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## RNA Slide Preparation Protocol (FFPE) for nanostring DSP - GeoMx - Human Breast samples

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**Protocol status:** Working

We use this protocol and it's working

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**Keywords:** rna slide preparation protocol, rna slide preparation for formalin, rna slide preparation, nanostring dsp, human breast samples this protocol, rna, ffpe, human breast sample

## Disclaimer

The steps of the protocol are from Nanostring MAN-10150-03 June 23 version.

## Abstract

This protocol is designed for RNA slide preparation for formalin-fixed tissue.



## Guidelines

This is the default protocol for slide preparation by Nanostring.

### IMPORTANT:

Take care to maintain nuclease-free conditions. The most significant 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, GeoMxprobes 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.

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, ensuring the slide gasket and tip calibration area are tissue-free. Scraping off tissue before slide preparation could generate tissue folds that may result in staining or binding artifacts.

### Selecting and Sectioning FFPE Samples

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

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

### Selecting FFPE blocks

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

1. Blocks should be fixed in 10% neutral buffered formalin for 18 to 24 hours at room temperature. This applies to tissues less than 0.5 cm in thickness. Larger tissues have not been tested by NanoString and may require longer fixation times.

2. Tissues should be fixed immediately after excision for best results. Fixation within one hour post-excision is acceptable

3. Tissues should be thoroughly dehydrated in ethanol gradients prior to embedding in paraffin.
4. FFPE blocks should be stored at room temperature and ambient humidity.
5. For best results, do not use FFPE blocks that are more than 10 years old.

### Sectioning FFPE blocks

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

Due to oxidation at the surface, discard the first few sections cut from the block face.

NanoString recommends SuperFrost™ Plus slides (for manual slide preparation) or Apex BOND® or BOND Plus slides (for BOND automated slide preparation or manual slide preparation for tissues that exhibit poor adhesion).

FFPE tissue sections should be cut 5 µm thick on a calibrated microtome and mounted on the slide immediately, without scratches or folds.

Tissue sections must be placed in the **Scan Area** (shown in green in [Figure 1](#)) 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 ScanArea.

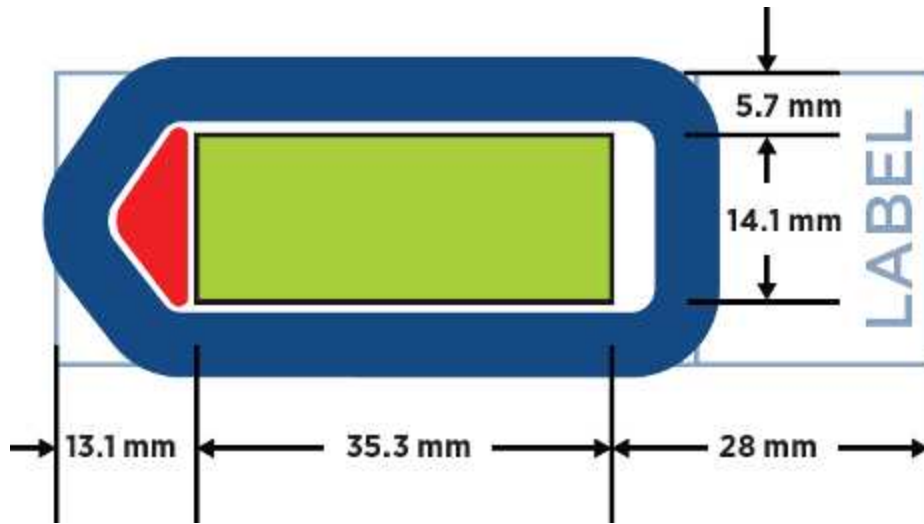


Figure 10: Slidedimensions. Measure from label edge of slide as reference point.

Any water trapped under the wax or tissue section should be removed by gently touching a folded Kimwipe to the corner of the wax section. The Kimwipe should not contact the tissue.

Air dry mounted slides overnight prior to use. Store slides in a vertical position such that any remaining water can drain away from the tissue section.

Slides stored in a desiccator (or in a sealed container with a desiccant pouch) at 4°C or room temperature have been shown to yield quality results for up to 3 months. Quality of results is tissue and block-dependent and should be tested empirically.



## Materials

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

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

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

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



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

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

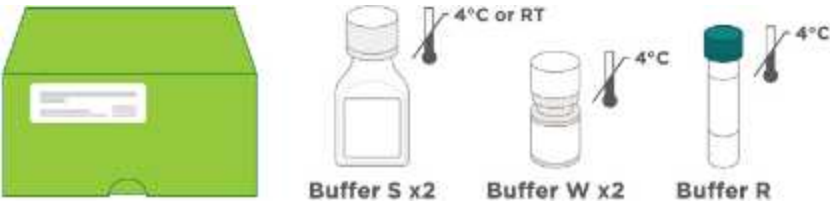
Table 3: Reagents for RNA slide prep not supplied by NanoString. RT = room temperature

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

|  | A                                  | B                              | C  |
|--|------------------------------------|--------------------------------|----|
|  | 100% ethanol (ACS grade or better) | Various                        | RT |
|  | 10% Tween20                        | Teknova, T0710 (or comparable) | RT |

NanoString Reagents

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



GeoMx RNA Slide Prep Kit



GeoMx Morphology Kit – Human or Mouse Protein compatible



GeoMx Nuclear Stain Morphology Kit





## Slides sectioning

- 1 Tissue sections should be **5  $\mu\text{m}$**  thick and mounted on **Superfrost Plus or BOND Plus slides**. Tissue sections must be placed in the **Scan Area** (shown in green in [Figure 3](#)) in the center of the slide and be **no larger than 35.3 mm long by 14.1 mm wide**. 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.

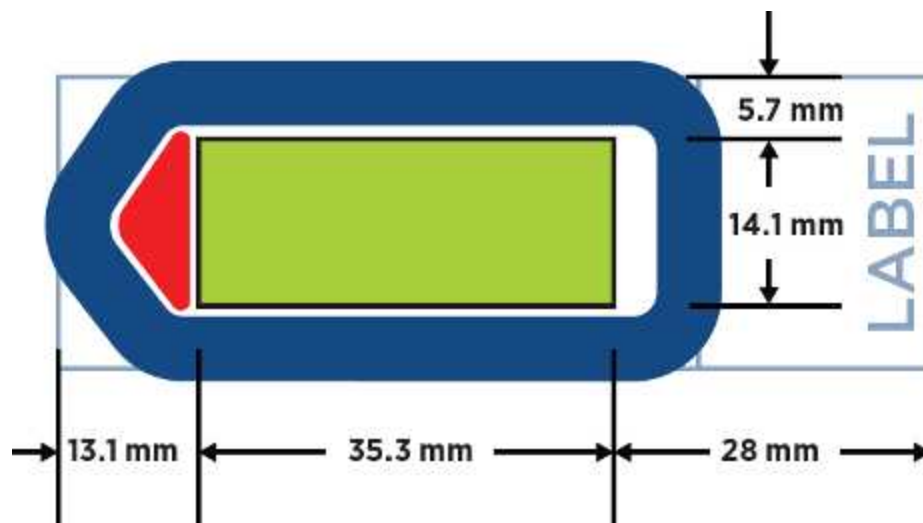


Figure 3: Slide dimensions. Measure from labeled edge of slide as reference point.

If sections are too large and/or placed off-center, continue with slide preparation as usual. Just before loading the slide in the instrument slide tray, scrape off the parts of the tissue exceeding the scan area, ensuring the slide gasket and tip calibration area are tissue-free. 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.

## Slides baking

- 2 **Bake slides with mounted sections** in a 60°C drying oven for 30 minutes to 3 hours prior to deparaffinization. Stand slides vertically during baking to allow excess paraffin to flow off. Longer baking times may be necessary for some tissues to



sufficiently adhere to the slide (e.g., overnight at 37°C followed by 2–4 hr at 60°C); this should be empirically tested.

## Deparaffinize and rehydrate FFPE tissue sections (31 minutes),

- 3 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).

**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. Check on the steamer **every 30 minutes**.

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

Alternatively, a pressure cooker may be used for antigen retrieval. However, the pressure cooker must be used without pressure (i.e. pressure valve open) during the incubation time.

**Prepare the pressure cooker** by adding water to the correct level per the manufacturer's instructions, level 6 and preheating to 115°C with the 1X Tris EDTA (pH9.0) and DEPC water. Once the temperature reaches 110°C, turn off the pressure cooker and release the pressure by leaving the valve open.

- 4 **Deparaffinize and rehydrate FFPE tissue sections.** Place slides in a rack and perform the following washes in staining jars ([see Figure 2](#)). 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 2: Wash steps

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

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**

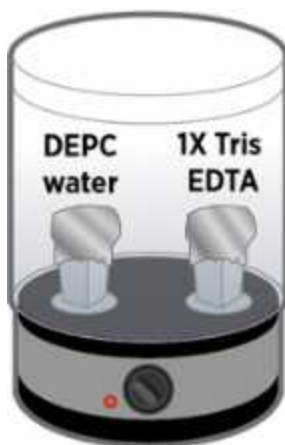
**to preheat to 37°C**. Refer to [Table 2](#) for the recommended Proteinase K concentration for your tissue type.

## Perform target retrieval (25 minutes)

- 5 Needed for this step: **Steamer, Staining jars, 1X Tris-EDTA (pH 9.0)** (antigen retrieval solution), **DEPC- treated water** (all preheated in the step 2) and **1X PBS**.

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

- 5.1 Place an instant-read digital thermometer through the vents in the steamer lid without removing the 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.

- 5.2 Once the 1X Tris-EDTA reaches 99°C, carefully remove the steamer lid, pressure cooker 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/ pressure cooker lid.

If using a pressure cooker, ensure that the pressure valve is open and the temperature of the pressure cooker is above 110°C. Once the pressure is fully release, open the pressure cooker and 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/ pressure cooker lid.

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

- 5.3 Incubate the slides according to the conditions for different tissue types in **Table 2**. Incubation times and temperatures may need to be empirically determined. If the tissue type you wish to use is not listed, start with 15 minutes in your empirical testing.

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

- 5.4 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 for up to 1 hour in 1X PBS



## Expose RNA target (10-30 minutes)

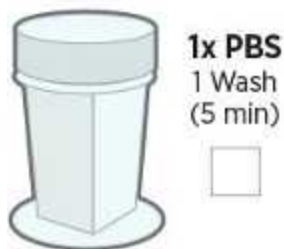
### 6 Expose RNA targets (10–30 minutes).

Needed for this step: preheated water bath, preheated Proteinase K dilution, and 1X PBS.

- 6.1 Incubate slides in Proteinase K solution at 37°C according to the conditions for different tissue types in [Table 2](#). 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.

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

- 6.2 **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.**



## Post-fixation (20 minutes)

### 7 Postfix: Preserve tissue morphology for soft tissues (20 minutes).

**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.

#### 7.1

Post-fix the tissue by performing these washes (see Figure 3):



Figure 3: 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.

## *In situ* hybridization (overnight)

### 8 Needed for this step: hybridization chamber, hybridization oven, Buffer R, RNA Probe Mix, and 2X SSC or DEPC-treated water.

#### 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 decontaminated with UV light. Change gloves after handling any probe mixes to avoid cross-contamination.

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

8.1 **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, flick to mix, then spin down. **Store unused RNA detection probes at 4°C for up to 6 months or re-freeze.**

8.2 **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 the liquid should not pool. If your chamber is light permeable, wrap the lid in aluminum foil to minimize light exposure.

8.3 **Make hybridization solution** following for NGS assays or Table 4 for nCounter assays.

Confirm that you use 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). Write down the part and lot numbers of the probe set you are using. You will need the part number when loading the slides on the instrument.

Table 4: Hybridization solution for assays with NGS readout(Atlas = WholeTranscriptome Atlas (WTA), n = number of slides.

|  | A                   | B               | C               | D                | E               |
|--|---------------------|-----------------|-----------------|------------------|-----------------|
|  | Panel Configuration | Buffer R        | Atlas Probe Mix | DEPC-treated H2O | Final Volume    |
|  | Atlas (WTA)         | 200 $\mu$ L x n | 25 $\mu$ L x n  | 25 $\mu$ L x n   | 250 $\mu$ L x n |

8.4 **One at a time, remove slides from 1X PBS**, wipe away excess liquid, and set in a hybridization chamber in a horizontal position. **Take care not to let the slides dry out.**

8.5 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.

- 8.6 **Add 200  $\mu$ L hybridization solution** to each slide. Take care not to introduce any bubbles.

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

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

- 8.7 **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 4).

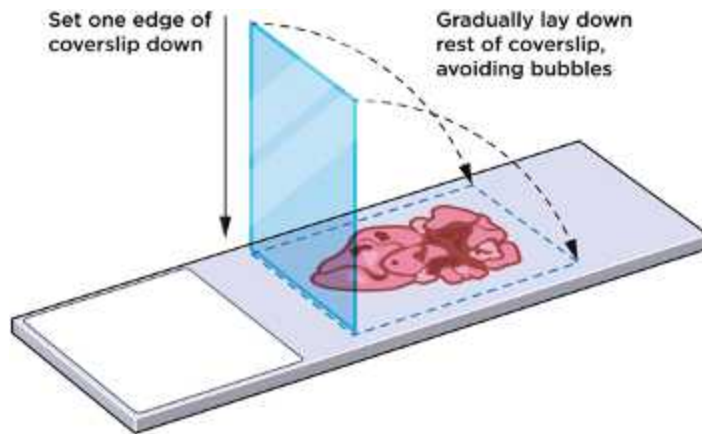


Figure 4: Applying coverslip

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



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



- 10 Needed for this step: **water bath, 4X SSC, 100% formamide, 2X SSC**, and (optional) **2X SSC-T**

**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.

- 10.1 Preheat water bath to 37°C.

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



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

- 10.3 **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.

## 10.4

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 5). After the last wash, slides can be stored in 2X SSC for up to 1 hour.



Figure 5: Stringent washes to remove off-target probes

## Add morphology markers (100 minutes)

- 11 Needed for this step: **humidity chamber, Buffer W, SYTO 13 nuclear stain, morphology markers**, and **2X SSC**.

**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.

## 11.1

**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.

## 11.2

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 or hydrophobic pen can be used at this step if desired but must be carefully removed with a razor blade before loading on the instrument.

## 11.3 Prepare 220 $\mu\text{L}$ of morphology marker solution per slide (see Table 4).

| Nuclear stain<br>(SYTO 13) | Morphology<br>Marker 1 | Morphology<br>Marker 2 | Other<br>Markers* | Buffer W**            | Final Volume          |
|----------------------------|------------------------|------------------------|-------------------|-----------------------|-----------------------|
| 22 $\mu\text{L}$ x n       | 5.5 $\mu\text{L}$ x n  | 5.5 $\mu\text{L}$ x n  | ...               | 187 $\mu\text{L}$ x n | 220 $\mu\text{L}$ x n |

Table 4: Morphology marker solution (n = number of slides)

\* 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  $\mu\text{L}$  per slide.

## 11.4 Mix morphology marker solution by flicking and briefly picofuging.

## 11.5 Remove Buffer W from one slide at a time by tapping slide onto a Kimwipe, then return the slide to the humidity chamber.

## 11.6 Cover tissue with morphology marker solution (up to 200 $\mu\text{L}$ ).



- 11.7 Repeat steps 11.5 and 11.6 for each slide.
- 11.8 Incubate for **1 hour** in the humidity chamber at room temperature, protected from light. 
- 11.9 After staining, **remove solution** by tapping slide on a clean, absorbent surface.
- 11.10 **Wash in 2X SSC**, 2 times for 5 minutes each.



## Add nuclei morphology markers (25minutes)

- 12 Cover tissue with diluted nuclei morphology marker solution (200-1000  $\mu$ L of the diluted nuclei marker).
- Syto 13 FITC 1:10,000  
Syto 83 Cy3 1:10,000  
Syto 59 Texas Red 1:10,000  
Syto 62 Cy 5 1:10,000
- 13 Incubate for **10 min** in the humidity chamber at room temperature, protected from light. 
- 14 **Wash in DEPC water**, 3 times for 5 minutes each.



## Stripping and re-probing procedure for RNA slides

- 15 GeoMx RNA assay slides are reusable and can be restained with a different commercial or custom panel following this procedure. This procedure requires a UV light box or transilluminator capable of emitting 302/312 nm UV light (**example**), 2X SSC-T, 1X Tris-EDTA, and 2X SSC.
- 15.1 Place the slide flat on the surface of a UV transilluminator.
- 15.2 Apply enough **2X SSC-T** to completely cover the tissue (50–200µL depending on the size of the tissue).
- 15.3 **Expose to UV light for 3 minutes** to cleave tags from bound probes.
- 15.4 Carefully tap each slide on a clean, disposable surface (e.g., paper towel) to remove liquid and avoid oligo contamination.
- 15.5 **Wash slides** by dipping in a staining jar with **2X SSC-T**.
- 15.6 Transfer to another staining jar with fresh **2X SSC-T**.
- 15.7 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.
- 15.8 Wash 3 times in **2X SSC**.
- 15.9 Proceed to In situ hybridization (overnight) section 8.



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

16 Follow instruction from User Manual

## Protocol references

GeoMx® DSP Manual Slide Preparation User Manual (MAN-10151-03, June 2023).