



GeoMx[®] DSP

Manual Slide Preparation

User Manual

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MAN-10150-01 | April 2022

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Changes in This Revision

The GeoMx DSP Slide Preparation User Manuals were restructured for clarity and simplicity. 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[®]. These manuals replace the GeoMx-NGS Slide Preparation User Manual (SEV-00153-05) and GeoMx-nCounter Slide Preparation User Manual (SEV-00172-08).

NGS-specific information is separated from nCounter-specific information with colored text boxes, as described in [Conventions on page 7](#).

Other changes in this manual revision include:

- Updated items and links in Equipment, Materials, and Reagents lists [on page 12](#) and [on page 27](#)
- Updated slide dimensions diagrams (example [on page 17](#)) to clarify GeoMx DSP scan area
- Added guidance for epitope retrieval and Proteinase K conditions for mouse tissue [on page 36](#) and [on page 37](#), and updated cell pellet Proteinase K digestion conditions [on page 37](#)
- Extended safe storage guidelines to 3 months for protein slides [on page 25](#) and 7 days for RNA slides [on page 44](#)
- Revised storage and handling guidance for RNA probe mixes in step 1 [on page 39](#)
- Updated fresh frozen sample preparation guidance [on page 48](#)
- Updated fixed frozen sample preparation guidance [on page 52](#)
- Improved guidance for including custom barcoded antibodies in GeoMx protein assays [on page 57](#)
- Added guidance to use secondary antibodies in morphology marker labeling [on page 60](#)
- Improved guidance for tyramide signal amplification (TSA) in morphology marker labeling [on page 62](#)
- Added a section on Troubleshooting [on page 64](#)

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Conventions

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

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

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

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



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



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

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

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

GeoMx DSP Workflow

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

- **Day 1: Slide Staining.** Prepare slides and incubate biological targets with UV-cleavable probes. Prepare manually or using the BOND RX/RX™ Fully Automated IHC/ISH Stainer from Leica Biosystems®.
- **Day 2: Process Slides on GeoMx DSP.** Load prepared slides into the GeoMx DSP instrument and enter slide/study information. Slides are scanned to capture fluorescent images used to select regions of interest (ROIs). The instrument collects UV-cleaved oligos from the ROIs into the wells of a collection plate.

For NGS readout:

Day 3: Transfer the collected aspirates to a PCR plate and perform **Library Prep** with Seq Code primers. Pool and purify the products, then **Sequence** on an Illumina NGS instrument.

Day 4: Process FASTQ sequencing files into digital count conversion (DCC) files using **NanoString's GeoMx NGS Pipeline** on Illumina DRAGEN™ accessed via BaseSpace™ Sequence Hub, or using GeoMx NGS Pipeline standalone software. Upload DCC files on to the GeoMx DSP.

For nCounter readout:

Day 2, continued: Transfer the collected aspirates to a hybridization plate along with GeoMx Hyb Code reagents. Hybridization occurs overnight.

Day 3: Pool wells and **Process on an nCounter MAX/FLEX or Pro Analysis System or SPRINT Profiler.** Upload reporter count conversion (RCC) files to the GeoMx DSP.

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

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GeoMx DSP Workflow

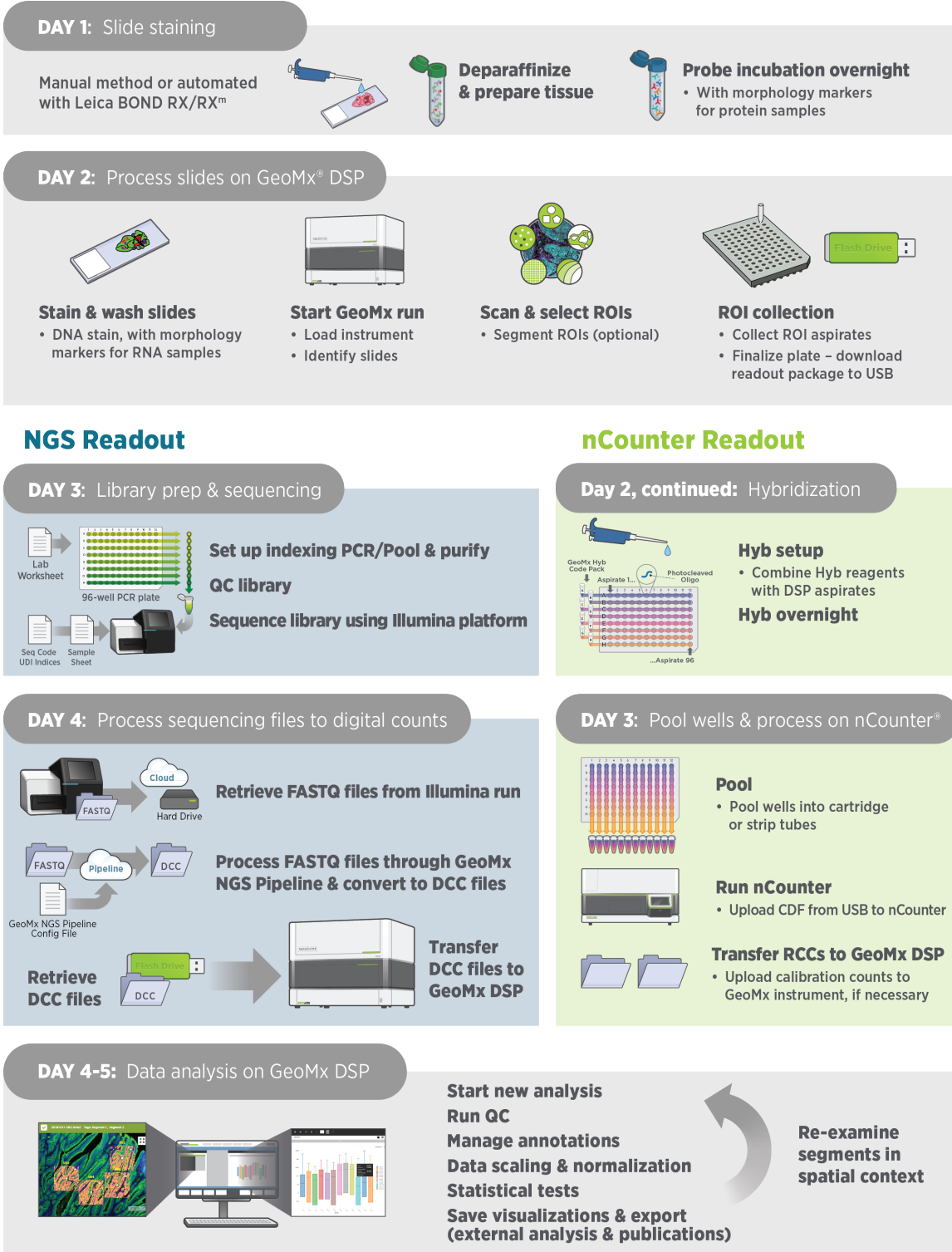


Figure 1: GeoMx DSP workflow summary

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User Manuals and Resources

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

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

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

Instrument and workflow training courses are available in NanoString University.

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

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 ([see 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.

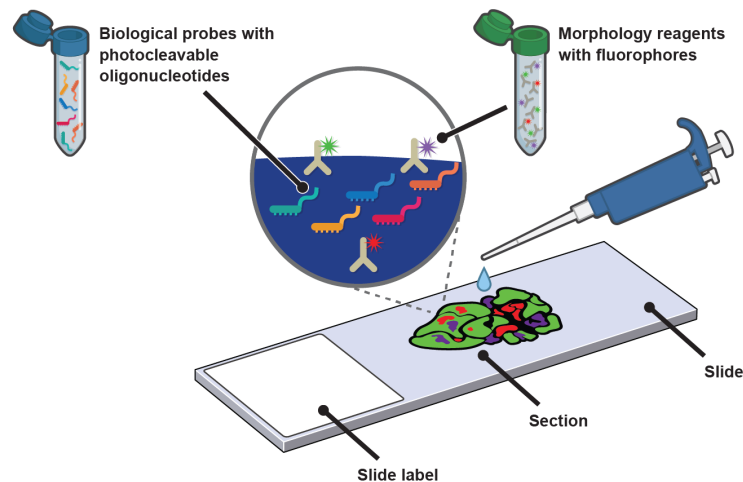


Figure 2: Slide preparation with ISH probes

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>).

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Protein Slide Preparation Protocol (FFPE)

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).

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 provided by NanoString.

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

*A TintoRetriever pressure cooker is recommended for this protocol. These alternatives are acceptable and may be more easily purchased outside the U.S., but have not been validated by NanoString: [Tefal CY505E 6 Liter](#); [AmazonBasics multipurpose pressure cooker 5.5L](#).

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




Materials	Source	Part No.
Pipettes for 5–1,000 µL	Various	Various
12-channel P20 multi-channel pipetter	Various	Various
Filter tips (DNase/RNase free)	Various	Various
Microcentrifuge tubes (DNase/RNase free)	Various	Various
Superfrost Plus microscope slides or Leica BOND Plus microscope slides	Fisher Scientific or Leica Biosystems	12-550-15 S21.2113.A
Slide staining jars (Coplin jars) (recommended number: 12) and slide holder inserts	VWR (or comparable)	25608-904, 25608-868
Humidity chamber	Simport (or comparable)	M920-2 (select black lid)
Hydrophobic barrier pen	Vector Labs (or comparable)	H-4000
RNase AWAY® or 10% Bleach (RNaseZap® is not a substitute)	Thermo Fisher	7003PK
Razor blades	Various	Various
Coverslips (optional)	Various	Various

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Protein FFPE Equipment, Materials, Reagents

Materials	Source	Part No.
Heat/cold protectant handling glove	Various	Various
USB drive v3.0, 64 GB or higher (able to be NTFS formatted)	SanDisk (or comparable)	SDCZ800-128G-G46

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

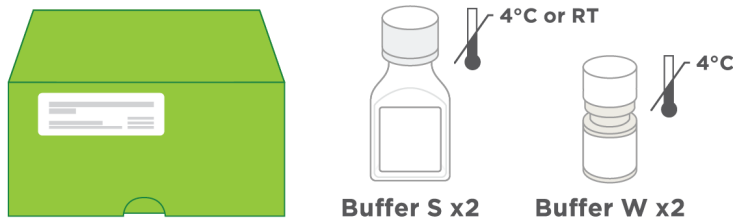
Reagents	Source, Part No.	Storage
DEPC-treated water	Thermo Fisher, AM9922 (or comparable) NOTE: As an alternative to commercial DEPC-treated water, prepare your own following standard protocols.	RT
10X tris-buffered saline (TBS)	Cell Signaling Technologies, 12498S	RT
10X TBS with Tween- 20 (TBS-T)	Cell Signaling Technologies, 9997S	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) NOTE: Used <i>only</i> to dilute 16% PFA	Thermo Fisher, 10010031KU (or comparable)	RT
Fluoromount-G mounting media (optional)	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

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NanoString Reagents

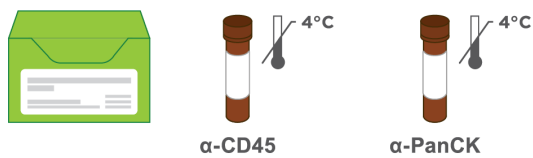
The following reagents are **supplied by NanoString**. Contact your NanoString Sales Representative to use our reagent planning tools to calculate required quantities.

GeoMx Protein Slide Prep Kit

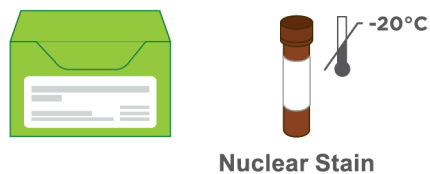


GeoMx Morphology Kit — Human or Mouse Protein compatible (various available)

Example morphology kit (Solid Tumor TME)



GeoMx Nuclear Stain Morphology Kit



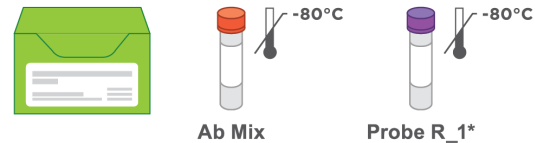
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Protein FFPE Equipment, Materials, Reagents**For NGS readout:**

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

**For nCounter readout:**

GeoMx Protein Cores and optional 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 56](#) for more information and to plan Probe R usage in your experiment.

Each tube of detection antibody (Ab) mix contains sufficient reagent for 12 slides (112 μ L). If you are using the entire Ab mix in one week, then store at 4°C. If not, **aliquot the Ab mix** into 4-slide aliquots (37 μ L + 37 μ L + 38 μ L) and freeze unused aliquots at -80°C. Do not exceed more than 2 freeze/thaw cycles and do not freeze diluted antibody.

The morphology marker antibodies are stored at 4°C. Aliquoting is not required.

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Prepare reagents

Prepare your reagents using the dilution instructions ([see Table 4](#)).

Table 4: Reagent prep for protein slide preparation

Reagent	Dilution	Storage
1X citrate buffer (pH 6)	Dilute 10X citrate buffer (pH 6) in DEPC-treated water. Must be prepared on the day of slide prep. Do not prepare ahead of time.	4°C
95% ethanol (EtOH)	Prepare 500 mL of 95% ethanol by adding 25 mL of DEPC-treated water to 475 mL of 100% ethanol.	RT
4% paraformaldehyde (PFA)	NOTE: Use <i>only</i> for post-fixation step If using 16% stock, dilute from 16% to 4% in 1X PBS, aliquot, and store.	4°C (or manufacturer instructions)
1X tris-buffered saline with Tween-20 (TBS-T)	Prepare 1 L by diluting 100 mL of 10X TBS-T in 900 mL DEPC-treated water. Total volume needed for slide prep depends on volume on staining jars to be used.	RT
1X TBS	Dilute 500 µL of 10X TBS in 4.5 mL DEPC-treated water in order to prepare a total of 5 mL 1X TBS.	RT

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2 Prepare tissue samples

[Appendix I: Selecting and Sectioning FFPE Samples on page 46](#) 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 greater 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) (see [Figure 3](#)) in the center of the slide and be **no larger than 35.3 mm long by 14.1 mm wide**. Mounted material 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.

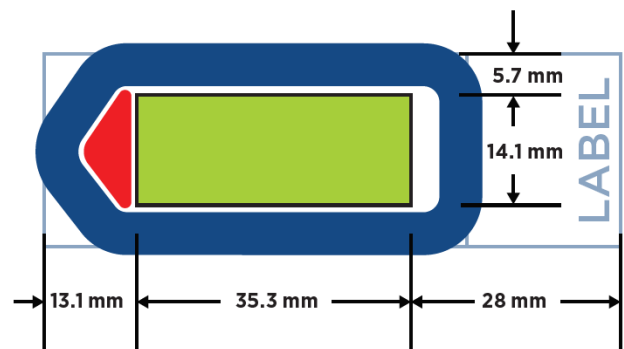


Figure 3: Slide dimensions

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; this should be empirically tested.

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3 Deparaffinize and rehydrate FFPE tissue sections (45 minutes)

You will need the following items and reagents 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 12](#) for more details.

1. **Prepare the pressure cooker** 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.**
2. **Deparaffinize and rehydrate FFPE tissue sections.** Place slides in a rack and perform the following washes in staining jars ([see Figure 4](#)). 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.



Figure 4: Wash steps



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

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*Protein FFPE Slide Prep Protocol***4 Perform antigen retrieval (1 hour)**

You will need the following items and reagents for this step: **staining jars, pressure cooker, 1X Citrate Buffer** and **1X TBS-T**. See the Equipment, Materials, and Reagents lists [on page 12](#) and Prepare Reagents steps [on page 16](#).

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.**



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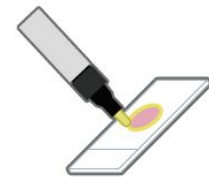
5 Blocking (1 hour)

You will need the following items and reagents for this step: **hydrophobic pen**, **humidity chamber**, and **Buffer W**. See the Equipment, Materials, and Reagents lists [on page 12](#) 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. **Fill a humidity chamber** with enough water to cover the bottom. Avoid overfilling to prevent splashing while moving 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 size of the area within the barrier.
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 μ L** per slide, depending on the size of the tissue). Repeat steps 2-4 for any additional slides.
5. **Incubate slides with Buffer W for 1 hour** at room temperature in the closed humidity chamber.
6. **Thaw detection probe antibody (Ab) mix** (cores and modules) on ice. Keep tube protected from light.



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6 Primary antibody incubation (overnight)

You will need the following materials and reagents 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 12](#) for more details.

i 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. Gloves should be changed 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 56](#).

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

Each tube of detection antibody (Ab) mix contains sufficient reagent for 12 slides (112 μ L). If you are using the entire Ab mix in one week, then store at 4°C. If not, **aliquot the Ab mix** into 4-slide aliquots (37 μ L + 37 μ L + 38 μ L) and freeze unused aliquots at -80°C. Do not exceed more than 2 freeze/thaw cycles and do not freeze diluted antibody.

The morphology marker antibodies are stored at 4°C. Aliquoting is not required.

2. **Make a working Ab solution** by diluting detection antibodies and morphology markers into Buffer W (n = number of slides) ([see 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 200 μ L per slide**). [Table 5](#) addresses both NGS assays and nCounter assays.

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

Core Mix	Module 1	Module 2	Other Modules*	Morph Marker1**	Morph Marker2	Other Markers	Buffer W***	Final Volume
8 μ L x n	8 μ L x n	8 μ L x n	...	5 μ L x n	5 μ L x n	...	(up to 200 μ L) x n	200 μ L x n

* If adding a custom-barcoded detection antibody, follow instructions in [Appendix V: Adding Custom Barcoded Antibodies on page 57](#).

** 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 **200 μ L/slide**.

3. **Remove slide from humidity chamber and remove Buffer W** 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 μ L 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.

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*Protein FFPE Slide Prep Protocol***7 Postfix (70 minutes)**

You will need the following materials and reagents for this step: **staining jars, 1X TBS-T, and 4% PFA**. See the Equipment, Materials, and Reagents lists [on page 12](#) 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. Staining jars should be cleaned with RNase AWAY before each use.

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 several paper towels.
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 the next 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**.



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8 Nuclei staining (20 minutes)

You will need the following materials and reagents for this step: **humidity chamber, staining jars, razor, SYTO 13 nuclear stain, 1X TBS, and 1X TBS-T**. See the Equipment, Materials, and Reagents lists [on page 12](#) and Prepare Reagents steps [on page 16](#).

IMPORTANT: Before using the humidity chamber in the following steps, clean it with RNase AWAY. Prep 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**. Prepare a sufficient volume per slide to completely cover tissue (~200 μ L 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 in 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. **Store stained slides in 1X TBS-T**. If it is necessary to re-stain with SYTO 13, re-draw the hydrophobic barrier, then repeat steps 1-8.



Once the slides are prepared, load them onto the GeoMx DSP (see the [GeoMx DSP Instrument User Manual](#) (MAN-10152)) or store slides according to guidelines on next page. **DO NOT let slides dry out.**

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Protein FFPE Slide Prep Protocol**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.

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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 protocol. This protocol requires a UV light box or transilluminator capable of emitting 302/312 nm UV light ([example](#)) and 1X TBS-T.

1. Place the 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 each slide on a clean, absorbent surface (e.g. paper towel) to remove liquid and avoid oligo contamination.
5. **Wash slides** 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)

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).

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 provided by NanoString.

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

*NanoString recommends the listed hybridization ovens for this protocol. The following alternatives are acceptable, but have not been validated by NanoString. Test to ensure slides remain hydrated overnight. [Abbott ThermoBrite](#), [Leica ThermoBrite](#)

**NanoString recommends the listed steamers for this protocol. These alternatives are acceptable, but have not been validated by NanoString: [Philips HD9140](#).






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

Materials	Source	Part No.
Pipettes for 5–1,000 µL	Various	Various
12-channel P20 multi-channel pipette	Various	Various
Filter tips (DNase/RNase free)	Various	Various
Microcentrifuge tubes (DNase/RNase free)	Various	Various
Superfrost Plus microscope slides or Leica BOND Plus microscope slides	Fisher Scientific or Leica Biosystems	12-550-15 S21.2113.A
Slide staining jars (Coplin jars) (recommended number: 16) and slide holder inserts	VWR (or comparable)	25608-904, 25608-868
Humidity chamber	Simport	M920-2
Benchtop protector sheet (fits inside the hybridization oven, optional)	Fisher Scientific (or comparable)	14-206-62
HybriSlip hybridization covers (22 mm x 40 mm x 0.25 mm)	Grace Bio-Labs	714022
Note: Other products have not been validated by NanoString.		
RNase AWAY® or 10% Bleach (RNaseZap® is not a substitute)	Thermo Fisher	7003PK
Heat/cold protectant handling glove	Various	Various
Forceps (for slide handling)	Various	Various
Aluminum foil	Various	Various
USB drive v3.0, 64 GB or higher (able to be NTFS formatted)	SanDisk (or comparable)	SDCZ800-128G-G46

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RNA FFPE Equipment, Materials, Reagents

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

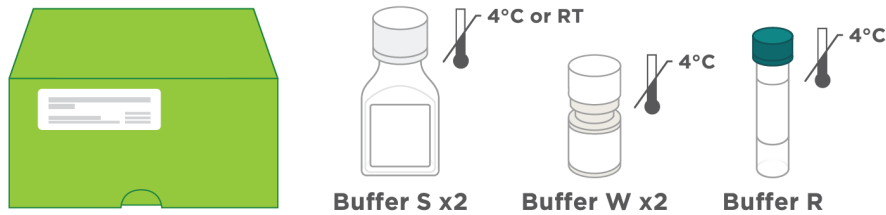
Reagents	Source, Part No.	Storage
DEPC-treated water	Thermo Fisher, AM9922 (or comparable) NOTE: As an alternative to commercial DEPC-treated water, prepare your own following standard protocols.	RT
10X phosphate buffered saline pH 7.4 (PBS)	Sigma-Aldrich, P5368-10PAK , P5368-5X10PAK (or comparable)	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 	Ambion, 2546 Thermo Fisher, AM2548 or 25530049 Note: Use of Proteinase K from any other vendor will require optimization of incubation times and concentration.	See manufacturer's instructions
Antigen Retrieval Solution (10X Tris-EDTA pH 9.0)	eBioscience™ IHC Antigen Retrieval Solution - High pH, 00-4956-58	4°C
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% Tween- 20	Teknova, T0710 (or comparable)	RT

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NanoString Reagents

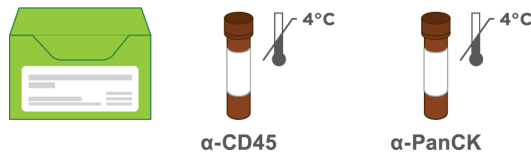
The following reagents are **supplied by NanoString**. Contact your NanoString Sales Representative to use our reagent planning tools to calculate required quantities.

GeoMx RNA Slide Prep Kit

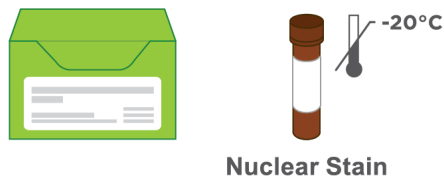


GeoMx Morphology Kit - Human or Mouse RNA compatible (various available)

Example morphology kit (Solid Tumor TME)



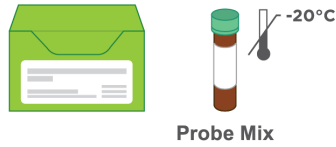
GeoMx Nuclear Stain Morphology Kit



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For NGS readout:

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

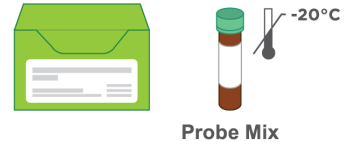


Custom RNA-NGS Probe Mix (optional)



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 ([see Table 9](#)). Use DEPC-treated water for all dilutions.

i **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. Don't 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 11 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 24.5 g Tris base and 15 g Glycine to 2 L DEPC-treated water. Do not reuse. Solution will lose efficacy with repeated use.	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% Tween-20, 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 9.0 (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

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2 Prepare tissue samples

[Appendix I: Selecting and Sectioning FFPE Samples on page 46](#) 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 greater 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) (see [Figure 5](#)) in the center of the slide and be **no larger than 35.3 mm long by 14.1 mm wide**. Mounted material 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.

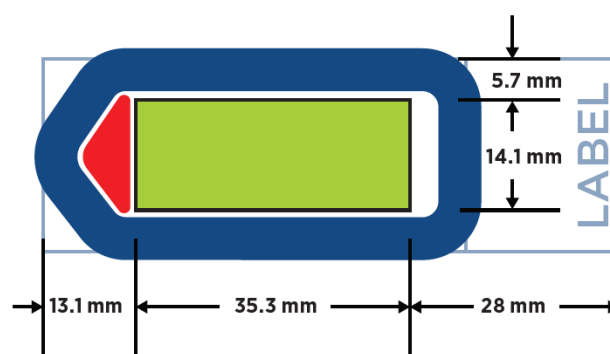


Figure 5: Slide dimensions

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; this should be empirically tested.

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3 Deparaffinize and rehydrate FFPE tissue sections (35 minutes)

You will need the following materials and reagents 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 ([see Figure 6](#)). 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.

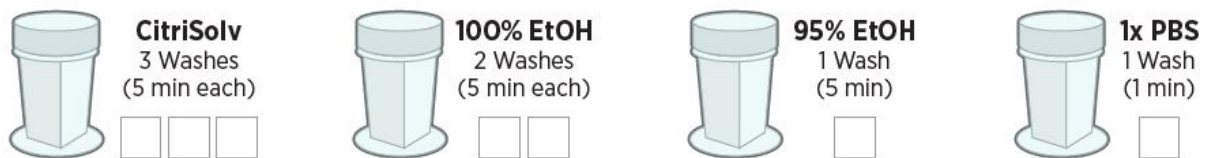


Figure 6: Wash steps

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 11](#) for the recommended Proteinase K concentration for your tissue type.

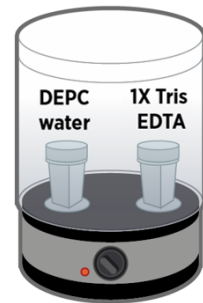
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4 Perform target retrieval (25 minutes)

You will need the following materials and reagents 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 32](#).

(To prepare cell pellet samples, use a hot plate set to 85°C, rather than the steamer, to preheat buffers and for target retrieval ([see 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 start to fall rapidly. Try to limit the time the steamer is uncovered to 30 seconds (maximum uncovered time is 2 minutes). Reproducible results rely upon minimizing this variation in temperature.

3. **Incubate the slides.** Incubation times and temperatures may differ by tissue and may need to be empirically determined. Conditions for certain tissues are listed below ([see Table 10](#)). If the tissue type you wish to use is not listed, start with 15 minutes.

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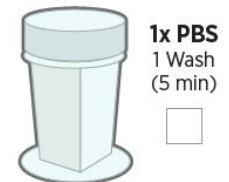
Epitope retrieval times were determined based on FFPE tissue blocks meeting the constraints outlined in the sample guidance section with minimal normal adjacent tissue. These conditions may vary by sample, the amount of normal adjacent tissue, and other factors. These conditions were optimized for large 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 min. See [Appendix II: Modifications to Protocol for Fresh Frozen Samples on page 48](#) or [Appendix III: Modifications to Protocol for Fixed Frozen Samples on page 52](#) for more information.

Table 10: Target retrieval times by tissue type

Tissue Type (FFPE)	Target Retrieval in Tris-EDTA
Breast	20 min
Cell pellets	15 min at 85°C (use hot plate instead of steamer)
Colorectal	20 min
Melanoma	20 min
Mouse tissue	20 min
NSCLC	20 min
Prostate tumor	20 min
Tonsil	15 min

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



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5 Expose RNA targets (10–30 minutes)

You will need the following materials and reagents 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 for the time specified for the tissue type ([see Table 11](#)). 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**.

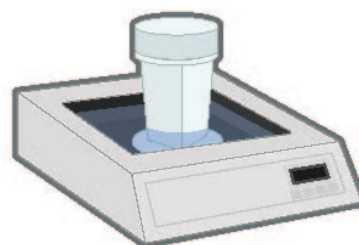


Table 11: Proteinase K digest concentrations and times by tissue type

Tissue Type	Proteinase K Digest
Breast	0.1 µg/mL for 15 min
Cell pellets	1 µg/mL for 5 min
Colorectal	1 µg/mL for 15 min
Melanoma	1 µg/mL for 15 min
Mouse tissue	1 µg/mL for 15 min
NSCLC	1 µg/mL for 15 min
Prostate tumor	1 µg/mL for 15 min
Tonsil	1 µg/mL for 15 min

Proteinase K digestion times and concentrations were determined based on FFPE tissue blocks meeting the constraints outlined in the sample guidance section with minimal normal adjacent tissue. The conditions were optimized for large tumor sections and may not apply to arrayed tissues, cored tissues, and needle biopsies. The conditions required may vary by sample, the amount of normal adjacent tissue, and other factors. Use of Proteinase K from any other vendor 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 min. See [Appendix II: Modifications to Protocol for Fresh Frozen Samples on page 48](#) or [Appendix III: Modifications to Protocol for Fixed Frozen Samples on page 52](#) for more information.

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

If preparing fixed frozen samples instead of FFPE, DO NOT perform this Postfix step. Proceed to [In situ hybridization \(overnight\) on page 39](#). See [Appendix III: Modifications to Protocol for Fixed Frozen Samples on page 52](#) for more information.

You will need the following materials and reagents 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 32](#).



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 ([see Figure 7](#)):



Figure 7: 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)

You will need the following materials and reagents for this step: **hybridization chamber**, **hybridization oven**, **Buffer R**, **RNA Probe Mix**, and **2X SSC** or **DEPC-treated water**. See the Equipment, Materials, and Reagents lists [on page 27](#) for more details.

i **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. Gloves should be changed 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 detection probes reduces viscosity, improving pipetting accuracy.) Before use, briefly vortex, then spin down. Store unused RNA detection probes 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 12](#) for NGS assays or [Table 13](#) 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).

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For NGS readout:

Table 12: Hybridization solution for assays with NGS readout (Whole Transcriptome Atlas (WTA), Cancer Transcriptome Atlas (CTA)). *n* = number of slides

Panel Configuration	Buffer R	Atlas Probe Mix	Custom assay 1	Custom assay 2	DEPC-treated H ₂ O	Final Volume
CTA or WTA	200 μ L x <i>n</i>	25 μ L x <i>n</i>	0	0	25 μ L x <i>n</i>	250 μ L x <i>n</i>
CTA or WTA + 1 custom assay	200 μ L x <i>n</i>	25 μ L x <i>n</i>	12.5 μ L x <i>n</i>	0	12.5 μ L x <i>n</i>	250 μ L x <i>n</i>
CTA or WTA + 2 custom assays	200 μ L x <i>n</i>	25 μ L x <i>n</i>	12.5 μ L x <i>n</i>	12.5 μ L x <i>n</i>	0	250 μ L x <i>n</i>
1 custom assay, standalone (no CTA or WTA)	200 μ L x <i>n</i>	0	12.5 μ L x <i>n</i>	0	37.5 μ L x <i>n</i>	250 μ L x <i>n</i>
2 custom assays, standalone (no CTA or WTA)	200 μ L x <i>n</i>	0	12.5 μ L x <i>n</i>	12.5 μ L x <i>n</i>	25 μ L x <i>n</i>	250 μ L x <i>n</i>

For nCounter readout:

Table 13: Hybridization solution for assays with nCounter readout . *n* = number of slides

Panel Configuration	Buffer R	Immune Pathways Panel	Custom assay*	DEPC-treated H ₂ O	Final Volume
RNA Immune Pathways Panel	200 μ L x <i>n</i>	37.5 μ L x <i>n</i>	0	12.5 μ L x <i>n</i>	250 μ L x <i>n</i>
RNA Immune Pathways Panel + 1 custom assay	200 μ L x <i>n</i>	37.5 μ L x <i>n</i>	12.5 μ L x <i>n</i>	0	250 μ L x <i>n</i>

*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 μ L hybridization solution** to each slide. Take care not to introduce any bubbles.

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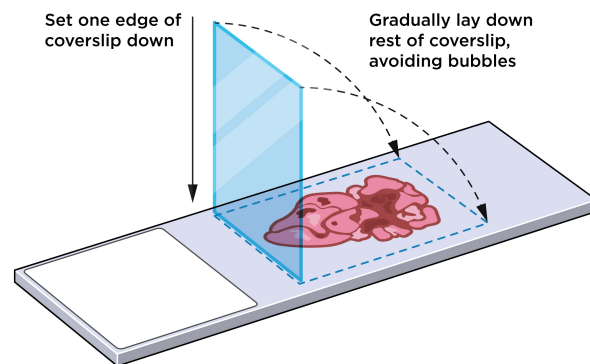
RNA FFPE Slide Prep Protocol

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 ([see Figure 8](#)).



8. Repeat steps 5–8 for each slide.

Figure 8: Applying coverslip

9. Close hybridization chamber, insert into hybridization oven, and clamp into place ([see Figure 9](#)). **Incubate at 37°C overnight (16–24 hr).**



Figure 9: Placing chamber in oven

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

You will need the following materials and reagents 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 32](#).

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 buffer **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** (see [Figure 10](#)). After the last wash, slides can be stored in 2X SSC for up to 1 hour.



Figure 10: Stringent washes to remove off-target probes

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9 Add morphology markers (100 minutes)

You will need the following materials and reagents 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 32](#).

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 they don't dry out. 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** ([see Table 14](#)) ($n = \text{number of slides}$).



Table 14: Morphology marker solution equation

Nuclear stain (SYTO 13)	Morphology Marker 1	Morphology Marker 2	Other Markers*	Buffer W**	Final Volume
22 µL x n	5.5 µL x n	5.5 µL x n	...	187 µL x n	220 µL x n

* 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 needs to be adjusted to bring total volume up to 220 µL per slide.

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

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7. **Cover tissue with morphology marker solution (up to 200 μ L).**
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 min each.



2x SSC
2 Washes (5 min 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 hr: submerge in **2X SSC and store at room temperature, protected from light.**
- Storage from 6 hr to 7 days: submerge in **2X SSC and store at 4°C, protected from light.**
- Slides may be stored longer than 7 days, but counts will decrease as a function of days stored. For best results, minimize storage time between slide preparation and loading on the GeoMx DSP.

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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 protocol. This protocol 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 39](#).

Appendix I: Selecting and Sectioning FFPE Samples

When preparing, sectioning, and storing FFPE blocks for use in the GeoMx DSP instrument 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 greater 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. Up to 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 greater than 10 years old.

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Appendix I. Selecting, Sectioning FFPE Samples

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.

- Always discard the first few sections from the block face.
- NanoString recommends SuperFrost™ Plus slides (for manual slide preparation) or Apex BOND® slides (for BOND automated slide preparation or manual slide preparation for tissues that are prone to falling off the slide).
- Tissue sections should be cut at 5 µm thickness 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) (see [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.

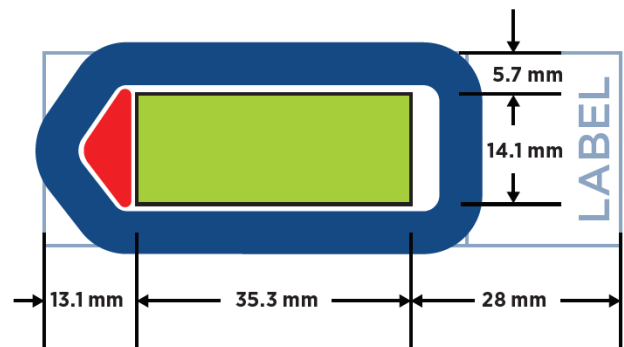


Figure 11: Slide dimensions



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 onto the corner of the wax section. The Kimwipe should not contact the tissue.
- Mounted slides should be allowed to air dry 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 dessicator (or in a sealed container with a dessicant pouch) at 4°C yield quality results for up to 3 months. Quality of results is tissue and block dependent and should be tested empirically.

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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 that you use only one sample type preparation method (e.g., use only FFPE or only fresh frozen samples).

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 [Cryosectioning technique on page 51](#).

- Always discard the first few sections from the block face.
- NanoString recommends SuperFrost Plus slides (for manual slide preparation) or Apex BOND Adhesive slides (for BOND automated slide preparation, or manual slide preparation for tissues that are prone to falling off the slide).
- Sections should be cut at 5–10 µm thickness on a calibrated cryostat and mounted immediately on the slide, without scratches or folds.

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Appendix II: Fresh Frozen Samples

- Tissue sections must be placed in the **Scan Area** (shown in green) (see [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.

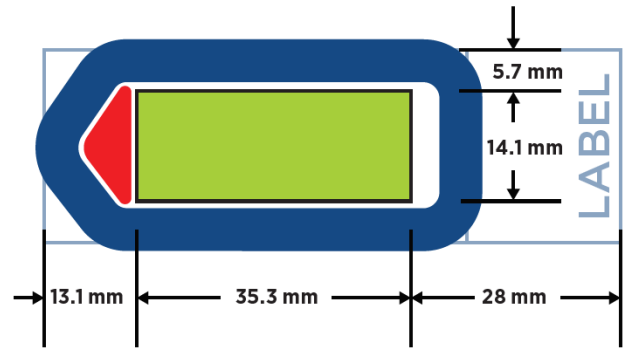


Figure 12: Slide dimensions



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.

- In a BSL2 room, handle fresh frozen slides in a biosafety cabinet.** Remove the slides from storage at -80°C. **Submerge in 10% NBF overnight (12–16 hours) at room temperature** to thaw and fix tissue. Thorough fixation is required to maintain tissue integrity.
- After fixation, wash the slides 3 times in 1X TBS-T for 5 min each wash, using a new staining jar for each wash.
- 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.

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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. 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 min.
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 35](#). 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.

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Appendix II: Fresh Frozen Samples

Cryosectioning technique

This is not meant to be an all-inclusive guide on cryosectioning. Please refer to your local pathologist or core facility for training on cryosectioning.

Prior to cryosectioning, read the user manual for the cryostat model being used, making sure to review the safety guidelines. Follow that manual's temperature recommendations for different tissue types. Recommendations should be used as a starting point but may require adjustments for your samples.

1. Set the specimen head temperature following recommendation noted above. If the cryostat allows the temperature of the blade to be set separately from that of the specimen head, set the blade temperature to be 2°C warmer than the specimen head.
2. Equilibrate the frozen tissue block to the intended cryosectioning temperature, either in the cryostat chamber or in a -20°C freezer (not frost-free).
3. Use OCT to mount the frozen tissue block to the cryostat chuck: dispense a small amount of OCT onto a room temperature chuck and place the tissue block onto the liquid OCT. Keep in mind the desired sectioning plane when mounting the frozen tissue block onto the chuck. To freeze the OCT, place it in an environment that is equivalent to or colder than that of the cryosectioning temperature for ~5 minutes. It may be beneficial to first trim the block to facilitate the mounting of cryosections onto the microscope slides. One preferred method of trimming a square or rectangular block is to cut the corners.
4. Cut 5 -10 µm-thick sections and mount on Superfrost Plus or BOND Plus microscope slides. Personal preference plays into the technique used for generating sections, and there are many tutorials and resources on the web that discuss how to cryosection. Make sure to mount tissue sections in the GeoMx DSP Scan Area (see diagram [on page 49](#)). After the tissue section has been mounted onto the slide, inspect the section, and make sure it does not contain visible scratches or folds, which will negatively impact the performance in the workflow. Once mounted, move slide to dry ice and let sit for at least one hour and then store sections in a -80°C freezer for at least 12 hours.

If sections rip or fail to stay intact during sectioning, try adjusting the sectioning temperature by a few degrees. Softer or fat-containing tissue may benefit from colder sectioning temperatures, while tissues with a high water content may benefit from warmer sectioning temperatures.

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Appendix III: Modifications to 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.

Preparing fixed frozen tissue block from harvested tissue

Reagents required for this protocol: 10% NBF, 10X PBS, DEPC-treated water, 10% Tween-20, sucrose, OCT compound.

Prepare reagents (1 hour)

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

- Prepare **30% Sucrose** as follows:
 1. Weigh out 15 g sucrose and place 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 the tube.
 3. Centrifuge conical tube.
- Prepare **20% Sucrose** as follows:
 1. Weigh out 10 g sucrose and place in a 50 mL conical tube.
 2. With a tissue culture-grade serological pipette, add DEPC-treated water up to the 50 mL mark of the tube.
 3. Centrifuge conical tube.
- Prepare **10% Sucrose** as follows:
 1. Weigh out 5 g sucrose and place in 50 mL conical tube.
 2. With a tissue culture-grade disposable pipette, add DEPC-treated water up to the 50 mL mark of the tube.
 3. Centrifuge conical tube.

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Appendix III: Fixed Frozen Samples

- 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.
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.

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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 [Cryosectioning technique on page 51](#)

- Always discard the first few sections from the block face.
- NanoString recommends SuperFrost Plus slides (for manual slide preparation) or Apex BOND slides (for BOND automated slide preparation, or manual slide preparation for tissues that are prone to falling off the slide).
- 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) (see [Figure 13](#)) 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.

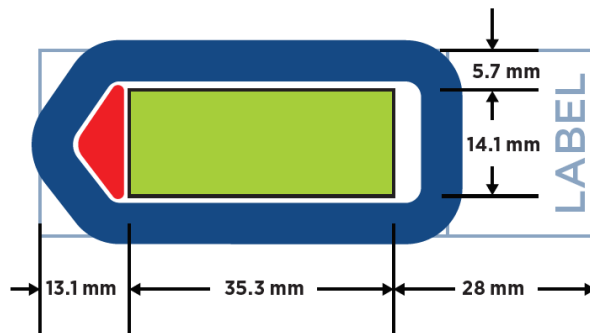


Figure 13: Slide dimensions



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.

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Appendix III: Fixed Frozen Samples

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 35](#) 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 39](#) and follow all subsequent steps from the RNA FFPE slide preparation protocol.

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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., Human IO, Mouse IO, or Human Neuroscience).

Each panel is assigned a Probe R space for nCounter readout ([see Table 15](#)). **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 15: 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

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

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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 μ L antibody** to **6 μ L of 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 = $(1.1 \mu\text{L} \times n)$, where n = number of slides

For barcoded antibodies that required a higher concentration to pass the post-conjugation IHC QC test, add $(2.2 \mu\text{L} \times n)$, where n = number of slides, to the working antibody solution. See the NanoString whitepaper **Selection and Validation of GeoMx Custom Antibody Spike-ins (MK3600)** for details.

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

Appendix VI: RNAscope[®] and GeoMx RNA Assays

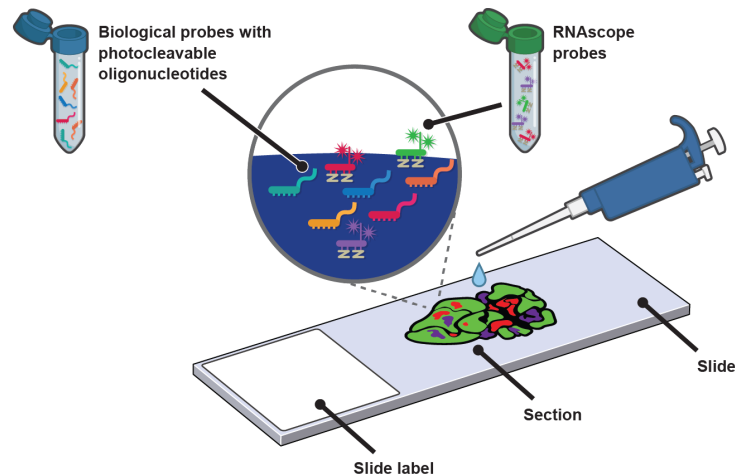


Figure 14: RNAscope probes applied to RNA slide

IMPORTANT: 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.

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.

Refer to documentation from ACD™ for protocols and materials required to run the RNAscope 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 counterstain with DAPI**. Refer to [Table 16](#) for recommended TSA fluorophore dilutions.

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Appendix VI: RNAscope & GeoMx RNA Assays

Table 16: TSA fluorophore dilutions

Fluorescence	Product number (PerkinElmer)	Recommended dilution
PerkinElmer TSA Plus Cyanine 3 System	NEL744001KT	1:1500
PerkinElmer TSA Plus Cyanine 5 System	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.

- After the wash step following HRP signal development, proceed to step 6 [on page 38](#), **Postfix**, by incubating slides in 10% NBF for 5 min. Proceed through subsequent washes and overnight **in situ hybridization**.
- The next day, continue following the 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.
- 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 Immunofluorescence Staining for RNA Assays

Secondary antibody immunofluorescence staining is a viable alternative to using fluorescently conjugated primary antibodies in GeoMx RNA assays. It is **not viable** for protein assays since 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.

The following secondary antibodies are recommended for this protocol:

Table 17: Recommended secondary antibodies

Target	Channel	Host	Source	Part No.
Mouse	647	Goat	Thermo Fisher	A21236
Mouse	594	Goat	Thermo Fisher	A11032
Rabbit	647	Goat	Thermo Fisher	A21245
Rabbit	594	Goat	Thermo Fisher	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 up to and including Blocking with Buffer W [on page 43](#).
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. Prepare 220 µL per slide.
3. Incubate slide(s) for **1 hr** (minimum) with **200 µL** of diluted primary antibody solution made in step 2, in humidity chamber at room temp. If your fluorescently conjugated primary antibodies are of a different host species than the unconjugated primary antibody, they can be added at this step along with the nuclear stain, and you can skip Step 8 below.
4. Wash slide(s) in **2X SSC** for 1 min, then wash in **2X SSC** 4 times for 3 min each.
5. Prepare solution of secondary antibody (with fluorescent label) diluted to 5 µg/mL (1:400 for secondary antibodies listed in [Table 17](#)) in Buffer W. Secondary antibody target must match host of primary antibody.

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Appendix VII: Secondary Antibody Staining

6. Incubate slide(s) for 30 min with **200 µL** of diluted secondary antibody solution made in step 5, in humidity chamber at room temp.
7. Wash slide(s) in fresh **2X SSC** for 1 min, then wash in **2X SSC** 4 times for 3 min 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:
 - a. Perform an additional **Blocking** step using goat- or other host species–specific serum for 30 min.
 - b. Wash slide(s) in fresh **2X SSC** for 1 min, then wash in **2X SSC** 4 times for 3 min each.
 - c. Incubate for **1 hr** (minimum) with your fluorescently conjugated primary antibodies and nuclear stain diluted in Buffer W, as described in step 4 [on page 43](#).
 - d. Wash slide(s) in fresh **2X SSC** for 1 min, then wash in **2X SSC** 4 times for 3 min 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 44](#)).

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

TSA can be used to enhance a weak immunolabeling signal and provides an alternative to directly conjugated fluorescent antibody morphology markers in the GeoMx DSP workflow. Please note that this protocol is not yet validated and is offered only as guidance.

This protocol applies to TSA in combination with **GeoMx protein assays**. For TSA in combination with GeoMx RNA assays (validated only for the semi-automated method), please refer to the [GeoMx DSP Automated Slide Preparation User Manual](#) (MAN-10151).

Reagents required for this protocol: Tyramide SuperBoost™ Kit (ThermoFisher, [various available](#) to target Mouse or Rabbit antibodies); unconjugated primary antibody (host species must match TSA Kit).

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 and including [Perform antigen retrieval \(1 hour\) on page 19](#).
3. First blocking step:
 - a. Apply sufficient Kit Component C2 (3% H₂O₂) 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:

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Appendix VIII: TSA with Protein Assays

- 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 of primary antibody.
- c. During the incubation, prepare the tyramide working solution ([see Table 18](#)). Prepare fresh each time.

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

Reagents prepared from kit in Step 1			
AlexaFluor Tyramide Reagent	H ₂ O ₂ Solution	Reaction Buffer	Total Volume
1 μL x n	1 μL x n	100 μL x n	102 μL x n

- d. Once the poly-HRP-conjugated secondary antibody incubation is done, 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.
 - 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 at [Primary antibody incubation \(overnight\) on page 21](#) and proceed as directed through the end of the slide preparation workflow.

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Troubleshooting

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

Issue	Possible Cause	Suggested Actions
Equipment, materials, or reagents are not available	Supply chain issues or regional variability	Contact GeoMxSupport@nanosttring.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 falls off slide	Tissue did not adhere sufficiently during baking	Try Leica BOND Plus Slides as an alternative to Superfrost Plus slides. Extending slide baking time, up to overnight at 37°C.
Some sections of tissue are not in focus	Tissue may be separating from the slide in places or have folds	See above “Tissue falls off slide”. Ensure tissue does not have folds.

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Troubleshooting

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; not more) 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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