Manual FFPE Protein Slide Preparation Protocol

Equipment, Materials, and Reagents

The following equipment **(see Table 1)**, materials **(see Table 2)**, and reagents **(see Table 3)** are recommended for this protocol but are **not supplied by NanoString**. See **Equipment, Materials, & Reagents for Protein nCounter Assays on GeoMx DSP** (available under **Support Documents** at www.nanostring.com/GeoMxDSP) for a complete list of vendor and product number information for the GeoMx protein assay.

Equipment

Table 1: Equipment not provided by NanoString.

	Equipment	Source	Part Numbers
General	Baking oven	Quincy Lab, Inc. (or comparable)	Various GC models
(optional)	UV Light Box	VWR	Various
Manual Slide Prep only	TintoRetriever Pressure Cooker **Note**: A TintoRetriever Pressure Cooker is recommended for this protocol. Other alternatives are acceptable, but have not been validated by NanoString and will require optimization. The TintoRetriever is rated for **110V**; **a transformer is required for 220V.**	BioSB	BSB 7008

Materials

The following materials are recommended for the GeoMx DSP Manual Protein workflow but are not provided by NanoString.

Table 2: Materials not provided by NanoString.

Materials	Source	Part Numbers

	Materials	Source	Part Numbers
General	Pipettes for 5–1,000 µL	Various	Various
	12-channel P20 multi-channel pipetter	Various	Various
	Filter Tips (RNase/DNase free)	Various	Various
	Microtubes	Sarstedt (or comparable)	72.785.005
	SuperFrost Plus microscopic slides	Fisher Scientific (or comparable)	12-550-15
	Tissue-Tek Staining Dish (plastic Coplin Jars) or equivalent	Sakura (or comparable)	25608-904 or 25608-906
	Humidity Chamber	Simport	M920-1
	Hydrophobic Barrier Pen	Vector Labs (or comparable)	H-4000
	Razor Blades	Various	Various
(optional)	Cover slips	Various	Various
Manual Slide Prep only	Heat/cold protectant handling glove	Various	Various

Reagents

The following reagents are recommended for the GeoMx DSP Manual Protein workflow but are not provided by NanoString.

Table 3: Reagents not provided by NanoString.

	Reagent	Source/Part Number	Storage
General	DEPC-treated water	ThermoFisher, AM9922 (or comparable) Various	RT
	10X tris buffered saline (TBS)	Cell Signaling Technologies, Cat # 12498S	RT
	10X TBS with tween 20 (TBS-T)	Cell Signaling Technologies, Cat # 9997S	RT
	4 or 16% paraformaldehyde (PFA) **Note**: Use *only* for post-fix step	Thermo Scientific, 4% concentration, Cat # FB002, R37814 16% concentration (must be diluted to 4%), Cat # 28906, 28908 (or comparable)	-20°C

	Reagent	Source/Part Number	Storage
	10X phosphate buffered saline pH 7.4 (PBS) **Note**: Used *only* to dilute PFA	Sigma Aldrich, SKU P5368-10PAK, P5368-5X10PAK (or comparable)	RT
(optional)	Fluoromount-G mounting media	SouthernBiotech, Cat. No. 0100-01	RT
Manual Slide Prep only	CitriSolv **or** Xylene	Fisher Scientific, Cat # 04-355-121 Sigma Aldrich, SKU 183164-100ML or 183164-500ML (or comparable)	RT
	100% ethanol (EtOH): ACS grade or better	Various	RT
	10X citrate buffer pH 6	Sigma Aldrich,SKU C9999-100ML or C9999-1000ML (or comparable)	4°C

NanoString Reagents

The following reagents are **supplied by NanoString**. Find them in the kits listed below.

Table 4: NanoString-provided reagents for protein slide prep

NanoString Commercial Kit	Reagent	Storage
GeoMx Protein Slide Prep Kit for FFPE	Buffer S (instrument-loading buffer)	4°C or RT
	Buffer W (blocking buffer)	4°C
GeoMx Morphology Kit for Protein	Morphology markers	4°C
	Nuclear stain (SYTO 13)	-20°C
GeoMx Core and Module Kits for nCounter	Antibody (Ab) mix	-80°C See note below.

IMPORTANT: Not all modules are compatible with each other. Some combinations of modules require a substitute probe R. Contact your NanoString representative for information on the combinations of modules that require a Substitute Probe R.

NOTE:

Each tube of antibody contains sufficient material for 12 slides. If you are using the entire Ab mix in one week, then store at 4°C. If not, **aliquot the antibody mix** and refreeze unused aliquots. Do not exceed more than 2 freeze/thaw cycles with the Ab mix and do not freeze diluted antibody.

- The total volume of detection Ab (stored at -80°C) is **112 µL**. Make aliquots to prepare 4 slides at a time, e.g., aliquot **37 µL** + **37 µL** + **38 µL** (the latter to leave in the original tube).
- The visualization Ab is stored at 4°C, so there is no need to aliquot this.

Prepare Reagents

Prepare your reagents to the following concentrations using the dilution instructions **(see Table 5)**.

Table 5: Reagent prep for protein slide preparation

Reagent	Dilution	Storage
1X Citrate Buffer (pH 6)	Dilute 10X Citrate Buffer (pH 6) in DEPC-treated water.	4°C
CitriSolv or Xylene	No dilution necessary.	RT
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 *only* for post-fix step	If using 16% concentration, dilute from 16% to 4% in 1X PBS, aliquot, and store.	-20°C
1X Phosphate-buffered saline pH 7.4 (PBS)	Dilute 100 mL of 10X PBS in DEPC-treated water per liter.	RT
1X Tris Buffered Saline with Tween 20 (TBS-T)	Dilute 100 mL of 10X TBS-T in DEPC-treated water. Prepare 5 L per Slide Preparation.	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

Prepare Tissue Samples

The **GeoMx DSP Protein and RNA Sample Prep Guidelines** cover FFPE block selection and sectioning in detail. Please review that document prior to beginning the Protein assay.

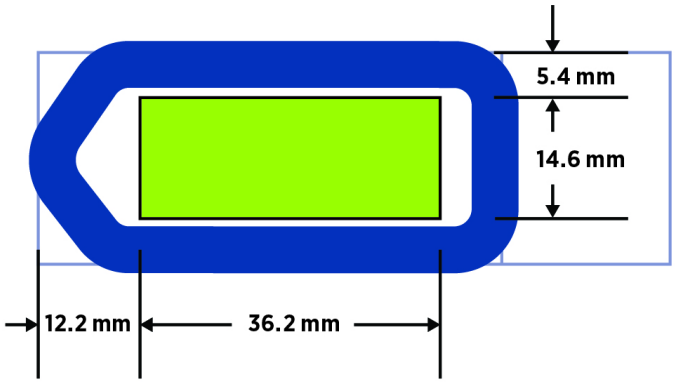
NOTE: GeoMx has been validated for samples up to 3 years old prepared from tissue with a cold ischemic time of less than 1 hour using 10% NBF or a similar fixative. Assay performance will be influenced by tissue block age and treatment conditions such as cold/warm ischemic time, fixative, and storage.

Sectioning FFPE Tissues

- Slides should be stored at room temperature in a desiccator prior to processing.
- Mounted slides should be allowed to air dry overnight.

Slide Preparation

For Slide Preparation, unstained tissue sections should be **5 µm** thick on **SuperFrost Plus slides**. Tissue sections **must be placed in the Scan Area** (the green area in the slide diagram) in the center of the slide and be **no larger than 36.2 mm long by 14.6 mm wide**. They should not overlap the slide gasket or the Tip Calibration area (this is the triangular region to the left of the green scan area in the slide diagram). If sections are larger than this size and/or placed off-center, it is likely that the tissue located outside the Scan Area will not be measured by the GeoMx DSP instrument.



- For DSP processing, ensure that **no hydrophobic pen or tissue** is in the **Scan** or **Tip Calibration areas** before placing your slides in the DSP slide holder.
- **Bake sections on slides** in a 60°C drying oven for a minimum of 30 minutes prior to deparaffinization. Longer baking times may be necessary for some tissues to adhere to the slide; this should be empirically tested. If tissue falls off, then baking longer *could* help.

> **NOTE:** In the event that sections are larger than the indicated size and/or placed off-center, continue with the slide preparation as usual. Just before loading the slide in the instrument slide tray, scrape 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 the slide preparation could generate tissue folds that may result in staining/binding artifacts, while suboptimal scraping may result in leaks in the hydrophobic barrier.

Deparaffinize and rehydrate FFPE tissue sections (45 minutes)

You will need the following items/reagents for this step: **Staining jars, Citrisolv/Xylene, 100% EtOH, 95% EtOH,** and **DEPC-treated water**. See **Equipment, Materials, and Reagents** for more details.

1. **Prime the pressure cooker** up to 80° C for Step 4 (Perform Antigen Retrieval). Before turning on the pressure cooker, ensure that the water is at the correct level per the manufacturer's instructions (for the TintoRetriever, above 4 cups).

2. **Prepare the humidity chamber** according to product instructions. If your chamber is light-permeable, minimize light exposure (e.g., by wrapping the lid in aluminum foil).
3. **Deparaffinize and rehydrate FFPE tissue sections**. Gently place the slides in a rack, and gently perform the following washes using staining jars (45 min). Ensure you have sufficient buffer to cover all slides in container for the washes below . In the first wash, you may use CitriSolv or Xylene.

Perform antigen retrieval (1 hour)

You will need the following items/reagents for this step: **staining jars, pressure cooker, 1X Citrate Buffer** and **1X TBS-T**. See **Equipment, Materials, and Reagents** for more details.

1. **Place FFPE slides in a staining jar containing 1X Citrate Buffer pH6**. Place a lid on the staining jar to prevent evaporation; to prevent pressurization, do *not* seal the jar.
2. Place the staining jar containing the slides and lid **into the pressure cooker**.
3. Attach the pressure cooker lid and run on **high pressure and high temperature for 15 minutes.**

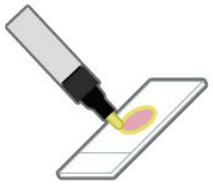
4. When the timer reaches zero, **carefully release the pressure** and transfer the staining jar with slides to room temperature, remove the staining jar lid, and **let stand for approximately 25 minutes (or up to one hour).**

5. **Wash** the slides **in 1X TBS-T** using staining jars **for 5 minutes**.
6. Using an absorbent wipe, carefully **remove the excess buffer** from the slide around the tissue.

Blocking (1 hour)

You will need the following items/reagents for this step: **hydrophobic pen, humidity chamber,** and **Buffer W**. See **Equipment, Materials, and Reagents** for more details.

1. **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 to be stained.



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

2. **Place the slides in the humidity chamber** in a horizontal position and add enough Buffer W to completely fill the hydrophobic barrier (up to **200 µL** per slide, depending on the size of the tissue).
3. **Incubate in Buffer W for 1 hour** at room temperature (RT) in a closed humidity chamber.
4. **Thaw detection antibody (Ab) mix** on ice, keeping the aliquot protected from light.

Primary antibody incubation (Overnight)

You will need the following items/reagents for this step: **Humidity chamber, detection antibodies (core and module), morphology markers,** and **Buffer W**. See **Equipment, Materials, and Reagents** for more details.

> **NOTE:** Due to the high sensitivity of this assay, it is highly recommended that you change pipette filter tips for every step, change gloves frequently, and use fresh wipes for liquid removal.

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

> **NOTE:** Each tube of antibody contains sufficient material for 12 slides. If you are using the entire Ab mix in one week, then store at 4°C. If not, **aliquot the antibody mix** and refreeze unused aliquots. Do not exceed more than 2 freeze/thaw cycles with the Ab mix and do not freeze diluted antibody.
>
> - The total volume of detection Ab (stored at -80°C) is **112 µL**. Make aliquots to prepare 4 slides at a time, e.g., aliquot **37 µL** + **37 µL** + **38 µL** (the latter to leave in the original tube).
> - The visualization Ab is stored at 4°C, so there is no need to aliquot this.

2. **Make a working antibody solution** by diluting detection and visualization antibodies into Buffer W (*n* = number of slides) **(see Table 6)**. Adjust to reflect the number of core, module, and morphology reagents and

to cover the number of slides to be prepared (up to 200 µL per slide).

- Detection antibody mixes (1:25 for each mix)
- Visualization antibodies (1:40 each marker). Additional visualization markers should be used at appropriate concentration.

> **NOTE:** For custom antibody mixes, a final concentration of 0.25 µg/mL is recommended but may need to be adjusted based on results.

Table 6: Working antibody mix equation for protein slide prep where n = # slides

Core Ab	Module Ab1	Module Ab2	Other Modules	Morph Marker1	Morph Marker2	Other Markers	Buffer W	Total Volume
(8 µL x *n*) _____	(8 µL x *n*) + _____	(8 µL x *n*) + _____	…	(5 µL x *n*) + _____	(5 µL x *n*) + _____	…	(up to 200 µL)* x *n* + _____	(200 µL x *n*) = ____

*If a different number of detection or visualization antibody tubes are used, Buffer W amount needs to be adjusted to bring total volume up to **200 µL/slide.**

> **IMPORTANT:** Not all modules are compatible with each other. Some combinations of modules require a substitute probe R. Contact your NanoString representative for information on the combinations of modules that require a Substitute Probe R.

3. **Blot the excess buffer** from the slide with an absorbent wipe.
4. **Place the slide into the humidity chamber**. **Cover the tissue with the diluted antibody solution**. Make sure the entire tissue is covered (up to approximately 200 µL, depending on the size of the tissue).

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

Post-fix (70 minutes)

Next day:

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

IMPORTANT: When tapping off slides, use a clean disposable surface to avoid protein barcode contamination.

You will need the following items/reagents for this step: **staining jars, 1X TBS-T,** and **4% PFA**. See **Equipment, Materials, and Reagents** for more details.

1. **Carefully tap off each slide** on a fresh, clean, disposable surface (e.g., several paper towels) to remove excess solution.
2. Place the tissue sections in a rack, and gently wash using staining jars; **3 changes of 1X TBS-T for 10 minutes each**.
3. **Carefully tap off each slide** on a clean, disposable surface to remove excess solution.

4. Ensure that the hydrophobic barrier is still intact; otherwise, draw a fresh hydrophobic barrier over the old one using the hydrophobic pen.

NOTE: In the next step (Nuclei Staining), you will use DNA stain, SYTO 13; it may be ideal to remove it from the freezer (stored at -20°C) at this time and allow it to warm to room temperature on the bench.

5. Cover the sample with up to **200 µL 4% PFA and incubate for 30 minutes** in the humidity chamber at RT.

6. Wash with **2 changes of 1X TBS-T for 5 minutes** each.

Nuclei staining (20 minutes)

You will need the following items/reagents for this step: **humidity chamber, staining jars, razor, DNA stain SYTO 13, 1X TBS, and 1X TBS-T**. See **Equipment, Materials, and Reagents** for more details.

1. If you have not already, **remove DNA stain** (SYTO 13) from the freezer (stored at -20°C) and allow to **warm to room temperature** on bench.
2. **Picofuge for at least 1 minute** to bring the solution to the bottom of the vial and precipitate unsoluble particles. Do not pipet from the bottom of the vial. Before refreezing, close all vials tightly.

3. **Dilute 5 µM SYTO 13 1:10 to 500 nM using 1X TBS** in a similar volume per slide as used in the blocking and post-fix steps (up to 200 µL per slide). Mix by pipetting up and down.
4. **Place slides in humidity chamber** and **cover tissue with diluted DNA stain**, up to 200 µL.
5. **Stain for 15 minutes** at RT in the humidity chamber.

6. **Wash** slides by dipping in a jar with **1X TBS-T**.
7. **Transfer** to another staining jar with fresh **1X TBS-T**.
8. Carefully **scrape off the hydrophobic pen** with a razor, making sure to remove all of the wax. Dip slide back into TBS-T if it is drying out. Work on one slide at a time, leaving the others in TBS-T.

9. **Store stained slides in 1X TBS-T**. If it is necessary to re-stain with nuclear stain (SYTO 13), re-mark the tissue with a hydrophobic pen, add the stain, and again be sure to completely remove the wax.

When the slides are prepared, load them immediately on the GeoMx DSP (see **GeoMx DSP Run Introduction**).

Safe Storage Guidelines

If not immediately loading prepared slides into a GeoMx DSP slide holder, you must adhere to the following guidelines:

- Slides must never be stored dry; they may be submerged in TBS-T if being loaded onto the GeoMx DSP instrument within ~6 hrs of slide prep. For longer term storage, mounting a coverslip is required.
- Slides must be stored at 4°C.
- Slides must be stored in the dark (avoiding light is crucial since UV light cleaves DSP tags).

Slide Mounting Procedure for Long-Term Storage (>3 days)

1. **Rinse slide** to be mounted with TBS-T or PBS-T, touch the edges of slide on a paper towel to remove excess liquid. Place slides 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. Optional: let stand approximately 5 minutes.
3. **Mount coverslip** (hold one slide to align, then drop from one side to the other) and remove excess mounting medium.
4. **Allow slide to dry** at ambient temperature in a dark area overnight (bench drawer).
5. Store slide at ambient temperature or at 4°C. Avoid light exposure.

Slide Unmounting Procedure

1. Submerge mounted slide in TBS-T or PBS-T until cover slip has been loosened or has fallen off (>30-60 min).
2. If coverslip has fallen off, slide is ready for use. If not, gently remove coverslip using tweezers.
3. Wash slide with 1X fresh TBST for 5 minutes to ensure removal of mounting media.

Stripping and Re-probing Procedure

1. Place the slide in a horizontal position and **apply enough TBS-T to completely cover the tissue** (approximately 50–200 µL, depending on the size of the tissue).
2. Place slides directly (or on a clear tray) onto a **UV transilluminator and expose to 302/312 nm UV light** for **3 minutes** to cleave tags from bound antibodies.
3. Carefully **tap off each slide** and remove excess solution on a fresh, clean, disposable surface (e.g., several paper towels) to avoid protein barcode contamination.
4. **Wash slides** by dipping in a jar with **TBS-T.**
5. Transfer to another staining jar with fresh **TBS-T**.
6. Continue with **Perform antigen retrieval (1 hour)** in this protocol.
