Manual RNA Slide Preparation Protocol

Equipment, Materials, and Reagents

The following equipment **(see Table 15)**, materials **(see Table 16)**, and reagents **(see Table 17)** are recommended for this protocol but are **not supplied by NanoString**. See **Equipment, Materials, & Reagents for RNA 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 RNA assay.

Equipment

The following equipment is recommended for the GeoMx DSP Manual RNA workflow but is not supplied by NanoString.

Table 15: Equipment not provided by NanoString.

	Equipment	Source	Part Numbers
General	Baking oven	Quincy Lab, Inc. (or comparable)	Various GC models
	Hybridization oven including hybridization chamber*:		
	Boekel Scientific RapidFISH Slide Hybridizer **or**	Boekel Scientific	240200 for 120V or 240200-2 for 230V

	Equipment	Source	Part Numbers
	HybEZ II Hybridization System • HybEZ oven • Humidity control tray • EZ-Batch wash tray • EZ-Batch slide holder • HybEZ humidifying paper (2 sheets) * These hybridization ovens are designed to keep the slides hydrated and maintain a precise temperature overnight. NanoString does not recommend the use of any other hybridization ovens for GeoMx RNA DSP. Please contact NanoString with any concerns.	ACDBio	321710/321720 310012 321717 321716 310025
	Water bath (up to at least 37°C)	Various	Various
(optional)	UV Light Box	VWR	Various
Manual Slide Prep only	TintoRetriever Pressure Cooker (for manual slide prep only) **Note**: A TintoRetriever Pressure Cooker is recommended for this protocol. Other alternatives may be acceptable, but have not been validated by NanoString and will require optimization. Any model used needs to have a temperature gauge. 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 RNA workflow but are not supplied by NanoString.

Table 16: Materials not provided by NanoString.

	Materials	Source	Part Numbers
General	Pipettes for 5–1,000 µL	Various	Various
	12-channel P20 multi-channel pipette	Various	Various
	Filter Tips (RNase/DNase free)	Various	Various
	Microtubes	Sarstedt (or comparable)	72.785.005
	SuperFrost Plus microscope 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
	Benchtop protector sheet (fits inside the hyb oven, optional)	Fisher Scientific (or comparable)	15235101
	Hybrislip hybridization covers (22 mm x 40 mm x 0.25 mm) **Note:** this has been validated by NanoString. Do not use other products	Grace Biolabs	714022
	RNase AWAY	ThermoFisher	7003PK
Manual Slide Prep only	Heat/cold protectant handling glove	Various	Various

Reagents

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

Table 17: Reagents not provided by NanoString.

	Reagent	Source/ Part Number	Storage
General	DEPC-treated water	ThermoFisher, AM9922 (or comparable)	RT
	10X phosphate buffered saline pH 7.4 (PBS)	Sigma Aldrich, SKU P5368-10PAK, P5368-5X10PAK (or comparable)	RT
	10% neutral buffered formalin (NBF) 	EMS Diasum, Cat # 15740-04 (or comparable)	RT

	Reagent	Source/ Part Number	Storage
	100% deionized formamide 	ThermoFisher , AM9342 **or** VWR, VWRV0606 (or comparable)	4°C (bring to RT before opening)
	20X SSC (DNase, RNase free)	Sigma Aldrich, S6639	RT
	Proteinase K	Proteinase K from Ambion, 2546, **or** Thermo Fisher, AM2548 **Note**: Use of Proteinase K from any other vendor will require optimization of incubation times and concentration.	-20°C
	1X Tris-EDTA pH 9.0	eBioscience™ IHC Antigen Retrieval Solution - High pH, 00-4956-58 (10X - will need to be diluted with DEPC-treated water)	4°C
	Tris base	Sigma, 10708976001 (or comparable)	RT
	Glycine	Sigma, G7126 (or comparable)	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
	10% Tween 20	Teknova, T0710 (or comparable)	RT

NanoString Reagents

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

Table 18: NanoString-provided reagents for RNA slide prep

NanoString Commercial Kit	Reagent	Storage
GeoMx DSP RNA Slide Prep Kit for FFPE	Buffer R	4°C

NanoString Commercial Kit	Reagent	Storage
	Buffer S (instrument-loading buffer)	4°C or RT
	Buffer W (blocking buffer)	4°C
GeoMx Morphology Kit for RNA	Morphology markers	4°C
	Nuclear stain (SYTO 13)	-20°C
GeoMx Core and custom module Kits for nCounter	RNA Probe Mix	-20°C Do not freeze after initial thaw. Store at 4°C for up to 3 months.

Prepare Reagents

Prepare your reagents to the following concentrations using the dilution instructions **(see Table 19)**.

IMPORTANT: Take care to maintain nuclease-free conditions. The greatest risk of contamination comes from GeoMx RNA DSP probes and other oligos. We recommend the use of RNase AWAY (Thermo Scientific™ 7002), as it will limit contamination from oligos, GeoMx RNA detection probes, and nucleases. After using RNase AWAY, allow to air dry completely, or rinse with DEPC-treated water.

See manufacturer's instructions for details.

NOTE: All of these buffers can be made in bulk ahead of time unless otherwise noted. If buffers have a shelf life, it is noted.

NOTE: Use DEPC-treated water for all dilutions.

Table 19: 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. Change at least weekly.	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.	4°C

Reagent	Dilution	Storage
10% neutral buffered formalin (NBF)		RT
	Caution: NBF is hazardous, handle with care and minimize inhalation risks. Work with 10% NBF in the fume hood. OK to reuse.	
Proteinase K	Prepare **1 µg/mL** by adding **10 µL** of **20 mg/mL** Proteinase K to **200 mL** of **1X PBS** made with DEPC-treated water **Note:** Should be prepared fresh daily; don't reuse. Take care to pipette accurately. Inaccurate concentration of Proteinase K will affect assay performance. Serial dilutions are recommended.	RT
NBF stop buffer	Prepare **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	Prepare **100 mL** of 2X SSC by combining **10 mL** of 20X SSC, **1 mL** of 10% Tween-20, and **89 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

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, particularly for RNA, will be influenced by tissue block age and treatment conditions such as cold/warm ischemic time, fixative, and storage.

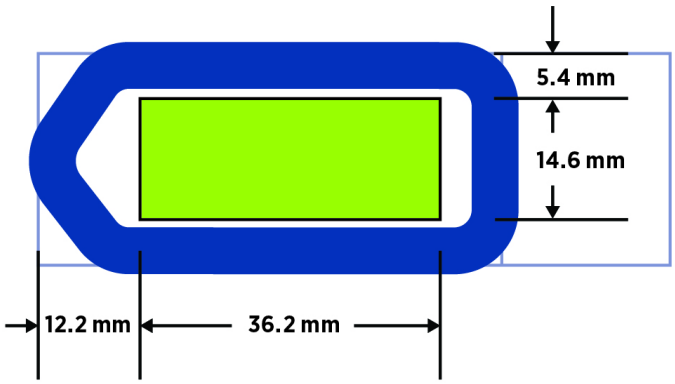
Sectioning FFPE Tissues

- Due to oxidation, always discard the first few sections from the block face when cutting sections.

- It is recommended to use mounted sections within two weeks for best results. Older sections (1-2 months) may produce reasonable results, but this may be tissue or block dependent and should be tested empirically. Slides should be stored at room temperature in a desiccator or at 4°C 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.

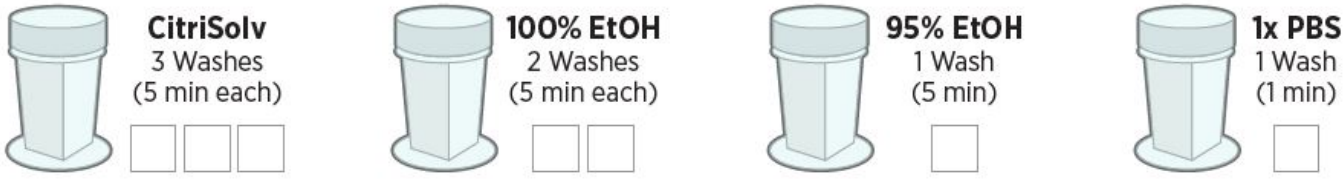
Deparaffinize and rehydrate FFPE tissue sections (31 minutes)

You will need the following items/reagents for this step: **Staining jars, Citrisolv/Xylene, 100% EtOH, 95% EtOH,** and **1x PBS**. See **Equipment, Materials, and Reagents** for more details.

1. **Preheat the pressure cooker and water bath** to the settings listed for your tissue type in step 4 **(see Table 20)**. 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. **Deparaffinize and rehydrate FFPE tissue sections**. Place the slides in a rack and gently perform the following washes using staining jars . Ensure you have sufficient buffer to cover all slides in container for the washes below. After the last wash, slides can be stored in the 1x PBS for up to one hour.



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

Perform target retrieval (15 - 25 minutes)

You will need the following items/reagents for this step: **Staining jars, pressure cooker, 1x Tris EDTA,** and **1x PBS**. See **Equipment, Materials, and Reagents** for more details.

1. Place the slide rack in a staining jar containing **1x Tris EDTA (pH 9.0)**. **Cover (but do not seal) jar**; this prevents evaporation and light exposure.
2. Once the pressure cooker has reached the appropriate temperature, **carefully release the lid** and place the slide rack inside. Seal the pressure cooker and start the timer.

3. **Incubate the slides**. Incubation times and temperatures may differ by tissue and may need to be empirically determined. The table below lists starting conditions **(see Table 20)**. If the tissue type you wish to use is not listed, start with the default conditions: 15 min @ 100°C (low pressure, medium temp).

IMPORTANT: Monitor the pressure cooker temperature closely, as set temperatures may not be reliable. Temperatures over 100°C will lead to boiling, which can pull the tissue off of the slide surface. If the temperature climbs over 100°C, shut the cooker off (but do not release the pressure). Pressure and temperature will fall very slowly. Turn the pressure cooker back on if temperature reaches 90°C before time is up.

NOTE: Epitope retrieval times and temperatures were determined based on FFPE tissue blocks meeting the constraints outlined in the sample guidance document 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.

Table 20: Target retrieval times and temps by tissue type

Tissue Type	Target Retrieval in Tris-EDTA
Breast	10 min @ 100°C (low pressure, medium temp)
Cell pellets	10 min @ 85°C (low pressure, low temp)
Colorectal	20 min @ 100°C (low pressure, medium temp)
Melanoma (skin)	20 min @ 100°C(low pressure, medium temp)
NSCLC	20 min @ 100°C(low pressure, medium temp)
Prostate tumor	20 min @ 100°C(low pressure, medium temp)
Tonsil	10 min @ 100°C (low pressure, medium temp)

4. When the timer is finished, **release the pressure** and **move slides to PBS immediately**.
5. **Wash in PBS for 5 minutes** Slides can be stored up to 1 hour in the PBS.

Expose RNA targets (10 - 30 minutes)

You will need the following items/reagents for this step: **Staining jars, water bath, Proteinase K,** and **1x PBS**. See **Equipment, Materials, and Reagents** for more details.

1. **Dilute proteinase K**. Proteinase K concentration and incubation times differ by tissue and may need to be empirically determined. Modifications for certain tissues listed below **(see Table 21)**. If the tissue type you wish to use is not listed, start with the **default conditions: 1 µg/mL for 15 min.**

> **NOTE:** Proteinase K digestion times and concentrations were determined based on FFPE tissue blocks meeting the constraints outlined in the sample guidance document 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. Use of Proteinase K from any other vendor will require optimization of incubation times and concentration.

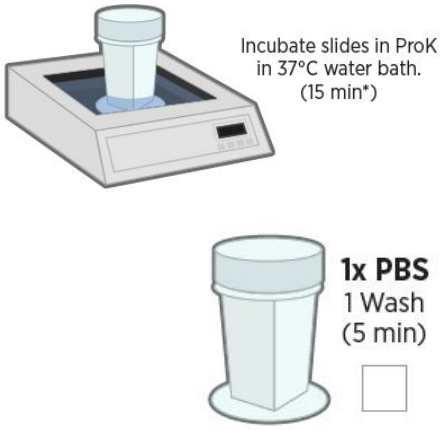
Table 21: ProK digest concentrations and times by tissue type

Tissue Type	Proteinase K Digest
Breast	0.1 µg/mL for 15 min
Cell pellets	1 µg/mL for 5 min

Tissue Type	Proteinase K Digest
Colorectal	1 µg/mL for 15 min
Melanoma (skin)	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

Example: For 1 µg/mL PK, add **10 µL** of **20 mg/mL** proteinase K into **200 mL** 1X PBS made with DEPC-treated water (make a minimum of 200 µL to avoid pipetting small volumes of proteinase K).

2. Place **proteinase K** in a Coplin jar and **warm it to 37°C** in water bath.
3. **Incubate slides** in proteinase K at 37°C using the default time and concentration or tissue-dependent conditions listed in the table above **(see Table 21)**.
4. **Wash in 1X PBS** for 5 minutes. Proceed to the next step **immediately**.



Postfix—Preserve tissue morphology for soft tissues (20 min)

You will need the following items/reagents for this step: **Staining jars, 10% NBF, NBF Stop Buffer** and **1x PBS**. See **Equipment, Materials, and Reagents** for more details.

NOTE: Ensure that you have prepared **NBF stop buffer** before commencing.

Post-fix the tissue by performing the washes listed in the figure below .

WARNING: Used NBF Stop Buffer contains NBF and must be disposed of in the same manner as the NBF.

NOTE: Slides can be stored in the final PBS wash up to 1 hour at RT or up to 6 hours at 4°C.

In Situ Hybridization (overnight)

You will need the following items/reagents for this step: **Hybridization chamber, oven, Buffer R, RNA Probe Mix,** and **DEPC-treated water**. See **Equipment, Materials, and Reagents** for more details.

IMPORTANT: Probe mixes should be assembled in an area separate from any nCounter work, NGS library prep or other GeoMx workflows. GeoMx detection reagents can contaminate results. Areas should be cleaned thoroughly with *RNase AWAY* after probe mix formulation (*RNaseZap* is only effective for enzymes, not oligos, and should *not* be used in place of *RNase AWAY*). Alternately, mixes can be made in PCR workstations and decontaminated with UV light. Gloves should also be changed after handling any probe mixes to avoid contamination.

NOTE: Prepare buffers: Warm Buffer R to room temperature (RT) before opening. Thaw RNA detection probes on ice. Before use, mix thoroughly by pipetting. Once thawed, ***do not re-freeze probes.*** Refrigerate at 4°C for up to 3 months.

NOTE: Prepare the hybridization chamber according to product instructions. The hyb ovens listed in the Equipment section are not light-permeable. If your chamber is light-permeable, minimize light exposure (e.g., by wrapping the lid in aluminum foil).

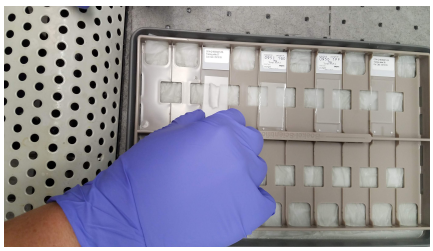
1. Make hybridization solution (*n*= number of slides) **(see Table 22)**.

   If adding custom targets to the RNA assay, add the appropriate volume of custom RNA probes (see below); if not, add DEPC water in lieu of the custom spike-in.

Table 22: Hybridization solution equation

Buffer R	RNA Probe Mix	Custom RNA Probe (if applicable) / DEPC H_2O	Final Volume
(200 µL x *n*)	(37.5 µL x *n*)	(12.5 µL x *n*)	(250 µL x *n*)
_____	+ _____	+ _____	= _____

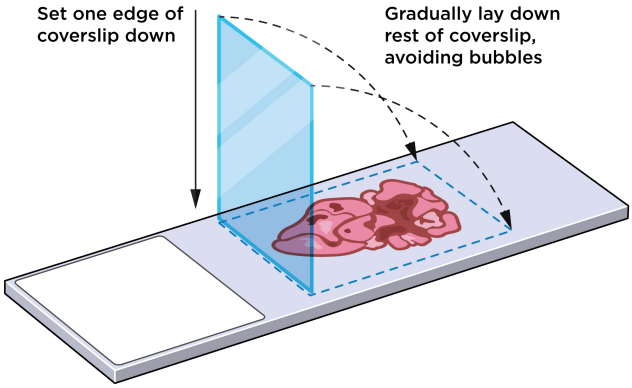
2. **Clean all equipment** with RNase AWAY and allow to dry or rinse with DEPC-treated water (see **Important** note, above). The hybridization chamber can be a **key source of contamination** by oligos. **Arrange fresh kimwipes** on bottom of the chamber.
3. **Wet the Kimwipes with 2x SSC or DEPC-treated water**. Take care that the Kimwipes and 2x SSC do not contact the slides. Hybridization solution can wick off of the slides if in contact with Kimwipes or liquid. Kimwipes should be thoroughly damp, but standing buffer should not be present.



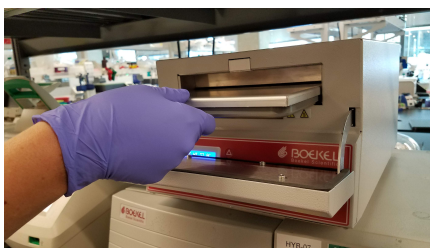
4. **One at a time**, remove slides from PBS, **wipe away excess liquid**, and **set in hybridization chamber**. **Take care not to let the slides dry out.**
5. **Add 200 µL hybridization solution** to each slide. Take care not to introduce any bubbles.

> **NOTE:** Avoid introducing bubbles by leaving a small residual volume in the pipette tip. In the event that a bubble is introduced, it can be effectively gently aspirated with a pipette tip. Avoid contact between the tissue and the tip, as this could result in tearing. When removing air bubbles, removing small amounts of hybridization solution (as long as sufficient solution remains to cover the slide after the cover slip is applied) is preferable to having bubbles.

6. **Gently apply a Grace Biolabs 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 .
7. Repeat steps 4–6 for each slide.



8. Close hybridization chamber, insert into oven, and clamp into place. **Incubate at 37°C overnight** (16–24 hours).



NOTE: If your oven does not seal (with a gasket) you may seal your hyb chamber in a ziplock bag to simulate a sealed chamber (chambers sealed in this manner should be tested to ensure they maintain humidity for 24 hours before use). Unsealed conditions lead to evaporation of the hyb solution.

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

You will need the following items/reagents for this step: **Water bath, 4x SSC,** and **formamide**. See **Equipment, Materials, and Reagents** for more details.

Before you begin, ensure water bath is set to 37°C for later use. **Warm 100% formamide** to room temperature before opening. Once formamide is at room temperature, make **Stringent Wash** by mixing equal parts **4x SSC** and **100% formamide**. Fill two staining jars with **Stringent Wash** and preheat them in 37°C water bath.

NOTE: One way to minimize formamide use: fill one of your staining jars with 4x SSC and a second with 100% formamide. Pour these quantities into a third container to mix, then redistribute back into the two staining jars.

1. **Dip** the slides in **2x SSC-T** and allow the coverslips to slide off by themselves. **Minimize the time in SSC-T**; continue to the wash steps within **5 minutes**. Slides with coverslips that **have not come off after 5 minutes can be moved to stringent wash**. Coverslips should come off in the first stringent wash.
2. Perform the washes listed in the figure below . After the last wash, slides can be stored in 2x SSC for up to one hour.

IMPORTANT: Forcibly removing coverslips will damage the tissue. Place slides in SSC-T to help loosen the coverslips. Allow the coverslips to slide off freely without contacting the sides of the container.

NOTE: Use separate staining jars for different probe mixes. Staining jars should be cleaned with *RNase AWAY* before use.

IMPORTANT: Anything coming into contact with hyb solution (which contains probes), such as containers for ProK and SSC-T, needs to be exclusive for this purpose and thoroughly washed and cleaned with RNase AWAY, as probes may contaminate later runs.

Add morphology markers (100 minutes)

You will need the following items/reagents for this step: **Humidity chamber, Buffer W, SYTO 13, morphology markers,** and **2x SSC**. See **Equipment, Materials, and Reagents** for more details.

Before you begin: Remove DNA stain (SYTO 13) from the freezer (stored at -20°C) and allow to warm to room temperature on bench.

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.

1. **Block with Buffer W.** Move one slide at a time to the tray. Transfer tray to the humidity chamber for antibody staining. Cover tissue with up to **200 µL Buffer W** and leave at RT for 30 minutes. **Cover to prevent light exposure**.

NOTE: 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, but must be carefully removed with a razor blade after antibody incubation.

2. Picofuge thawed DNA stain (SYTO 13, see above) for at least 1 minute to bring the solution to the bottom of the vial and precipitate insoluble particles. Do not pipette from the bottom of the vial. Before refreezing, close all vials tightly.

3. **Prepare morphology marker solution** (*n* = number of slides)**(see Table 23)**. Prepare up to 200 µL per slide (total of 220 µL including 10% overage per slide).

Table 23: Morphology marker solution equation

SYTO 13	Morph Marker 1	Morph Marker 2	Other Marker (optional)	Buffer W	Total Volume
(22 µL x *n*)	(5.5 µL x *n*)	(5.5 µL x *n*)	…	(187 µL x *n*)*	(220 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 **220 µL per slide**.

4. **Spin morphology marker solution and antibodies** by flicking and spinning down.
5. **Remove Buffer W** by tapping slide onto a kimwipe.
6. **Stain with antibodies for 1 hour** in the humidity chamber at room temperature (RT).

7. **Wash 2 times for 5 min in 2x SSC**.

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

Safe Storage Guidelines for RNA Slides

- Slides must never be stored dry; they may be submerged in 2X SSC-T if being loaded onto the GeoMx DSP instrument within ~6 hrs of slide prep. They can be stored submerged in 2x SSC at 4°C for up to 48 hours. For best results, minimize storage time between slide preparation and loading on the DSP.
- Slides must be stored in the dark (avoiding light is crucial since UV light cleaves DSP tags).

Stripping and Re-probing Procedure

1. Place the slide in a horizontal position and cover the tissue with enough 2x SSC-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 oligo contamination.
4. Wash slides by dipping in a jar with 2x SSC-T.
5. Transfer to another staining jar with fresh 2x SSC-T.
6. Continue with Step 4 of the RNA Slide Preparation procedure, **Perform target retrieval (15 - 25 minutes)**, then proceed directly to Step 7 of the RNA Slide Preparation procedure, **In Situ Hybridization (overnight)**.
