash slide** by dipping in a staining jar with **1X TBS-T.**
6. Transfer to another staining jar with fresh **1X TBS-T**.
7. To apply new probes, proceed to Perform antigen retrieval (1 hour) on page 19.

RNA Slide Preparation Protocol (FFPE)

1 Equipment, Materials, and Reagents

The following tables list equipment, materials, and reagents that are required for this protocol but are **not supplied by NanoString**.

Table 6: Equipment for RNA slide prep not supplied by NanoString.

Equipment	Source, Catalog No.
Baking oven	Quincy Lab, Inc., various GC models (or comparable)
Hybridization oven including hybridization chamber*: HybEZ II Hybridization System **or** RapidFISH Slide Hybridizer	ACDBio, 321710/321720 Boekel Scientific, 240200 for 120V
Water bath (programmable to at least 37°C)	Various
5-quart steamer**	Hamilton Beach, 37530Z Nesco, ST-25F
Hot plate programmable up to 85°C NOTE: only needed for preparation of cell pellet tissue type	Various
Digital thermometer	Various including ThermoPro, TP01A or 1EasyLife
Picofuge	Various
Vortex	Various

*The listed hybridization ovens are validated for this protocol. These alternatives have been recommended by GeoMx DSP users, but have not been validated by NanoString: Abbott ThermoBrite, Leica ThermoBrite. Test to ensure slides remain hydrated overnight.

**The listed steamers are validated for this protocol. This alternative has been recommended by GeoMx DSP users, but has not been validated by NanoString: Russell Hobbs 19270-56.

Table 7: Materials for RNA slide prep not supplied by NanoString.

Materials	Source, Catalog No.
Pipettes for 5–1,000 µL	Various
Filter tips (DNase/RNase free)	Various
Microcentrifuge tubes (DNase/RNase free)	Various
Superfrost Plus microscope slides or Leica BOND Plus microscope slides (more adhesive; recommended for tissues prone to detaching from slides)	Fisher Scientific, 12-550-15 or Leica Biosystems, S21.2113.A
Slide staining jars (Coplin jars) (qty 16; recommend at least 2 of plastic for use in the steamer) and slide holder inserts	VWR, 25608-904, 25608-868 (or comparable)
Humidity chamber	Simport, M920-2 (select black lid) (or comparable)
HybriSlip hybridization covers (22 mm x 40 mm x 0.25 mm) NOTE: Other products have not been validated by NanoString.	Grace Bio-Labs, 714022
RNase AWAY® or 10% Bleach (RNaseZap® is not a substitute)	Thermo Fisher, 7003PK
Heat/cold protectant handling glove	Various
Forceps (for slide handling)	Various
Aluminum foil	Various
Kimwipes™	Various
USB drive v3.0, 64 GB or higher (able to be NTFS formatted)	SanDisk, SDCZ800-128G-G46 ; AmazonBasics LS21USB128G1; or comparable

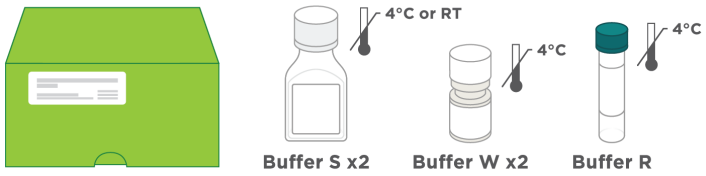
Table 8: Reagents for RNA slide prep not supplied by NanoString. RT = room temperature

Reagents	Source, Catalog No.	Storage
DEPC-treated water	Thermo Fisher, AM9922 (or comparable) NOTE: As an alternative to commercial DEPC-treated water, prepare your own following standard protocols.	RT
10X phosphate buffered saline pH 7.4 (PBS)	Sigma Aldrich, P5368-10PAK, P5368-5X10PAK (or comparable)	RT
10% neutral buffered formalin (NBF) 	EMS Diasum, 15740-04 (or comparable)	RT
100% deionized formamide 	Thermo Fisher, AM9342 or VWR, VWRV0606 (or comparable) NOTE: If deionized formamide is unavailable, molecular grade formamide may be substituted.	4°C (bring to RT before opening)
20X SSC (DNase/RNase free)	Sigma Aldrich, S6639	RT
Proteinase K 	Thermo Fisher, AM2546, AM2548 or 25530049 NOTE: Use of Proteinase K from any other vendor will require optimization of incubation times and concentration.	See manufacturer's instructions
10X Tris-EDTA pH 9.0 (Antigen Retrieval Solution, 10X concentrate)	Thermo Fisher (eBioscience™), 00-4956-58	RT
Tris base	Sigma Aldrich, 10708976001 (or comparable)	RT
Glycine	Sigma Aldrich, G7126 (or comparable)	RT
CitriSolv or Xylene or D-Limonene ((R)-(+)-Limonene) 	Fisher Scientific, 04-355-121 Sigma Aldrich, 183164-100ML or 183164-500ML (or comparable)	RT
100% ethanol (ACS grade or better) 	Various	RT
10% Tween20	Teknova, T0710 (or comparable)	RT

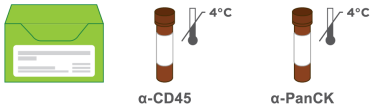
NanoString Reagents

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

GeoMx RNA Slide Prep Kit



GeoMx Morphology Kit - Human or Mouse RNA compatible (various available)



GeoMx Nuclear Stain Morphology Kit

For NGS readout:

GeoMx Probe Mix for NGS readout (various available; green or white cap)

Custom RNA-NGS Probe Mix (optional)

For nCounter readout:

GeoMx Probe Mix for nCounter readout

Custom RNA-nCounter Probe Mix (optional)

Be sure to use probe mixes for manual (non-automated) RNA slide preparation. Probe mixes for fully automated slide preparation on BOND RX/RX^m (yellow label, red cap) are not compatible with this manual RNA slide preparation protocol. Refer to GeoMx DSP Automated Slide Preparation User Manual (MAN-10151) for information about automated protocols.

Prepare reagents

Prepare the reagents using the dilution instructions (Table 9). **Use DEPC-treated water for all dilutions.** The actual volume of reagents used in the protocol will vary – the volumes to prepare in Table 9 are suggestions.

> **IMPORTANT:** Take care to maintain nuclease-free conditions. The greatest risk of contamination comes from GeoMx probes and other oligos. We recommend the use of RNase AWAY (Thermo Fisher 7002) for cleaning of all surfaces and equipment, as it will limit contamination from oligos, GeoMx probes and nucleases. After using RNase AWAY, allow area/items to air dry completely, or rinse with DEPC-treated water. See manufacturer's instructions for details.

Table 9: Reagent prep for RNA slide preparation.

Reagent	Dilution	Storage
95% EtOH	Prepare **500 mL** of 95% ethanol by adding **25 mL** of DEPC-treated water to **475 mL** of 100% ethanol. NanoString recommends to prepare fresh each week.	RT
1X PBS pH 7.4	Prepare **1 L** of 1X PBS by combining **100 mL** of 10X PBS and **900 mL** of DEPC-treated water. Do not reuse.	RT
Proteinase K	Default concentration (**1 µg/mL**) is made by adding **10 µL** of **20 mg/mL** Proteinase K to **200 mL** of **1X PBS** made with DEPC-treated water. See Table 10 for alternative concentrations by tissue type. Note: Prepare fresh and do not reuse. Take care to pipette accurately. Inaccurate concentration of Proteinase K will affect assay performance.	n/a
NBF stop buffer	Add **12.12 g** tris base and **7.5 g** glycine to **1 L** DEPC-treated water to yield 0.1 M tris, 0.1 M glycine. Store at RT up to 1 month.	RT
2X SSC	Prepare **1 L** of 2X SSC by combining **100 mL** of 20X SSC and **900 mL** of DEPC-treated water. Do not reuse.	RT
2X SSC-T (optional)	Prepare **250 mL** of 2X SSC-T by combining **25 mL** of 20X SSC, **2.5 mL** of 10% Tween20, and **222.5 mL** of DEPC-treated water. Do not reuse.	RT
4X SSC	Prepare **1 L** of 4X SSC by combining **200 mL** of 20X SSC and **800 mL** of DEPC-treated water. Do not reuse.	RT
1X Tris-EDTA pH 9 (Antigen Retrieval Solution)	Prepare **1 L** of 1X Tris-EDTA pH 9.0 by combining **100 mL** of 10X Tris-EDTA pH 9.0 and **900 mL** of DEPC-treated water.	RT

2 Prepare tissue samples

Appendix I: Selecting and Sectioning FFPE Samples on page 45 covers FFPE block selection and sectioning in detail. Review it prior to beginning the RNA Slide Preparation protocol.

GeoMx has been validated for sample blocks up to 3 years old prepared from tissues with a cold ischemic time of less than 1 hour using 10% NBF or similar fixative. For best results, do not use FFPE blocks more than 10 years old. Assay performance will be influenced by tissue block age and treatment conditions such as cold/warm ischemic time, fixative, and storage.

Slide Preparation

1. Tissue sections should be **5 µm** thick and mounted on **Superfrost Plus or BOND Plus slides.** Tissue sections must be placed in the **Scan Area** (shown in green in Figure 4) in the center of the slide and be no larger than 35.3 mm long by 14.1 mm wide. Tissue should not overlap the slide gasket (shown in blue) or the tip calibration area (shown in red). If mounting multiple sections per slide, ensure that tissues are at least 2–3 mm apart and still fit within the Scan Area. Adhesive slide labels should not overlap Scan Area or slide gasket.

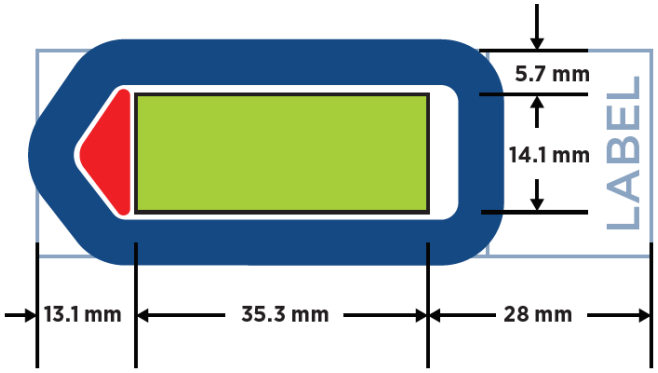


Figure 4: Slide dimensions. Measure from label edge of slide as reference point.

If sections are too large and/or placed off-center, continue with slide preparation as usual. Just before loading the slide in the instrument slide tray, scrape off the parts of the tissue exceeding the scan area, making sure the slide gasket and tip calibration area are free of tissue. Scraping off tissue before slide preparation could generate tissue folds that may result in staining or binding artifacts.

IMPORTANT: The GeoMx DSP instrument will only image the area inside the Scan Area. **Tissue outside of the Scan Area will not be imaged and may cause problems in tissue detection by the optical system.**

2. **Bake slides with mounted sections** in a 60°C drying oven for 30 minutes to 3 hours prior to deparaffinization. Stand slides vertically during baking to allow excess paraffin to flow off. Longer baking times may be necessary for some tissues to sufficiently adhere to the slide (e.g., overnight at 37°C followed by 2-4 hr at 60°C); this should be empirically tested.

3 Deparaffinize and rehydrate FFPE tissue sections (35 minutes)

Needed for this step: **Citrisolv** (or acceptable substitute)**, 100% EtOH, 95% EtOH,** and **1X PBS**.

The **steamer, staining jars, 1X Tris-EDTA (pH 9.0)** (Antigen Retrieval Solution), **DEPC-treated water, water bath** and **Proteinase K solution** are preheated here for their use in a later step. (To prepare cell pellet samples, use a hot plate set to 85°C, rather than the steamer, as described on next page). See the Equipment, Materials, and Reagents lists on page 27 for more details.

1. **Fill the steamer** reservoir up to the fill line with water. Place two staining jars inside, one containing **DEPC-treated water** and one containing **1X Tris-EDTA (pH 9.0)** (Antigen Retrieval Solution). Ensure sufficient reagent volume to cover slides up to the label. Loosely cover each jar with aluminum foil instead of the jar lid to allow for a thermometer reading in a later step. **Preheat the steamer to 100°C.** More water may need to be added to the steamer during preheating.

   The Nesco steamer takes 1 hour to heat the liquid in the jars to a stable maximum temperature near 100°C. Final temperature can be checked by inserting a digital thermometer through the hole in the lid of the steamer into the staining jars.

2. **Deparaffinize and rehydrate FFPE tissue sections**. Place slides in a rack and perform the following washes in staining jars (Figure 5). Ensure you have sufficient buffer volume to cover all slides. Slides should be dipped up and down gently several times when placing in and before removing from staining jars. After the last wash, slides can be stored in the 1X PBS for up to one hour.

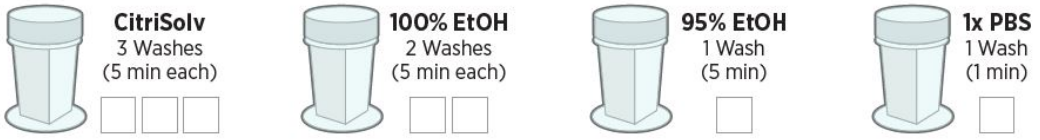


Figure 5: Wash steps

⚠ **WARNING:** Dispose of CitriSolv or its substitute in accordance with your lab's safety procedures.

3. During wash steps, **preheat the water bath to 37°C**. Prepare the Proteinase K dilution, if not yet done, and **add the diluted Proteinase K solution to a staining jar and place in the water bath to preheat to 37°C**. Refer to Table 10 for the recommended Proteinase K concentration for your tissue type.

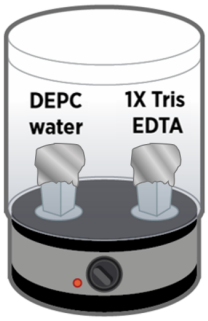
4 Perform target retrieval (25 minutes)

Needed for this step: **Steamer, Staining jars, 1X Tris-EDTA (pH 9.0)** (Antigen Retrieval Solution), **DEPC-treated water** (all preheated in the previous step) and **1X PBS**. See the Equipment, Materials, and Reagents lists on page 27 and Prepare Reagents steps on page 31.

Plastic staining jars are recommended for this step to avoid the risk of glass breaking. If using a glass jar, be aware of the risk of glass breaking during incubation or upon removal of the jar to room temperature.

(To prepare cell pellet samples, use a hot plate set to 85°C, rather than the steamer, to preheat buffers and for target retrieval (Table 10). Follow the steps as described below to move slides between buffers).

1. Without removing the lid, place an instant-read digital thermometer through the vents in the steamer lid and pierce the aluminum foil covering the 1X Tris-EDTA. Ensure the 1X Tris-EDTA has reached ~99°C. Reset the steamer's timer to ensure consistent heating during incubation and add more water as needed.



⚠ **WARNING:** Removing the steamer lid releases high-temperature steam. Use a thermal protection glove with full hand coverage and transfer slides using forceps or rack.

2. Once the 1X Tris-EDTA has reached 99°C, **carefully remove the steamer lid** and jar covers. Dip the slides into the **DEPC-treated water** for **10 seconds** to bring the slide temperature up to ~99°C. Quickly transfer the slides to the **1X Tris-EDTA**. Replace jar cover, then replace steamer lid.

ⓘ **IMPORTANT:** The steamer temperature will plateau at ~100°C. Once the lid is removed, the temperature of the buffers will fall rapidly. Try to limit the time the steamer is uncovered to 30 seconds (maximum uncovered time is 2 minutes). Reproducible results rely on minimizing this variation in temperature.

3. Incubate the slides according to the conditions for different tissue types in Table 10. Incubation times and temperatures may need to be empirically determined. If the tissue type you wish to use is not listed, start with 15 minutes in your empirical testing.

Epitope retrieval times were determined based on FFPE tissue blocks meeting the constraints outlined in the sample guidance section. Samples were primarily tumor with minimal normal adjacent tissue. These conditions may vary by sample, the amount of normal adjacent tissue, and other factors. These conditions were optimized for large tumor sections and may not apply to arrayed tissues, cored tissues, and needle biopsies.

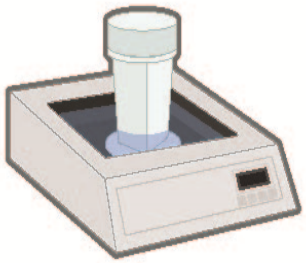
If preparing fresh frozen or fixed frozen samples instead of FFPE, target retrieval time is 15 minutes. See Appendix II: Modifications to Protocol for Fresh Frozen Samples on page 47 or Appendix III: Modifications to RNA Protocol for Fixed Frozen Samples on page 50 for more information.

4. When target retrieval time is up, **move slides to room temperature 1X PBS immediately**.
5. **Wash in room temperature 1X PBS for 5 minutes**. Slides can be stored up to 1 hour in 1X PBS.

5 Expose RNA targets (10–30 minutes)

Needed for this step: **preheated water bath, preheated Proteinase K dilution,** and **1X PBS**. See the Equipment, Materials, and Reagents lists on page 27 for more details.

1. **Incubate slides** in Proteinase K solution at 37°C according to the conditions for different tissue types in Table 10. Proteinase K concentration and incubation times may need to be empirically determined. If the tissue type you wish to use is not listed, start with a concentration of 1 µg/mL for 15 minutes.
2. **Wash slides in 1X PBS for 5 minutes.** During the wash, ensure that the **10% NBF** and **NBF Stop Buffer** needed in the next step are ready. **Proceed to the next step immediately.**



Proteinase K digestion conditions were determined based on FFPE tissue blocks meeting the constraints outlined in the sample guidance section. Samples were primarily tumor with minimal normal adjacent tissue. Optimization may be required for your sample types. The values listed above are recommended starting points. Use of Proteinase K from vendors other than those specified will require optimization of incubation times and concentration.

If preparing fresh frozen or fixed frozen samples instead of FFPE, digest with Proteinase K at 1 µg/mL for 15 minutes. See Appendix II: Modifications to Protocol for Fresh Frozen Samples on page 47 or Appendix III: Modifications to RNA Protocol for Fixed Frozen Samples on page 50 for more information.

Table 10: Conditions for target retrieval and Proteinase K digest by tissue type.

Tissue Type (FFPE)	Target Retrieval	Proteinase K Digest
Validated by NanoString		
Breast	20 min	0.1 µg/mL for 15 min
Cell pellet	15 min @ 85°C (use hot plate instead of steamer)	1 µg/mL for 5 min
Colorectal	20 min	1 µg/mL for 15 min
Melanoma	20 min	1 µg/mL for 15 min
Mouse tissue (see below for mouse brain/spinal cord)	20 min	1 µg/mL for 15 min
NSCLC	20 min	1 µg/mL for 15 min
Prostate tumor	20 min	1 µg/mL for 15 min
Tonsil	15 min	1 µg/mL for 15 min
Not validated - provided as suggestion for empirical testing		
Adipose	10 min	0.1 µg/mL for 15 min
Bone	10 min	0.1 µg/mL for 15 min
Bone marrow	20 min	0.1 µg/mL for 15 min
Brain	20 min	0.1 µg/mL for 15 min
Esophageal	20 min	1 µg/mL for 20 min
Gastric	20 min	1 µg/mL for 15 min
Kidney	20 min	1 µg/mL for 15 min
Liver	20 min	1 µg/mL for 15 min
Lung	20 min	1 µg/mL for 15 min
Lymph node	15 min	1 µg/mL for 15 min
Mouse brain/spinal cord	20 min	0.1 µg/mL for 15 min
Mucosa	20 min	0.1 µg/mL for 10 or 15 min
Olfactory bulb	20 min	0.1 µg/mL for 10 or 15 min
Ovarian cancer	20 min	1 µg/mL for 15 min
Pancreas	20 min	1 µg/mL for 15 min
Retina	10 min	0.1 µg/mL for 10 or 15 min
Sarcoma	20 min	1 µg/mL for 15 min
Skeletal muscle	20 min	1 µg/mL for 15 min
Skin	20 min	1 µg/mL for 15 min
Uterine	20 min	1 µg/mL for 15 min

6 Postfix: Preserve tissue morphology for soft tissues (20 minutes)

IMPORTANT: If preparing fixed frozen samples instead of FFPE, DO NOT perform this Postfix step. Proceed to In situ hybridization (overnight) on page 38. See Appendix III: Modifications to RNA Protocol for Fixed Frozen Samples on page 50 for more information.

Needed for this step: **Staining jars, 10% NBF, NBF Stop Buffer** and **1X PBS**. See the Equipment, Materials, and Reagents lists on page 27 and Prepare Reagents steps on page 31.

WARNING: Use of appropriate personal protective equipment is advised. Used NBF Stop Buffer contains NBF and must be disposed of in the same manner as the NBF.

Post-fix the tissue by performing these washes (Figure 6):

Figure 6: Post-fix wash steps

Slides can be stored in the final 1X PBS wash up to 1 hour at room temperature or 6 hours at 4°C.

7 *In situ* hybridization (overnight)

Needed for this step: **hybridization chamber, hybridization oven, Buffer R, RNA Probe Mix, DEPC-treated water** and (optional) **2X SSC.** See the Equipment, Materials, and Reagents lists on page 27 for more details.

> **IMPORTANT:** Probe mixes should be handled in an area separate from nCounter work, NGS library prep, or other GeoMx workflows. GeoMx detection reagents can cross-contaminate probe mixes and give misleading or incorrect results. Areas should be cleaned thoroughly with RNase AWAY after probe mix formulation. Alternatively, handle probe mixes in PCR workstations that are decontaminated with UV light. Change gloves after handling any probe mixes to avoid cross-contamination.

Due to the high sensitivity of this assay, it is recommended that you change pipette filter tips for every step, change gloves frequently, and use fresh wipes to remove excess liquids.

1. **Prepare reagents**: Warm Buffer R and RNA detection probes to room temperature before opening. (Warming RNA Probe Mix reduces viscosity, improving pipetting accuracy.) Before use, flick to mix, then spin down. Store unused RNA Probe Mix at 4°C for up to 6 months or re-freeze.
2. **Clean the hybridization chamber and other equipment with RNase AWAY** and allow to dry or rinse with DEPC-treated water. The hybridization chamber can be a key source of contamination by oligos. Arrange fresh Kimwipes on the bottom of the chamber and wet with 2X SSC or DEPC-treated water. Kimwipes should be thoroughly damp, but liquid should not pool. If your chamber is light-permeable, wrap the lid in aluminum foil to minimize light exposure.

   If your hybridization chamber does not seal with a gasket, place the chamber in a zip-lock bag to simulate a sealed chamber. Chambers sealed in this manner should be tested prior to use to ensure they maintain humidity (i.e. that slides do not dry out) for 24 hours. Unsealed chambers can result in evaporation of the hybridization solution.

3. **Make hybridization solution** following Table 11 for NGS assays or Table 12 for nCounter assays. Confirm that you are using probe mix for manual/semi- automated slide preparation (white label, green/white/amber cap) and not probe mix for fully automated slide prep (yellow label, red cap).

For NGS readout:

Table 11: Hybridization solution for assays with NGS readout (Atlas = Whole Transcriptome Atlas (WTA), Cancer Transcriptome Atlas (CTA), or Canine Cancer Atlas (CCA)). *n* = number of slides

Panel Configuration	Buffer R	Atlas Probe Mix	First Custom/Add-on Probe Mix	Second Custom/Add-on Probe Mix	DEPC-treated H_2O	Final Volume
Atlas (WTA, CTA, or CCA)	200 µL x *n*	25 µL x *n*	0	0	25 µL x *n*	250 µL x *n*
Atlas + 1 custom/add-on assay*	200 µL x *n*	25 µL x *n*	12.5 µL x *n*	0	12.5 µL x *n*	250 µL x *n*
Atlas + 2 custom/add-on assays*	200 µL x *n*	25 µL x *n*	12.5 µL x *n*	12.5 µL x *n*	0	250 µL x *n*
1 custom assay, standalone (no Atlas)	200 µL x *n*	0	12.5 µL x *n*	0	37.5 µL x *n*	250 µL x *n*
2 custom assays, standalone (no Atlas)	200 µL x *n*	0	12.5 µL x *n*	12.5 µL x *n*	25 µL x *n*	250 µL x *n*

**Custom/add-on assay* is a custom assay or add-on assay, such as the TCR Profiling Add-on. An Atlas may be combined with up to 1 custom and 1 add-on or 2 custom assays.

For nCounter readout:

Table 12: Hybridization solution for assays with nCounter readout. *n* = number of slides

Panel Configuration	Buffer R	Immune Pathways Panel	Custom Probe Mix	DEPC-treated H_2O	Final Volume
RNA Immune Pathways Panel	200 µL x *n*	37.5 µL x *n*	0	12.5 µL x *n*	250 µL x *n*
RNA Immune Pathways Panel + 1 custom assay	200 µL x *n*	37.5 µL x *n*	12.5 µL x *n* *	0	250 µL x *n*

*Only one custom assay may be added to the Immune Pathways Panel.

4. **One at a time, remove slides from 1X PBS,** wipe away excess liquid, and set in hybridization chamber in a horizontal position. **Take care not to let the slides dry out.**
5. Ensure that the Kimwipes and liquid do not contact the slides. Hybridization solution can wick off of the slides if it comes into contact with Kimwipes or liquid.
6. **Add 200 µL hybridization solution** to each slide. Take care not to introduce any bubbles.

To avoid bubbles, leave a small residual volume in the pipette tip. If a bubble forms, aspirate it gently with the pipette. Do not touch the tissue with the tip. It is preferable to lose some hybridization solution and remove bubbles than to have bubbles in the solution, as long as sufficient solution remains to cover the tissue after the coverslip is applied.

IMPORTANT: From this point on, minimize the slides' exposure to light to preserve the integrity of the photocleavable barcodes.

7. **Gently apply a Grace Bio-Labs HybriSlip**. Start by setting one edge of the coverslip down in solution on the slide, then gradually laying down the rest of the coverslip to avoid the formation of air bubbles (Figure 7).

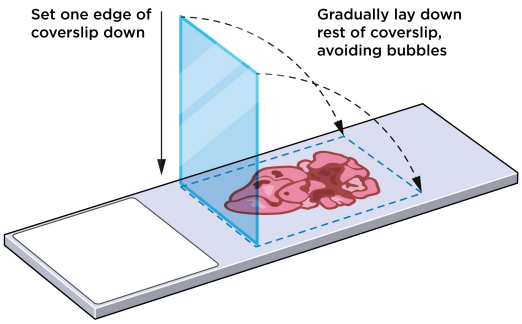


Figure 7: Applying coverslip

8. Repeat steps 4–7 for each slide.
9. Close hybridization chamber, insert into hybridization oven, and clamp into place (Figure 8). **Incubate at 37°C overnight** (16–24 hours).

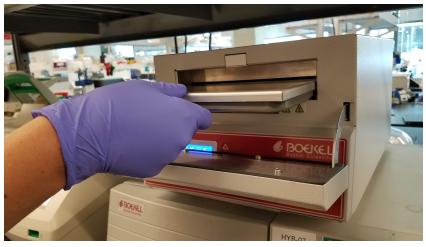


Figure 8: Placing chamber in oven

8 Perform stringent washes to remove off-target probes (90 minutes)

Needed for this step: **water bath, 4X SSC, 100% formamide**, **2X SSC**, and (optional) **2X SSC-T**. See the Equipment, Materials, and Reagents lists on page 27 and Prepare Reagents steps on page 31.

⚠ **WARNING:** Use of appropriate personal protective equipment is advised.

ⓘ **IMPORTANT:** Everything that comes into contact with the hybridization solution, such as containers for SSC, must be dedicated to this protocol and thoroughly cleaned with RNase AWAY, as probes may contaminate subsequent runs. Use separate staining jars for different probe mixes. Staining jars should be cleaned with RNase AWAY before each use.

1. Preheat water bath to 37°C.
2. **Warm 100% formamide** to room temperature before opening. Make **Stringent Wash** by mixing equal parts **4X SSC** and **100% formamide**. Fill two staining jars with **Stringent Wash** and preheat them in the 37°C water bath.

ⓘ **IMPORTANT:** The Stringent Wash **must** be at 37°C before washing the slides.

3. **Dip** slides in **2X SSC** allowing coverslips to slide off. Continue to wash steps within **5 minutes**.

If coverslips do not come off immediately, move them to 2X SSC-T for a maximum of 5 minutes. If coverslips have not fallen off in 5 minutes, proceed to the first Stringent Wash.

ⓘ **IMPORTANT:** Forcibly removing coverslips will damage the tissue. Allow the coverslips to slide off freely.

4. Perform **2 washes in Stringent Wash at 37°C for 25 minutes each**, then **2 washes in 2X SSC for 2 minutes each** (Figure 9). After the last wash, slides can be stored in 2X SSC for up to 1 hour.

Figure 9: Stringent washes to remove off-target probes

9 Add morphology markers (100 minutes)

Needed for this step: **humidity chamber, Buffer W, SYTO 13 nuclear stain, morphology markers,** and **2X SSC**. See the Equipment, Materials, and Reagents lists on page 27 and Prepare Reagents steps on page 31.

> **IMPORTANT:** Before using the humidity chamber in the following steps, clean it with RNase AWAY. Prep the humidity chamber by lining with Kimwipes wetted with 2X SSC or DEPC-treated water. Add just enough liquid to cover the bottom of the chamber.

1. **Remove SYTO 13 nuclear stain from -20°C** and allow it to warm to room temperature.
2. **Block with Buffer W:** Remove one slide at a time from 2X SSC and tap slide on clean, absorbent surface to remove excess liquid. Place slide in the humidity chamber. Cover tissue with up to **200 µL Buffer W** and leave at room temperature for 30 minutes, protected from light.

Ensure adequate Buffer W surrounds the edges of the tissues so the
Use a pipette tip to gently move the solution so there is a 2– 3 mm border around the tissue. A hydrophobic barrier can be used at this step if desired, but must be carefully removed with a razor blade before loading on the instrument.

3. **Once thawed, vortex then picofuge SYTO 13 for at least 1 minute** to bring the solution and insoluble particles to the bottom of the vial. When pipetting SYTO 13, pipette from the top of the vial.
4. **Prepare 220 µL of morphology marker solution per slide** (Table 13).

Table 13: Morphology marker solution (*n* = number of slides)

Nuclear stain (SYTO 13)*	Morphology Marker 1	Morphology Marker 2	Additional Markers**	Buffer W***	Final Volume
22 µL x *n*	5.5 µL x *n*	5.5 µL x *n*	5.5 µL x *n* per marker	(up to 220 µL) x *n*	220 µL x *n*

* NanoString-provided SYTO 13 is 5 µM stock; final concentration 500 nM in morphology marker solution.

** If using non-NanoString morphology markers, optimal concentration in the morphology marker solution must be determined by user testing.

*** If using a different number of morphology markers, Buffer W amount must be adjusted to bring total volume up to 220 µL per slide.

5. **Mix morphology marker solution** by flicking then briefly picofuge.
6. **Remove Buffer W** from one slide at a time by tapping slide onto a Kimwipe, then return the slide to the humidity chamber.

7. **Cover tissue with morphology marker solution (up to 200 µL).** Repeat steps 6 and 7 for each slide.
8. Stain for **1 hour** in the humidity chamber at room temperature, protected from light.

9. After staining, **remove solution** by tapping slide on a clean, absorbent surface. **Wash in 2X SSC**, 2 times for 5 minutes each.

Slides can remain in 2X SSC until loading on the GeoMx DSP (see safe storage guidelines, below). **DO NOT let slides dry out.** If a hydrophobic barrier was used, carefully remove it before loading on the instrument (see instructions on page 24).

Load slides onto the GeoMx DSP following the GeoMx DSP Instrument User Manual (MAN-10152).

Safe storage guidelines for RNA slides

- Storage up to 6 hours: submerge in **2X SSC** and store at **room temperature, protected from light.**
- Storage from 6 hours to 21 days: submerge in **2X SSC** and store at **4°C, protected from light.** For best results, minimize storage time between slide preparation and loading on the GeoMx DSP.
- If it is necessary to refresh morphology markers and/or nucleic acid stain: repeat step Add morphology markers (100 minutes) on page 42; skip the blocking step, and incubate marker solution for 30 minutes.

FFPE slides can be stored for 21 days in 2X SSC at 4°C without reducing the number of genes detected in tissue microarray and cell pellet array samples. Morphology marker signal remained functional for the duration of the study. SYTO 13 signal decreased, but remained functional. Nuclear or morphology marker stain can be repeated prior to scanning if deemed necessary (see bullet above).

Stripping and re-probing procedure for RNA slides

GeoMx RNA assay slides are reusable and can be restained with a different commercial or custom panel following this procedure. This procedure requires a UV light box or transilluminator capable of emitting 302/312 nm UV light (example), 2X SSC-T, 1X Tris-EDTA, and 2X SSC.

1. Place the slide flat on the surface of a UV transilluminator.
2. Apply enough **2X SSC-T** to completely cover the tissue (50–200 µL depending on the size of the tissue).
3. **Expose to UV light** for **3 minutes** to cleave tags from bound probes.
4. Carefully tap each slide on a clean, disposable surface (e.g., paper towel) to remove liquid and avoid oligo contamination.
5. **Wash slides** by dipping in a staining jar with **2X SSC-T.**
6. Transfer to another staining jar with fresh **2X SSC-T**.
7. Incubate the slides in **1X Tris-EDTA at 85°C** using either a hotplate or a pressure cooker on a low pressure setting for 15 minutes.
8. Wash 3 times in **2X SSC**.
9. Proceed to In situ hybridization (overnight) on page 38.

Appendices

Appendix I: Selecting and Sectioning FFPE Samples

When preparing, sectioning, and storing FFPE blocks for use in the GeoMx DSP Protein and RNA assays, take care to preserve sample integrity at all steps. The integrity of FFPE samples is impacted by many factors including time from excision to fixation, storage conditions, tissue type, and sample age. Samples with poor integrity are likely to give low signal, particularly in RNA assays.

GeoMx has been validated for sample blocks up to 3 years old prepared from tissues with a cold ischemic time of less than 1 hour using 10% NBF or similar fixative. For best results, do not use FFPE blocks more than 10 years old. Assay performance will be influenced by tissue block age and treatment conditions such as cold/warm ischemic time, fixative, and storage.

Selecting FFPE blocks

FFPE blocks should meet the following criteria for the best performance with GeoMx DSP assays.

1. Blocks should be fixed in 10% neutral buffered formalin for 18 to 24 hours at room temperature. This applies to tissues less than 0.5 cm in thickness. Larger tissues have not been tested by NanoString and may require longer fixation times.
2. Tissues should be fixed immediately after excision for best results. Fixation within one hour post-excision is acceptable.
3. Tissues should be thoroughly dehydrated in ethanol gradients prior to embedding in paraffin.
4. FFPE blocks should be stored at room temperature and ambient humidity.
5. For best results, do not use FFPE blocks that are more than 10 years old.

Sectioning FFPE blocks

The following are general guidelines for sectioning FFPE blocks for optimal GeoMx DSP assay performance. This is not meant to be an all-inclusive guide on sectioning. Please refer to your local pathologist or core facility for training on sectioning.

- Due to oxidation at the surface, discard the first few sections cut from the block face.
- NanoString recommends SuperFrost Plus slides (for manual slide preparation) or BOND Plus or Apex BOND slides (for BOND automated slide preparation or manual slide preparation for tissues that exhibit poor adhesion).
- FFPE tissue sections should be cut 5 µm thick on a calibrated microtome and mounted on the slide immediately, without scratches or folds.
- Tissue sections must be placed in the **Scan Area** (shown in green in Figure 10) in the center of the slide and be no larger than 35.3 mm long by 14.1 mm wide. They should not overlap the slide gasket (shown in blue) or the tip calibration area (shown in red). If mounting multiple sections per slide, ensure that tissues are at least 2–3 mm apart and still fit within the Scan Area. Adhesive slide labels should not overlap Scan Area or slide gasket.

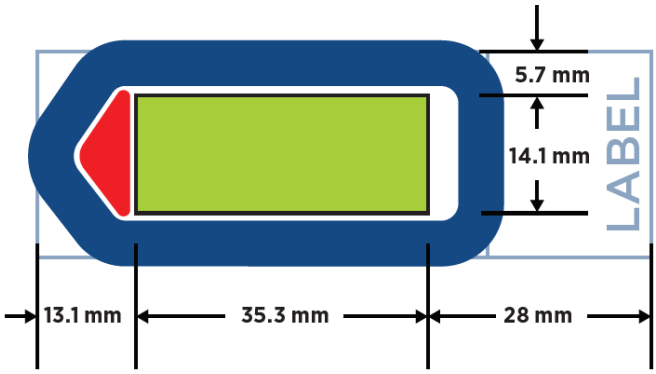


Figure 10: Slide dimensions. Measure from label edge of slide as reference point.

IMPORTANT: The GeoMx DSP instrument will only image the area inside the Scan Area. **Tissue outside of the Scan Area will not be imaged and may cause problems in tissue detection by the optical system.**

- Any water trapped under the wax or tissue section should be removed by gently touching a folded Kimwipe to the corner of the wax section. The Kimwipe should not contact the tissue.
- Air dry mounted slides overnight prior to use. Store slides in a vertical position such that any remaining water can drain away from the tissue section.
- Slides stored in a desiccator (or in a sealed container with a desiccant pouch) at 4°C or room temperature have been shown to yield quality results for up to 3 months. Quality of results is tissue and block dependent and should be tested empirically.

Appendix II: Modifications to Protocol for Fresh Frozen Samples

Disclaimers

- Human fresh frozen tissues can carry pathogens and should be handled according to your institution's lab safety guidelines.
- It is essential to minimize RNases when processing fresh frozen tissues for RNA assays. Ensure all buffers are made with DEPC-treated water and all equipment is RNase-free.
- NanoString has not optimized its assays for fresh frozen tissues and recommends that empirical optimization be performed for your samples.

> **IMPORTANT:** For any given study, NanoString recommends using only one sample type preparation method (e.g., FFPE or fresh frozen samples, but not a combination of sample types).

Selecting fresh frozen blocks

- Select tissues that are known to have been snap frozen in liquid nitrogen as quickly as possible after dissection. Any buffers used to wash or temporarily store tissues before fixation should be free of nuclease contamination.
- Frozen tissues should be embedded in Optimal Cutting Temperature (OCT) compound before sectioning.
- Blocks embedded in OCT should be stored at -80°C.

Sectioning fresh frozen blocks

The following are general guidelines for sectioning fresh frozen blocks for optimal GeoMx DSP assay performance. See also Sample Sectioning Tips and Tricks for CosMx SMI and GeoMx DSP Experiments (MAN-10175) or standard histopathology references.

- Due to oxidation at the surface, discard the first few sections cut from the block face.
- NanoString recommends SuperFrost Plus slides (for manual slide preparation) or BOND Plus or Apex BOND slides (for BOND automated slide preparation or manual slide preparation for tissues that exhibit poor adhesion).
- Fresh frozen sections should be cut at 5–10 µm thickness on a calibrated cryostat and mounted immediately on the slide, without scratches or folds.

- Tissue sections must be placed in the **Scan Area** (shown in green in Figure 11) in the center of the slide and be **no larger than 35.3 mm long by 14.1 mm wide**. They should not overlap the slide gasket (shown in blue) or the tip calibration area (shown in red). If mounting multiple sections per slide, ensure that tissues are at least 2–3 mm apart and still fit within the Scan Area. Adhesive slide labels should not overlap Scan Area or slide gasket.

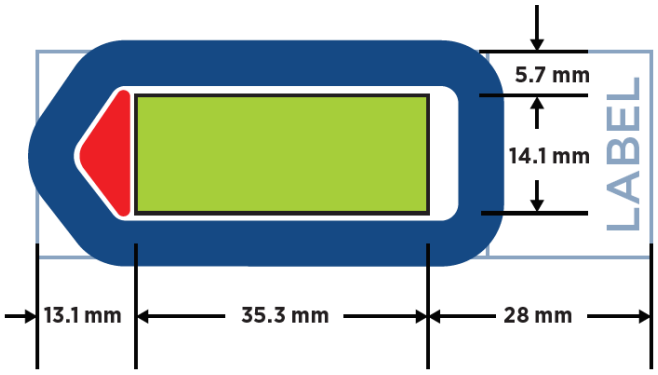


Figure 11: Slide dimensions. Measure from label edge of slide as reference point.

IMPORTANT: The GeoMx DSP instrument will only image the area inside the Scan Area. **Tissue outside of the Scan Area will not be imaged and may cause problems in tissue detection by the optical system.**

- After sectioning, the exposed block face should be covered with OCT to avoid desiccation of the sample.
- Slides can be stored at -80°C for several weeks before use.

Fresh frozen sample preparation for protein assays

Reagents required for this protocol: 10% NBF, 1X TBS-T.

1. **In a BSL2 room, handle fresh frozen slides in a biosafety cabinet.** Remove the slides from -80°C. **Submerge in 10% NBF overnight (12–16 hours) at room temperature** to thaw and fix tissue. Fixation at 4°C is also suitable. Thorough fixation is required to maintain tissue integrity.
2. After fixation, wash the slides 3 times in 1X TBS-T for 5 minutes each wash, using a new staining jar for each wash.
3. Turn to Step 4 of the **Protein Slide Preparation** protocol, Perform antigen retrieval (1 hour) on page 19. Proceed from that step forward without modification to the protocol.

NOTE: If fresh frozen tissue falls off the slide during the workflow, the tissue may benefit from the baking and ethanol wash steps described below for RNA assays. These steps have not yet been validated for protein assays, and are provided as guidance only.

Fresh frozen sample preparation for RNA assays

Reagents required for this protocol: 10% NBF, 1X PBS, 50% ethanol, 70% ethanol, 100% ethanol.

1. **In a BSL2 room, handle fresh frozen slides in a biosafety cabinet.** Remove the slides from -80°C. **Submerge in 10% NBF overnight (12–16 hours) at room temperature** to thaw and fix tissue. FIxation at 4°C is also suitable. Thorough fixation is required to maintain tissue integrity.
2. Transfer slides from 10% NBF to 1X PBS. Wash the slides 3 times, for 2 minutes each wash.
3. Transfer slides from 1X PBS to a slide rack and bake at 60°C for 30 minutes.
4. Wash the slides in 50% ethanol for 5 minutes.
5. Wash the slides in 70% ethanol for 5 minutes.
6. Wash the slides in 100% ethanol 2 times for 5 minutes each wash.
7. Let slides air dry for at least 5 minutes (but not more than 1 hour).

Turn to Step 4 of the **RNA FFPE Slide Preparation** protocol, Perform target retrieval (25 minutes) on page 34. Proceed from that step forward **with these modifications for your fresh frozen samples:**

- **Default target retrieval conditions are 100°C for 15 minutes**. 85°C for 15 minutes may improve tissue integrity in more delicate tissues, such as brain and cell pellets. It may also improve some morphology markers' performance, at the cost of a moderate reduction in the RNA assay's efficiency. Timing and temperature may need to be empirically determined for different tissue types and samples.
- **Default Proteinase K digestion conditions are 1 µg/mL Proteinase K for 15 minutes**. Proteinase K concentration and incubation time may need to be empirically determined for different tissue types and samples.

Appendix III: Modifications to RNA Protocol for Fixed Frozen Samples

This protocol is for the preparation of fixed frozen (FxF) samples for **RNA assays** only.

Tissue that has undergone an initial perfusion-based fixation is preferred, as that process rapidly inactivates endogenous RNases and preserves tissues in a uniform manner. Tissue immersed in fixative post-harvest is also acceptable.

> **IMPORTANT:** For any given study, NanoString recommends using only one sample type preparation method (e.g., FFPE or fresh frozen samples, but not a combination of sample types).

Preparing fixed frozen tissue block from harvested tissue

Reagents required for this protocol: 10% NBF, 10X PBS, DEPC-treated water, sucrose, OCT compound.

Prepare reagents (1 hour)

All buffers should be made with DEPC-treated water to minimize RNase contamination.

- Prepare sucrose solutions as follows:
    1. Weigh out sucrose:
        a. **30% Sucrose**: 15 g sucrose in a 50 mL conical tube
        b. **20% Sucrose**: 10 g sucrose in a 50 mL conical tube
        c. **10% Sucrose**: 5 g sucrose in a 50 mL conical tube
    2. With a tissue culture-grade disposable pipette, add DEPC-treated water up to the 50 mL mark of each tube.
    3. Centrifuge each tube.
- Prepare **50:50 solution of OCT : 30% sucrose** as follows:
    1. As OCT compound is too viscous to pipette, dispense 5 mL of OCT by pouring into a 15 mL conical tube up to the 5 mL mark.
    2. Add 5 mL of 30% sucrose (prepared above) using a tissue culture-grade serological pipette.
    3. Mix by inverting several times, then mix using a serological pipette until the two components have formed a homogenous mixture. Be careful not to introduce bubbles.

Prepare fixed frozen tissue block (2 days)

Incubations should be performed in a 50 mL conical tube. The volume of solution for each incubation should exceed the volume of the tissue by a factor of 15. Agitation should be gentle, and the tube should be positioned at an angle such that the entire solution volume is swirled over the tissue with each rotation.

1. After the mouse has been anesthetized (and ideally perfused with fixative) and euthanized according to an IACUC-approved protocol, harvest tissue. Tissue should be harvested quickly but gently using clean dissection tools. It is important to perform this step as quickly as possible to minimize RNA degradation, especially if the tissue has not undergone a perfusion-based fixation step.
2. Cut tissue such that the maximum size in one dimension does not exceed 10 mm. This size restriction enables adequate fixative penetration into the center of the tissue.
3. Fix tissues in 10% NBF at room temperature for 24 hours with gentle shaking. Fixation at 4°C is also suitable.
4. The next day, wash 2 times in 1X PBS for 30 minutes each wash.
5. Incubate in each of the following solutions, in order. For each incubation, shake on ice for 1.5 to 2 hours, or until the tissue sinks to bottom of tube (indicating tissue is adequately equilibrated).
    a. 10% sucrose
    b. 20% sucrose
    c. 30% sucrose
6. While processing tissue in 30% sucrose, prepare a dry ice/ethanol bath. Place an aluminum block with its top exposed in the center of the bath and let chill for 30 minutes.
7. Place tissue in 50:50 OCT:30% sucrose for 30 minutes on ice.
8. Place a few drops of OCT into a Tissue-Tek Cryomold.
9. Place the tissue into the cryomold and cover with OCT, minimizing the formation of bubbles.
10. Incubate for 15 minutes at room temperature.
11. Freeze samples in OCT by placing on the aluminum block in the dry ice/ethanol bath for 10 minutes.
12. Tightly wrap frozen sample in foil and store at -80°C.

Sectioning fixed frozen blocks

The following are general guidelines for sectioning fixed frozen blocks for optimal GeoMx DSP assay performance. See also Sample Sectioning Tips and Tricks for CosMx SMI and GeoMx DSP Experiments (MAN-10175) or standard histopathology references.

- Due to oxidation at the surface, discard the first few sections cut from the block face.
- NanoString recommends SuperFrost Plus slides (for manual slide preparation) or BOND Plus or Apex BOND slides (for BOND automated slide preparation or manual slide preparation for tissues that exhibit poor adhesion).
- Fixed frozen sections should be cut at 5–10 µm thickness on a calibrated cryostat and mounted immediately on the slide, without scratches or folds.
- Tissue sections must be placed in the **Scan Area** (shown in green in Figure 12) in the center of the slide and be **no larger than 35.3 mm long by 14.1 mm wide**. They should not overlap the slide gasket (shown in blue) or the tip calibration area (shown in red). If mounting multiple sections per slide, ensure that tissues are at least 2–3 mm apart and still fit within the Scan Area. Adhesive slide labels should not overlap Scan Area or slide gasket.

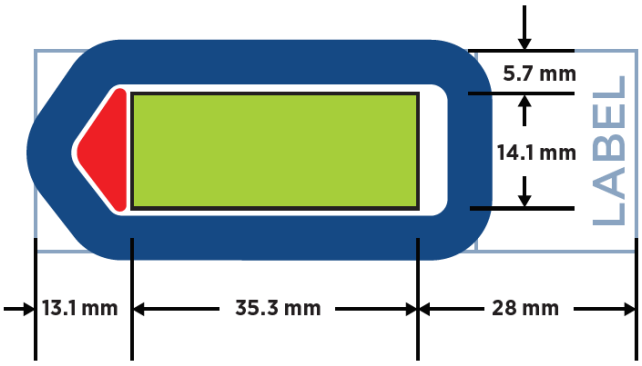


Figure 12: Slide dimensions. Measure from label edge of slide as reference point.

IMPORTANT: The GeoMx DSP instrument will only image the area inside the Scan Area. **Tissue outside of the Scan Area will not be imaged and may cause problems in tissue detection by the optical system.**

- After sectioning, the exposed block face should be covered with OCT to avoid desiccation of the sample.
- Slides can be stored at -80°C for several weeks before use.

Required fixed frozen sample preprocessing steps

Reagents required for this protocol: 1X PBS; 50%, 70%, and 100% ethanol solutions, prepared fresh.

All washes should be performed in staining jars treated with RNase AWAY and rinsed with DEPC-treated water.

1. Remove the fixed frozen tissue slides from -80°C and place face up on a clean surface.
2. Equilibrate to room temperature for 1–2 minutes. Condensation may form as slide warms.
3. Wash in 1X PBS for 5 minutes to remove OCT. Dip slide up and down every ~1 minute.
4. Remove from 1X PBS and use a Kimwipe to wick PBS from the edge of the slide. Place in a rack that orients mounted tissue vertically.

Optional: Bake at 60°C for 30 minutes. Perform this step if tissue is prone to detaching from the slide during slide prep. NanoString has not observed a negative effect from baking on the outcome of the GeoMx RNA assay.

5. Wash the slides in 50% ethanol for 5 minutes.
6. Wash the slides in 70% ethanol for 5 minutes.
7. Wash the slides in 100% ethanol 2 times for 5 minutes each.
8. Let slides air dry for at least 5 minutes (but not more than 1 hour).
9. Turn to the standard RNA FFPE slide preparation protocol beginning at Perform target retrieval (25 minutes) on page 34 **with these modifications for your fixed frozen samples:**
   - **Target retrieval should be performed at 100°C for 15 minutes**. 85°C for 15 minutes may improve tissue integrity in more delicate tissues, such as brain and cell pellets. It may also improve some morphology markers' performance, at the cost of a moderate reduction in the RNA assay's efficiency. Timing and temperature may need to be empirically determined for different tissue types and samples.
   - **Proteinase K digestion should be performed with 1 µg/mL of Proteinase K for 15 minutes.** Timing, temperature, and Proteinase K concentration may need to be empirically determined for different tissue types and samples.
   - Following Proteinase K digestion, **DO NOT perform the post-fixation step** with NBF and NBF Stop Buffer. Instead, proceed to In situ hybridization (overnight) on page 38 and follow all subsequent steps from the RNA FFPE slide preparation protocol.

Appendix IV: Substitute Probe R Guidance

For nCounter readout: This information applies only to GeoMx Protein assays with nCounter readout.

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., Mouse Immuno-oncology, Human Neuroscience).

Each panel is assigned a Probe R space for nCounter readout (Table 14). **Do not combine two modules with a common Probe R space (i.e., same Probe R_number) in the same experimental run, or the data cannot be interpreted.** Substitute Probe Rs are available from NanoString to allow the combination of modules that share Probe R space, such as MAPK Signaling and Immune Cell Typing.

IO Core and Modules

Table 14: Protein panels and their corresponding Probe R space

Panel	Probe R space	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

All Human Neuroscience modules are compatible with one another, and all Mouse Neuroscience modules are compatible with one another, without Substitute Probe R.

Appendix V: Adding Custom Barcoded Antibodies

These instructions were developed for custom barcoded antibodies obtained through NanoString's Protein Barcoding Service or prepared with the NanoString GeoMx Protein Barcoding Kit*. For custom barcoded antibodies obtained from Abcam®, skip steps 1–2 and begin at step 3. These instructions do not apply to morphology marker antibodies.

NOTE: For users of the GeoMx Protein Barcoding Kit, do not add the kit's control antibody to the GeoMx DSP assay.

1. Dilute the custom-barcoded antibody from its stock solution by adding **2 µL antibody** to **6 µL Buffer W**.
2. **Pipette up and down** at least 10 times to mix thoroughly.
3. **Add custom-barcoded antibody to the working antibody solution** prepared in Table 5 on page 22:

   Volume to add to working antibody solution = (1.2 µL x *n*), where *n* = number of slides

   For barcoded antibodies that required a higher concentration to pass the post-conjugation IHC QC test (as described in the NanoString whitepaper Selection and Validation of GeoMx Custom Antibody Spike-ins (MK3600)), add (2.4 µL x *n*), where *n* = number of slides, to the working antibody solution. See the whitepaper for details.

4. Store diluted antibody at 4°C and discard after 2 weeks.

*The GeoMx Protein Barcoding Kit is suitable to barcode antibodies for addition to nCounter assays or Seq Code Protein-NGS assays, but not the Pro Code Protein-NGS assay (Immuno-oncology Proteome Atlas). Please refer to the Protein Barcoding Service.

Appendix VI: RNAscope® and GeoMx RNA Assays

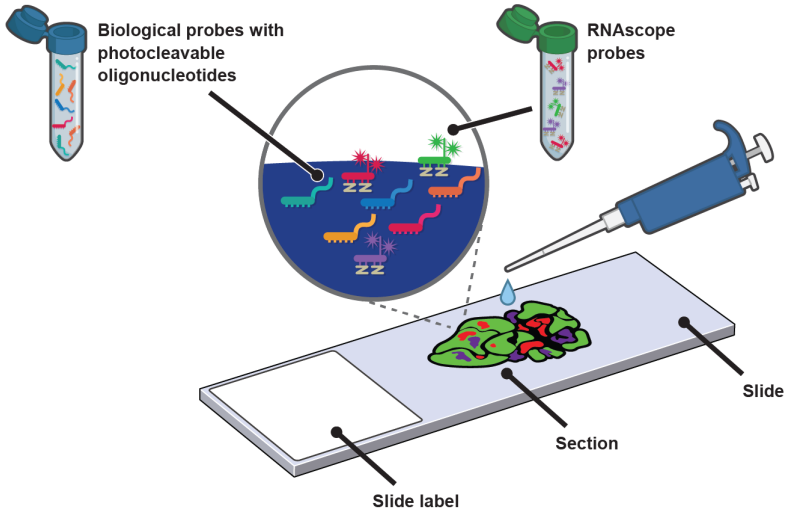


Figure 13: RNAscope probes applied to RNA slide

The following protocol applies only to slide preparation for GeoMx DSP **RNA assays**. (To use RNAscope with **protein** assays, prepare serial sections and use the Image Overlay feature during ROI selection (see GeoMx DSP Instrument User Manual (MAN-10152)). Overlay the RNAscope image over the slide labeled with protein assay reagents to guide ROI selection.)

RNAscope can be used to help set the location of ROIs (i.e., place ROIs where a particular RNA is enriched). However, the punctate signals from **most** RNAscope probes are not compatible with the segmentation algorithm on the GeoMx, meaning that the GeoMx may not be able to segment based on the RNAscope signal. Segmentation may be possible with highly abundant RNAscope targets, so it is recommended to test the segmentation tools with an RNAscope probe of interest in advance of your GeoMx experiments. If segmenting is not supported, consider using RNAscope in 1-2 channels and fluorescent antibody morphology markers in the other channel(s) to define the segments. Alternatively, prepare serial sections, one with RNAscope and one with fluorescent antibody morphology markers. During ROI selection on the antibody-labeled slide, overlay the image of the RNAscope slide to guide ROI selection. Proceed with segmentation and collection from the antibody-labeled slide.

Refer to documentation from ACD™ for protocols and materials required to run the RNAscope assay. To run RNAscope in combination with the GeoMx DSP RNA Assay:

1. Follow the RNAscope assay through developing the HRP signal (page 34 of RNAscope Multiplex Fluorescent Reagent Kit v2 User Manual (323100-USM)). **DO NOT proceed to counter-stain with DAPI.** Refer to Table 15 for recommended TSA fluorophore dilutions.

Table 15: TSA fluorophore dilutions

Fluorescence	Source, Catalog No.	Recommended dilution
TSA Plus Cyanine 3 Kit	Akoya Biosciences, NEL744001KT	1:1500
TSA Plus Cyanine 5 Kit	Akoya Biosciences, NEL745001KT	1:3000

Dilution of TSA fluorophores likely requires optimization by target and tissue type. Refer to RNAscope Multiplex Fluorescent Kit V2 User Manual (323100-USM) for additional details and guidance.

2. After the wash step following HRP signal development, proceed to step 6, Postfix, on page 37, by incubating slides in 10% NBF for 5 minutes. Proceed through subsequent washes and overnight **in situ hybridization**.
3. The next day, continue following the GeoMx RNA Slide Preparation protocol as written, staining slides with nuclear stain and any morphology markers to be used in addition to RNAscope. Ensure that RNAscope dyes and morphology marker fluorophores do not overlap in emission spectra.
4. When scanning slides labeled with RNAscope on the GeoMx DSP, scan parameters (exposure times) may need to be empirically determined. NanoString recommends setting 200 ms exposure time as a starting point.

Appendix VII: Secondary Antibody Staining for RNA Assays

Secondary antibody immunofluorescence staining is a viable alternative to using fluorescence conjugated primary antibodies in GeoMx RNA assays. It is **not viable** for protein assays as the secondary antibody may target the GeoMx DSP detection probe antibodies.

This protocol may require optimization to minimize cross-reactivity between primary and secondary antibodies. NanoString recommends running a pilot experiment before completing this protocol with your experimental samples and probe mix.

Table 16: Additional reagents required for secondary antibody IF staining.

Reagent	Concentration	Source, Catalog No.
Unconjugated primary antibody against target	1-10 µg/mL in Buffer W	Various suppliers; select an antibody raised in mouse or rabbit for compatibility with below products.
Fluorescence conjugated secondary antibody against primary antibody	4 µg/mL in Buffer W (or empirically determined concentration)	Various suppliers; the following are recommended:
		Goat anti-mouse, 647 nm channel, ThermoFisher A21236 or A21242
		Goat anti-mouse, 594 nm channel, ThermoFisher A11032 or A21125
		Goat anti-rabbit, 647 nm channel, ThermoFisher A21245
		Goat anti-rabbit, 594 nm channel, ThermoFisher A11037

This protocol extends Day 2 of Slide Preparation for RNA Assays by about 3 hours, prior to loading on the GeoMx DSP instrument.

1. Follow the RNA FFPE Slide Preparation protocol from page 27 through (including) Blocking with Buffer W (step 2 on page 42).
2. During blocking, prepare solution of **primary antibody (without fluorescent label) diluted in Buffer W**. Follow the vendor IHC recommended concentration (typically 1–10 µg/mL) or rely on empirical testing with the sample tissue and antibody of choice. If you are not using fluorescence conjugated primary antibodies in your staining, include your **SYTO nuclear stain** with this incubation. Prepare 220 µL per slide. (If you will add any fluorescence conjugated primary antibodies, and they are of a different host species than the unconjugated primary antibody, they can be included along with the **nuclear stain** in this incubation, and you can skip Step 8 below.)
3. Incubate slide(s) for **1 hour** (minimum) with **200 µL** of diluted primary antibody solution made in step 2, in humidity chamber at room temperature.
4. Wash slide(s) in **2X SSC** for 1 minute, then wash in **2X SSC** 4 times for 3 minutes each.

5. Prepare solution of secondary antibody (with fluorescent label) diluted to 4 µg/mL (1:500 for secondary antibodies listed in Table 16) in Buffer W. Secondary antibody target must match host of primary antibody.
6. Incubate slide(s) for 1 hour with **200 µL** of diluted secondary antibody solution made in step 5, in humidity chamber at room temperature.
7. Wash slide(s) in fresh **2X SSC** for 1 minute, then wash in **2X SSC** 4 times for 3 minutes each. Wash steps are critical to avoid high background staining.
8. If your fluorescently conjugated primary antibodies are of the same host species as the unconjugated primary antibody, perform the following steps. Please note that these steps have not been validated by NanoString.
   - Perform an additional **Blocking** step using serum from the same species as the primary antibodies, diluted in Buffer W, for 30 minutes.
   - Wash slide(s) in fresh **2X SSC** for 1 minute, then wash in **2X SSC** 4 times for 3 minutes each.
   - Incubate for **1 hour** (minimum) with your fluorescently conjugated primary antibodies and nuclear stain diluted in Buffer W, as described in step 4 on page 42.
   - Wash slide(s) in fresh **2X SSC** for 1 minute, then wash in **2X SSC** 4 times for 3 minutes each.
9. The slides are ready to be loaded on the GeoMx DSP instrument or stored (see Safe storage guidelines for RNA slides on page 43).

Appendix VIII: Tyramide Signal Amplification (TSA) of Morphology Markers

TSA enhances a weak immunolabeling signal and provides an alternative to directly conjugated fluorescent antibody morphology markers in the GeoMx DSP workflow. The below protocol is offered as guidance only and is not optimized for all tissues. NanoString recommends running a pilot experiment on your tissue with your selected antibodies. Variations such as longer incubation times or multiple antigen retrieval steps may help optimize the protocol for your application.

TSA with GeoMx Protein Assays

Table 17: Additional reagents required for TSA with Protein Assays.

Reagent	Source, Catalog No.	Storage
Tyramide SuperBoost™ Kit	ThermoFisher, various available to target mouse or rabbit antibodies	2-8°C
Unconjugated primary antibody (host species must match Tyramide Kit)	Various	-20°C

1. Prepare reagents of Tyramide SuperBoost Kit according to sections 1.1- 1.5 of the User Guide (MAN0015834_Tyramide_SuperBoost_Kits_UG). Some reagents can be prepared in advance and stored, while others must be prepared fresh the day of the labeling protocol.
2. Follow the standard Protein Slide Preparation protocol through (including) Perform antigen retrieval (1 hour) on page 19.
3. First blocking step:
    a. Apply sufficient Kit Component C2 (3% H_2O_2) to cover the tissue and incubate for 1 hour at room temperature.
    b. Wash slides in 1X TBS-T for 2 minutes, 3 times.
    c. Apply sufficient Kit Component A (Blocking Buffer) to cover the tissue and incubate for 1 hour at room temperature.
4. Primary antibody incubation:
    a. Dilute unconjugated primary antibody in Buffer W. The optimal concentration depends on the antibody. Rely on user testing or vendor- recommended concentration for immunofluorescence as a starting point.
    b. Apply sufficient unconjugated primary antibody dilution to cover the tissue and incubate overnight at 4°C in a humidity chamber prepared as in the standard Protein Slide Preparation workflow on page 20.

5. Poly-HRP-conjugated secondary antibody incubation:
    a. Wash slides with 1X TBS-T for 3 minutes, 5 times.
    b. Apply poly-HRP-conjugated secondary antibody from the kit to the tissue. Incubate for 1 hour at room temperature in the humidity chamber. Secondary antibody must target host species of primary antibody.
    c. During the incubation, prepare fresh tyramide working solution (Table 18).

Table 18: Tyramide working solution equation where *n* = # slides.

Reagents prepared from kit in Step 1			
AlexaFluor Tyramide Reagent	H_2O_2 Solution	Reaction Buffer	Total Volume
1 µL x *n*	1 µL x *n*	100 µL x *n*	102 µL x *n*

    d. After the poly-HRP-conjugated secondary antibody incubation, wash the slides with 1X TBS-T for 3 minutes, 5 times.
    e. Ensure that the Reaction Stop Reagent working solution is prepared since it will be needed promptly at Step h, below.
    f. Apply the tyramide working solution (100 µL/slide) and incubate for 8 minutes at room temperature.
    g. Remove the excess tyramide working solution.
    h. Apply Reaction Stop Reagent working solution (100 µL/slide) and incubate for a few seconds.
    i. Wash slides in 1X TBS-T for 2 minutes, 3 times.
6. Strip the primary antibody:
    a. Repeat the Antigen Retrieval step of the standard Protein Slide Preparation workflow on page 19.
    b. Wash the slides in 1X TBS-T for 2 minutes, 5 times.
7. Second blocking step:
    a. Apply sufficient Buffer W to cover the tissue and incubate in the humidity chamber for 1 hour at room temperature.
    b. Remove Buffer W and wash in 1X TBS-T for 2 minutes, 3 times.
8. Turn to the standard Protein Slide Preparation protocol beginning with Primary antibody incubation (overnight) on page 21 and proceed as directed through the end of the slide preparation workflow.

TSA with GeoMx RNA Assays

This dual channel TSA protocol was validated using two primary antibodies from different host species. If needed, modify and optimize the protocol for your experimental conditions.

Table 19: Additional reagents required for TSA with RNA Assays

Reagent	Source, Catalog No.	Storage
Unconjugated primary IgG antibody 1, against target 1	Various	-20°C
Unconjugated primary IgG antibody 2, against target 2, from a different host species than primary antibody 1	Various	-20°C
HRP-conjugated secondary antibody 1 (targeting primary antibody 1) HRP-conjugated secondary antibody 2 (targeting primary antibody 2)	Various, such as Goat anti-Rabbit IgG (H+L) Secondary Antibody HRP (Invitrogen G-21234) or Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP (Invitrogen G-21040)	-20°C
TSA Plus Cyanine 3 Kit	Akoya Biosciences, NEL744001KT	4°C in dark
TSA Plus Cyanine 5 Kit	Akoya Biosciences, NEL745001KT	4°C in dark
30% H_2O_2 	Sigma, H1009	4°C
10X TBS-T	Cell Signaling Technologies, 9997	RT

NOTE: Before starting the protocol with your experimental samples, determine the optimal concentration of secondary antibody solutions to be used in steps 6a and 9a. Rely on user testing or vendor-recommended concentration for immunofluorescence. Due to the Proteinase K digestion of the RNA slide preparation workflow, antibody concentrations may need to be higher than for normal immunofluorescence (2X is suggested starting point).

1. **Prepare the reagents** required for the standard RNA Slide Preparation workflow as described in Prepare reagents on page 31, except for NBF Stop Buffer. **In addition,** prepare the following reagents specific to the TSA workflow (Table 20).

Table 20: Reagent prep for RNA slide preparation

Reagent	Preparation	Storage
Cyanine 3 amplification reagent	Reconstitute as specified by the manufacturer	4°C
Cyanine 5 amplification reagent	Reconstitute as specified by the manufacturer	4°C
1X TBS-T	Prepare 1 L of 1X TBS-T by combining 100 mL of 10X TBS-T and 900 mL of DEPC-treated water. Total volume required in protocol depends on capacity of staining jars used; make 2 L to begin.	RT

2. **Prepare tissue samples** and **deparaffinize and rehydrate tissue sections** as described for the standard RNA Slide Preparation workflow on page 32 through 33. DO NOT pre-heat the Proteinase K solution at this point.

3. **Perform target retrieval** as described for the standard RNA Slide Preparation workflow on page 34; **however**, following target retrieval incubation, transfer slides to room temperature 1X TBS-T (instead of PBS) for 5 minutes.

   • During the target retrieval incubation, prepare the humidity chamber by cleaning it with RNase AWAY and lining it with Kimwipes wetted with 1X TBS-T or DEPC-treated water. Add just enough liquid to cover the bottom of the chamber.

   • While slides are in 1X TBS-T, prepare 3% H_2O_2: prepare 300 µL per slide by combining 30 µL 30% H_2O_2 with 270 µL DEPC-treated water.

4. **Perform blocking:**

   a. Remove one slide at a time from 1X TBS-T and tap slide on a Kimwipe to remove excess liquid. Place slide in humidity chamber and cover tissue with 200 µL 3% H_2O_2. Incubate at room temperature for 10 minutes.

   b. Remove 3% H_2O_2 from one slide at a time by tapping slide on a Kimwipe, then place slide in 1X TBS-T. Move slides up and down 3-5 times to wash.

   c. Transfer slides to a new jar of 1X TBS-T, and move slides up and down 3-5 times to wash. Repeat for a third wash.

   d. Remove one slide at a time from 1X TBS-T, tap slide on a Kimwipe, then wipe around the tissue to remove excess liquid. Place slide in humidity chamber and cover tissue with 200 µl Buffer W. Incubate at room temperature for 30 minutes.

5. **Introduce primary antibodies:**

    a. Prepare the primary antibody solution containing both primary antibodies, each at a concentration of 1 µg/mL, in Buffer W. Prepare 200 µl per slide. Flick tube to mix, then briefly spin down.

    b. Remove Buffer W from one slide at a time by tapping slide onto a Kimwipe, then return the slide to the humidity chamber. Cover tissue with 200 µL primary antibody solution. Repeat for each slide.

    c. Stain for 1 hour in the humidity chamber, at room temperature.

    d. During the final 10 minutes of primary antibody staining, perform step 6a.

6. **Introduce HRP for target 1:**

    a. Prepare the first HRP-conjugated secondary antibody solution by diluting the antibody in Buffer W to the optimal concentration, as determined previously (see NOTE under Table 17). Prepare 200 µL per slide. Flick to mix and briefly spin down.

    b. Remove primary antibody from the slide by tapping slide edge on a Kimwipe, then place slide in 1X TBS-T. Move slide up and down 3-5 times to wash.

    c. Transfer slide to a new staining jar of 1X TBS-T and repeat up-down washes.

    d. Transfer slide to a new staining jar of 1X TBS-T and let sit 1 minute. Transfer to two additional staining jars, each with fresh 1X TBS-T, for 1 minute each.

    e. Remove one slide at a time from 1X TBS-T, tap slide on a Kimwipe, then wipe around the tissue to remove excess liquid. Place slide in humidity chamber and cover tissue with 200 µL of the first secondary antibody solution. Leave at room temperature for 30 minutes.

    f. During the final 10 minutes of the incubation, perform step 7a and b.

7. **Tyramide signal amplification for target 1:**

    a. Allow Cyanine 5 to sit at room temperature for at least 5 minutes to ensure complete thawing.

    b. Dilute Cyanine 5 to 1:250 in 1X Plus Amplification Diluent provided in TSA Plus Cyanine Kit.

    c. Remove secondary antibody solution from slide by tapping slide edge on a Kimwipe, then place slide in 1X TBS-T. Move slide up and down 3-5 times to wash.

    d. Transfer slide to a new staining jar of 1X TBS-T and repeat up-down washes.

    e. Transfer slide to a new staining jar of 1X TBS-T and let sit 1 minute. Transfer to two additional staining jars, each with fresh 1X TBS-T, for 1 minute each.

    f. Remove one slide at a time from 1X TBS-T, tap slide on a Kimwipe, then wipe around the tissue to remove excess liquid. Place slide in humidity chamber and cover tissue with 200 µL Cyanine 5 solution. Leave at room temperature for 30 minutes.

    g. During the final 10 minutes of the incubation, prepare 0.3% H_2O_2: prepare 400 µL per slide by combining 40 µL of the 3% H_2O_2 prepared above with 360 µL DEPC-treated water.

8. **HRP inactivation for target 1:**

    a. Remove Cyanine 5 solution by tapping slide edge on a Kimwipe, then place slide in 1X TBS-T. Move slide up and down 3-5 times to wash.

    b. Transfer slide to a new staining jar of 1X TBS-T and repeat up-down washes.

    c. Transfer slide to a new staining jar of 1X TBS-T and let sit 1 minute. Transfer to two additional staining jars, each with fresh 1X TBS-T, for 1 minute each.

    d. Remove one slide at a time from 1X TBS-T, tap slide on a Kimwipe, then wipe around the tissue to remove excess liquid. Place slide in humidity chamber and cover tissue with 200 µL 0.3% H_2O_2 solution. Leave at room temperature for 15 minutes. Save remaining 200 µL 0.3% H_2O_2 solution for step 11.

    e. During the final 10 minutes of the incubation, perform step 9a.

9. **Introduce HRP for target 2:**

    a. Prepare the second HRP-conjugated secondary antibody solution by diluting the antibody in Buffer W to the optimal concentration, as determined previously (see NOTE above). Prepare 200 µL per slide. Flick to mix and briefly spin down.

    b. Remove 0.3% H_2O_2 from the slide by tapping slide edge on a Kimwipe, then place slide in 1X TBS-T. Move slide up and down 3- 5 times to wash.

    c. Transfer slide to a new staining jar of 1X TBS-T and repeat up-down washes.

    d. Transfer slide to a new staining jar of 1X TBS-T and let sit 1 minute. Transfer to two additional staining jars, each with fresh 1X TBS-T, for 1 minute each.

    e. Remove one slide at a time from 1X TBS- T, tap slide on a Kimwipe, then wipe around the tissue to remove excess liquid. Place slide in humidity chamber and cover tissue with 200 µL of the second secondary antibody solution. Leave at room temperature for 30 minutes.

    f. During the antibody incubation, preheat the Proteinase K solution in a 37°C water bath for 30-45 minutes.

    g. Toward the end of the antibody incubation, perform step 10a and b.

10. **Tyramide signal amplification for target 2:**

    a. Allow Cyanine 3 to sit at room temperature for at least 5 minutes to ensure complete thawing.

    b. Dilute Cyanine 3 to 1:250 in 1X Plus Amplification Diluent provided in TSA Plus Kit.

    c. Remove secondary antibody solution from slide by tapping slide edge on a Kimwipe, then place slide in 1X TBS-T. Move slide up and down 3-5 times to wash.

    d. Transfer slide to a new staining jar of 1X TBS-T and repeat up-down washes.

    e. Transfer slide to a new staining jar of 1X TBS-T and let sit 1 minute. Transfer to two additional staining jars, each with fresh 1X TBS-T, for 1 minute each.

f. Remove one slide at a time from TBS-T, tap slide on a Kimwipe, then wipe around the tissue to remove excess liquid. Place slide in humidity chamber and cover tissue with 200 µL Cyanine 3 solution. Leave at room temperature for 30 minutes.

11. **HRP inactivation for target 2:**

    a. Remove Cyanine 3 solution by tapping slide edge on a Kimwipe, then place slide in 1X TBS-T. Move slide up and down 3-5 times to wash.

    b. Transfer slide to a new staining jar of 1X TBS-T and repeat up-down washes.

    c. Transfer slide to a new staining jar of 1X TBS-T and let sit 1 minute. Transfer to two additional staining jars, each with fresh 1X TBS-T, for 1 minute each.

    d. Remove one slide at a time from 1X TBS-T, tap slide on a Kimwipe, then wipe around the tissue to remove excess liquid. Place slide in humidity chamber and cover tissue with 200 µL 0.3% H_2O_2 solution. Leave at room temperature for 15 minutes.

12. **Expose RNA targets:**

    a. Note that Proteinase K should be prepared and preheated in a 37°C water bath prior to this point (see Step 9f).

    b. Remove 0.3% H_2O_2 from the slide by tapping slide edge on a Kimwipe, then place slide in 1X TBS-T. Move slide up and down 3-5 times to wash.

    c. Transfer slide to a new staining jar of 1X TBS-T and repeat up-down washes.

    d. Transfer slide to a new staining jar of 1X TBS-T and let sit 1 minute. Transfer to two additional staining jars, each with fresh 1X TBS-T, for 1 minute each.

    e. Transfer slide to preheated Proteinase K solution in 37°C water bath and incubate for the length of time appropriate for your sample (Table 10 on page 32).

    f. Following incubation, transfer slide to fresh 1X TBS-T. Move slide up and down 3- 5 times to wash.

    g. Transfer slide to a new staining jar of 1X TBS-T and repeat up-down washes.

    h. Transfer slide to a new staining jar of 1X TBS-T and let sit 1 minute. Transfer to two additional staining jars, each with fresh 1X TBS-T, for 1 minute each.

    i. Transfer slide to 1X PBS.

13. **Resume** the standard RNA Slide Preparation workflow beginning with In situ hybridization (overnight) on page 38. Note that morphology marker blocking can be skipped, and the morphology marker solution prepared in Table 13 on page 42 should consist only of nuclear stain and no morphology markers. Incubate the stain on slides for 30 minutes instead of 1 hour.

Troubleshooting

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

Issue	Possible Cause	Suggested Actions
Equipment, materials, or reagents are not available	Supply chain issues or regional variability	Contact Support@nanostring.com or your Applications Scientist.
Tissue on slide extends beyond boundaries of scan area (see diagram on page 17)	Tissue slides were prepared outside of GeoMx guidance	Perform slide preparation steps as usual. Just before loading on instrument, scrape excess tissue away using a sharp, clean razor blade.
Coverslip is difficult to remove	Mounting medium evaporated or was insufficient in volume	Soak slides in 1X TBS-T with gentle agitation. Gently lift a corner of the coverslip with forceps.
Probe mix evaporated or did not stay within the barrier during overnight incubation	Improper conditions in humidity chamber or broken hydrophobic barrier (in protein workflow)	Check whether the entire tissue section still has liquid in contact with it. Areas that dried may lose signal. Check conditions of humidity chamber with a test slide and mock probe mix to prevent evaporation from reoccurring.
Tissue detaches from slide	Tissue did not adhere sufficiently during baking	Try Leica BOND Plus Slides as an alternative to Superfrost Plus slides. Try extending slide baking time, up to overnight at 37°C plus 2-4 hour at 60°C.
Some sections of tissue are not in focus	Tissue may be separating from the slide in places or have folds	See row above. Ensure tissue does not have folds.

Issue	Possible Cause	Suggested Actions
Fluorescent signal is not consistent across the tissue sample	Inconsistent tissue thickness	Ensure sections are cut with even thickness and don't have folds.
Streaks in scanned image	Smudges on bottom of glass slide	Clean bottom of slide with 70% ethanol and a Kimwipe thoroughly before and/or after loading in slide tray.
Morphology marker signal is weak	Morphology marker is simply a weak marker	Try adjusting exposure time on the GeoMx DSP (up to 300 ms per channel) or increasing the intensity using Render Settings in the Scan Workspace.
	Conditions have not been experimentally optimized	Optimize staining conditions by testing different antibody concentrations and/or incubation times following guidance in literature or previous experience with immunohistochemistry. For the RNA workflow, in certain cases, Proteinase K concentration may need to be adjusted.
Bright speckled spots in all channels across tissue	Autofluorescence from red blood cells (RBC) or dying cells	During segmentation, try adding a positive requirement for the nuclear stain signal to exclude RBC, or a negative requirement on a different channel to remove cells fluorescing in all channels. In certain cases, ImageJ can be used to define segmentation boundaries on a mask and import them to the GeoMx. See GeoMx DSP Instrument User Manual (MAN-10152).
	Dust or debris on slide	Follow washing and blocking steps to specifications in this user manual.

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