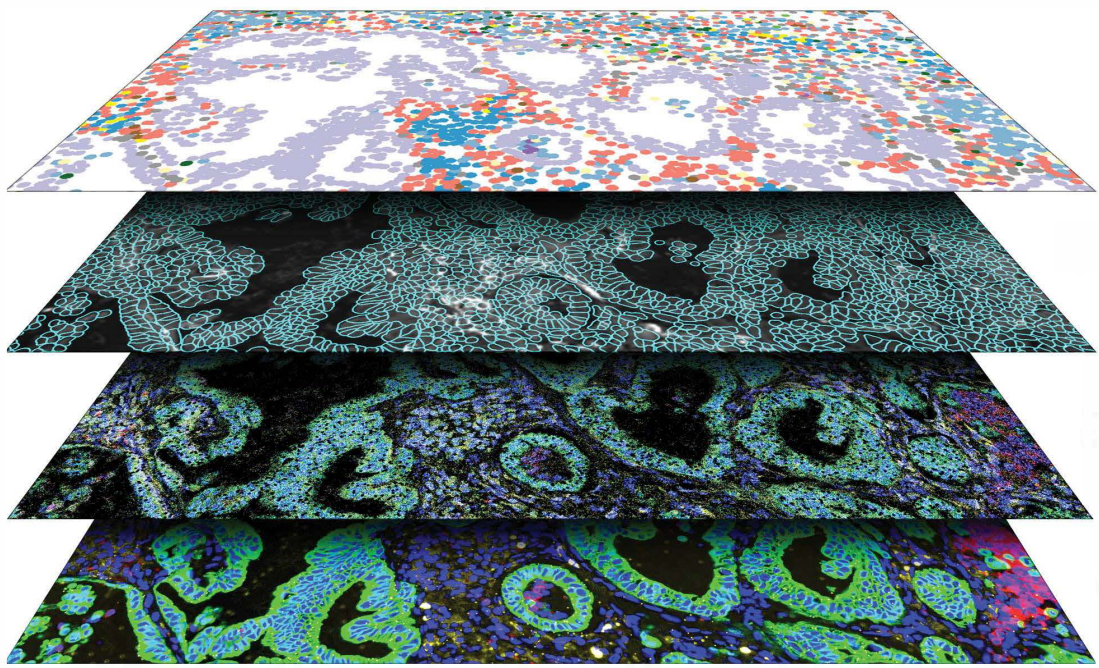
CosMx™ Spatial Molecular Imager

USER MANUAL

Manual Slide Preparation



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Conventions

The following conventions are used throughout this manual 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 and/or instructional material.

The gray box indicates general information that may be useful for improving assay performance. The notes may clarify other instructions or provide guidance to improve the efficiency of the assay work flow.

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.

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

Safety

WARNING: Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eye wear, clothing, and gloves. SDSs are available from www.nanostring.com/support/support-documentation.

IMPORTANT: Read all steps before you begin to familiarize yourself with this procedure.

Introduction to CosMx SMI Slide Preparation

The CosMx™ SMI platform is an integrated system with cyclic in situ hybridization chemistry, a high-resolution imaging readout instrument, and an interactive data analysis and visualization software. The CosMx SMI platform enables rapid quantification and visualization of up to 1,000 RNA and 64 validated protein analytes. This flexible spatial single-cell solution drives deeper insights into the cell atlas, cell-cell interaction, cellular processes, and bio-marker discovery.

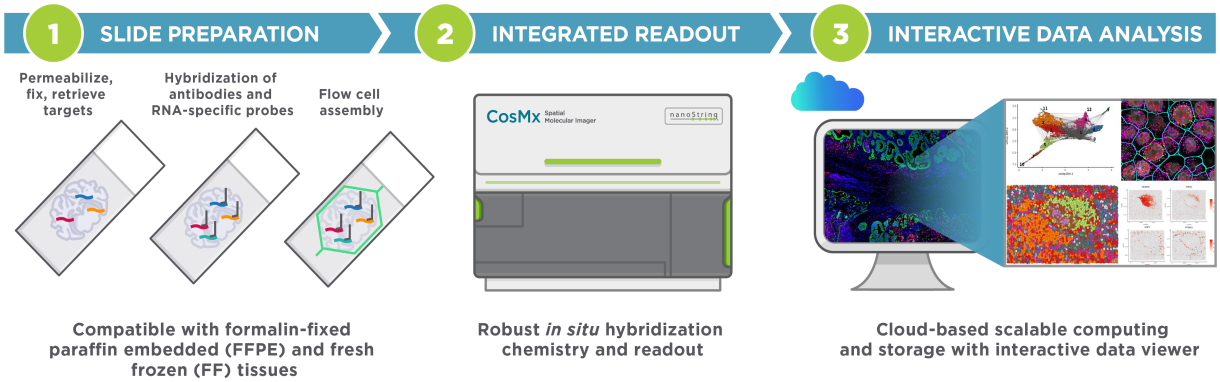


Figure 1: CosMx™ SMI is an integrated system that includes validated reagents and consumables, an instrument for chemistry and readout, and an interactive cloud-based software suite for data analysis.

Sample preparation involves basic in situ hybridization (ISH) processing steps. The protocols are compatible with the recommended glass pathology slides, and do not require complicated tissue expansion or clearing, cDNA synthesis or amplification.

RNA or protein targets in individual cells are identified via hybridization or binding with highly specific probes or antibodies labeled with a unique barcode system. Barcode readout occurs through multiple rounds of reporter probe binding and fluorescence imaging using the CosMx SMI instrument. Each RNA target appears as a distinct bright spot in the sample and is digitally quantified in the image. The data is then migrated to the cloud based AtoMx™ Spatial Informatics Platform for analysis and visualization. Within the AtoMx Platform, users can incorporate custom analysis workflows.

The CosMx™ Spatial Molecular Imager is the first platform to demonstrate simultaneous single-cell and sub-cellular detection of up to 64 proteins on standard, bio-banked, FFPE tissue samples. The CosMx Protein technology uses an antibody-oligonucleotide conjugate to detect each protein's (sub)-cellular localization and quantify its expression level. CosMx oligo-labeled antibodies undergo rigorous QC testing, and site-specific labeling chemistry to select for pure imaging reagents with no unconjugated antibody or free oligonucleotide contamination, which could lead to background noise.

CosMx SMI User Manuals and Resources

The CosMx SMI workflow is divided into the following user manuals:

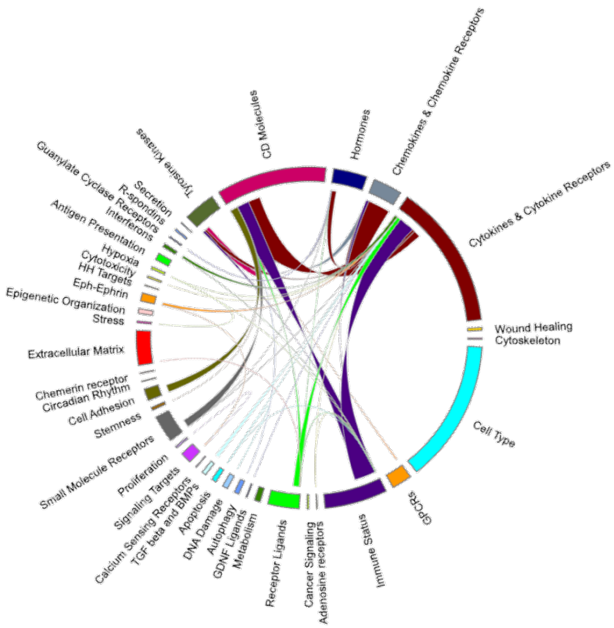
Workflow Step 1	CosMx SMI Manual Slide Preparation User Manual MAN-10159-01 CosMx SMI Semi-Automated Slide Preparation User Manual MAN-10160-01
Workflow Step 2	CosMx SMI Instrument User Manual MAN-10161-01
Workflow Step 3	CosMx SMI Data Analysis User Manual MAN-10162-01

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

Instrument and workflow training courses are also available in NanoString University.

For information about the AtoMx™ Spatial Informatics Platform, please refer to the AtoMx Spatial Informatics Platform User Manual (MAN-10170).

Panel and Cell Segmentation Marker Selection



NanoString currently provides **five (5) pre-defined panels** for use with CosMx SMI:

- Human Universal Cell Characterization Panel, 1000-plex, RNA
- Human Immuno-oncology Panel, 100-plex, RNA
- Mouse Neuroscience Panel, 1000-plex, RNA
- Human Immuno-oncology Panel, 64-plex, Protein

In addition, the following **custom add-on and stand-alone de novo custom panel** options are available:

- Add 7 to 50 user-defined genes to the 100-plex or 1000-plex RNA Panel
- Add up to 8 user-defined protein targets to the 64-plex Protein Panel (available at a later date)

De novo Custom Panels are also available and provide a turn-key solution that provides a ready-to-use assay for up to 300 targets.

- Collaborate with NanoString's Bio-informatics team to build a made-to-order custom panel.
- Email NanoString at Support@NanoString.com or visit https://nanostring.com/about-us/contact-us/ for help with custom panel design.

CosMx SMI Cell Segmentation Marker Selection

The following Cell Segmentation and Supplemental Marker kits are available for CosMx SMI.

Table 1: CosMx SMI Cell Segmentation and Supplemental Marker Kits

Kit Description	Kit Components
CosMx™ Human Universal Cell Segmentation Kit, (RNA) Channels 1 and 2	CosMx DAPI Nuclear Stain, Ch1
	CosMx Human CD298/B2M Segmentation Marker Mix, Ch2 (RNA)
CosMx™ Human IO PanCK/CD45 Supplemental Segmentation Kit (RNA) Channels 3 and 4 (Optional)	CosMx Hs PanCK/CD45 Marker, Ch3/Ch4 (RNA)
CosMx™ Mouse Neuroscience Cell Segmentation Kit, (RNA) Channels 1 and 2	CosMx DAPI Nuclear Stain, Ch1
	CosMx Mouse Neuro rRNA Neuro Marker, Ch2 (RNA)
CosMx™ Mouse Neuroscience Supplemental Segmentation Kit (RNA) Channels 3 and 4 (Recommended)	CosMx Mm Neuro Histone Marker, Ch3 CosMx Mm GFAP Marker, Ch4
CosMx™ Human Universal Cell Segmentation Kit, (Protein) Channels 1 and 2	CosMx DAPI Nuclear Stain, Ch1
	CosMx Human CD298/B2M Marker Mix, Ch2 (Protein)
CosMx™ Human IO PanCK/CD45 Supplemental Segmentation Kit (Protein) Channels 3 and 4 (Optional)	CosMx Human PanCK/CD45 Marker Mix, Ch3/Ch4 (Protein)

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The following markers are **available to order à la carte** for CosMx SMI.

Table 2: A La Carte Markers

Assay	Item Description
Human Universal RNA Channel 5 (optional)	CosMx Human CD68 A La Carte Marker, Ch5 (RNA)
	CosMx Human Cytokeratin 8/18 A La Carte Marker, Ch5 (RNA)
Human IO Protein Channel 5 (optional)	CosMx Human CD3 A La Carte Marker Ch5 (Protein)
	CosMx Human CD68 A La Carte Marker Ch5 (Protein)
	CosMx Human CD8 A La Carte Marker Ch5 (Protein)
	CosMx Human Cytokeratin 8/18 A La Carte Marker Ch5 (Protein)

Additional markers may be available to meet specific project needs.

Email NanoString at Support@NanoString.com or visit https://nanostring.com/about-us/contact-us/ for help with cell segmentation and supplemental marker selection.

RNA FFPE Manual Slide Preparation

CosMx SMI Slide Preparation Workflow

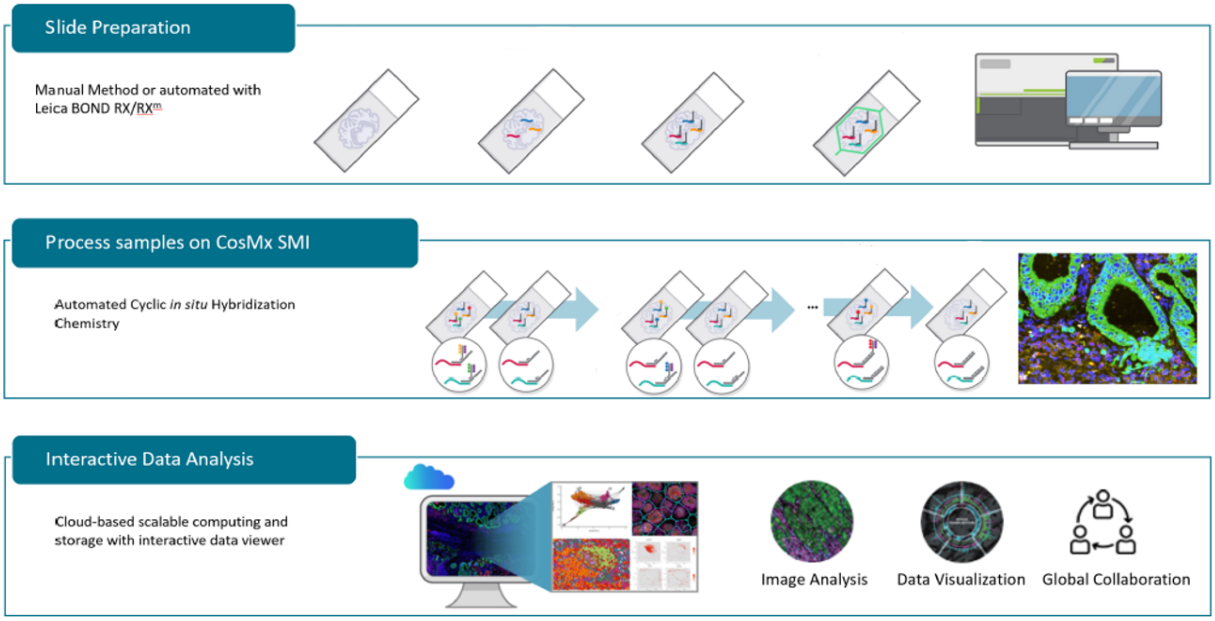

Figure 2: CosMx SMI Workflow Overview

Day 1: Slide Preparation. Prepare slides and incubate biological targets with ISH probes. Prepare manually or using the BOND RX/RX^m fully automated IHC/ISH stainer from Leica Biosystems (BOND RX/RX^m).

Day 2: Process Slides on CosMx SMI. Remove off-target probes and add cell segmentation markers to each slide. Load assembled flow cells into the CosMx SMI instrument and enter flow cell/study information. Tissue is scanned to capture RNA readout and morphology imaging within user-designated fields of view (FOVs).

After run completion: Create a **Data Analysis** study in the AtoMx Spatial Informatics Platform (SIP) and perform quality-control checks, data analysis, and generate analysis plots.

Equipment, Materials, and Reagents

The following equipment (see Table 3), materials (see Table 4), and reagents (see Table 5) are required for this protocol but are **not supplied by NanoString Technologies Inc.**

Equipment:

Table 3: Equipment not provided by NanoString

	Equipment	Source	Part Number(s)
☐	**Baking Oven**	Quincy Lab,Inc.® (or comparable)	Example
☐	**Hybridization Oven including hybridization chamber** • RapidFISH Slide Hybridizer **or** • HybEZ™ oven • Humidity control tray NOTE: 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 CosMx SMI slide preparation.	Boekel Scientific® or ACDBio™	240200 for 120V or 240200-2 for 230V 321710/321720 310012
☐	**6-quart Pressure Cooker** NOTE: Pressure Cooker must have a built in temperature gauge for validation NOTE: Products from other vendors may require testing and optimization OPTIONAL: A steamer may be used in lieu of a pressure cooker and may be preferred with more fragile tissues.	BioSB® TintoRetriever	BSB-7087
☐	**Ultrasonic Bath** (500 mL capacity) NOTE: 40kHz frequency with timer	General Lab Supplier	Example (CPX-952-118R)

	Equipment	Source	Part Number(s)
☐	**Vortex Mixer**	General Lab Supplier	Various
☐	**Micro Centrifuge** for 1.5 mL microcentrifuge tubes	General Lab Supplier	Various
☐	**Water Bath** Temperature setting of 37°C	General Lab Supplier	Various
☐	**Thermal Cycler** Must include a 96-well 200 µL tube block	General Lab Supplier	Various
☐	**Analytic Scale with draft shield** NOTE: ensure scale can measure in milligrams (mg) so that reagents can be weighed accurately.	Various	Example

Materials:

Table 4: Materials not provided by NanoString

	Materials	Source	Part Number (s)
☐	**Pipettes for 2.0 – 1,000 µL**	General Lab Supplier	Various
☐	**Filter tips** (RNase/DNase Free)	General Lab Supplier	Various
☐	**2.0 mL Centrifuge Tubes** (RNase/DNase Free)	General Lab Supplier	Various
☐	**0.2 mL PCR tubes** or PCR strip tubes	General Lab Supplier	Various
☐	**VWR™ Superfrost® Plus Micro Slide,**	VWR	48311-703

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	Materials	Source	Part Number (s)
	Premium or **Leica BOND PLUS slides** NOTE: these slides have been validated by NanoString. Do not use other products.	Leica® Biosystems	S21.2113.A
☐	**Slide Rack**	General Lab Supplier	Example
☐	**Polypropylene Slide Staining Jars** (24 required) or **Slide Staining Station** NOTE: due to the photo-sensitivity of this assay, the staining jars should be impermeable to light.	Ted Pella® Amazon® Fisher Scientific	21029 MH-SJ6302 NC1862866
☐	**Forceps** (for slide handling)	General Lab Supplier	Various
☐	**Razor Blades**	General Lab Supplier	Various
☐	**Timer**	General Lab Supplier	Various
☐	**RNase AWAY™** NOTE: RNaseZAP™ and other alternatives cannot be used as substitutes as they do not adequately remove both nucleic acid and nuclease contaminants.	ThermoFisher Scientific	7003PK
☐	**Kimwipes®** (large and small)	Various	Various
☐	**StainTray slide staining system with black lid**	Sigma-Aldrich®	Example

	Materials	Source	Part Number (s)
☐	**VWR® polyethylene slide holder** Optional - used during tissue sectioning	VWR	82024-524

Reagents:

Table 5: Reagents not provided by NanoString

	Reagent	Source/Part Number(s)	Storage Conditions
☐	**DEPC-treated water**	ThermoFisher Scientific, AM9922 (or comparable)	Room temperature
☐	**100% Ethanol** (EtOH): ACS grade or Better 	General Lab Supplier	Flammable Storage Room temperature
☐	**10X Phosphate Buffered Saline** pH 7.4 (PBS)	ThermoFisher Scientific, AM9625 (or comparable)	Room temperature
☐	**Xylene** NOTE: Citrisolv can be used, however, follow the alternative workflow for Citrisolv for **Day 1: Deparaffinize FFPE Tissue Sections (20 minutes) on page 30**.	General Lab Supplier	Flammable Storage Room temperature
☐	**20X SSC** (DNase, RNase free)	ThermoFisher Scientific, AM9763	Room temperature
☐	**Tris Base**	Sigma-Aldrich,	Room

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	Reagent	Source/Part Number(s)	Storage Conditions
		10708976001 (or comparable)	temperature
☐	**Glycine**	Sigma-Aldrich, G7126 (or comparable)	Room temperature
☐	**Sulfo-NHS-Acetate powder** NOTE: NHS-Acetate powder is shipped in a plastic bag with a desiccant and should be left in the bag and stored at -20°C until ready to use. NOTE: 100mg of NHS-Acetate is sufficient for 6-7 slides.	Fisher Scientific™, 26777	-20°C
☐	**10% Neutral Buffered Formalin** (NBF)	EMS Diasum®, 15740-04 (or comparable)	Room temperature
☐	**100% Deionized Formamide** NOTE: Deionized Formamide is optimal, however, formamide that is not deionized may also be used.	ThermoFisher Scientific, AM9342 or VWR, VWRV0606 (or comparable)	4°C (bring to RT for at least 30 minutes before opening)

OPTIONAL: Large volume stock solutions (>500 mL) of Deionized Formamide can be aliquoted into 50 mL conical tubes and stored, protected from light, at 4°C. This will save time during day 2 slide preparation. Bring stock to room temperature for at least 30 minutes before opening.

NanoString Supplied Reagents

CosMx FFPE Slide Preparation Kit (RNA)

Figure 3: RNA Slide Preparation Kit (Box 1/2)

Table 6: CosMx FFPE Slide Prep Kit (RNA) Box 1/2

Kit Contents (Store at 4°C)	
CosMx Target Retrieval Solution (10X)	Buffer R
CosMx RNA Blocking Buffer	CosMx Fiducials
NHS-Acetate Buffer	2X SSC-T Media
Incubation Frames	Incubation Frame Covers

Figure 4: CosMx FFPE Slide Prep Kit (RNA) Box 2/2

Table 7: CosMx FFPE Slide Prep Kit (RNA) Box 2/2

Kit Contents (Store at -20°C)	
CosMx RNase Inhibitor	CosMx Protease Solution (Proteinase K)

CosMx RNA Panel (see Panel and Cell Segmentation Marker Selection on page 11).

Figure 5: CosMx Human IO Panel (RNA)

Table 8: CosMx RNA Probe Mix

Kit Contents (Store at -20°C)	
CosMx RNA Panel Includes: RNA Probe Mix **and** RNA Add-On	Custom RNA Add-On Probes (if applicable) Custom RNA Add-On replaces off-the-shelf RNA Add-On

CosMx Segmentation and Supplemental Markers

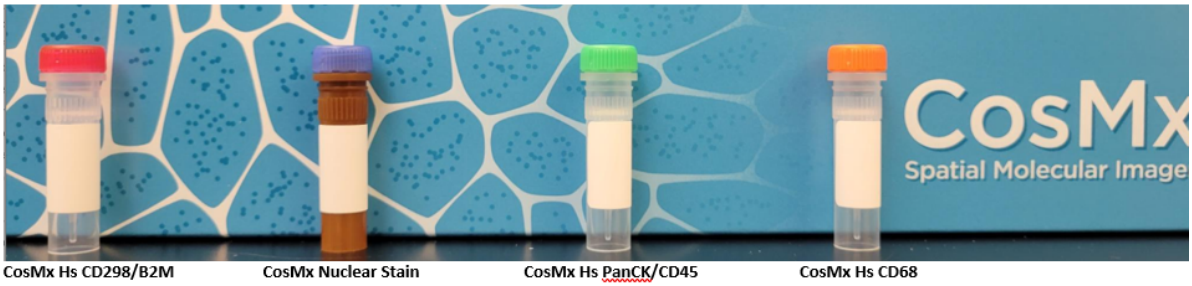

Figure 6: CosMx Segmentation and Visualization Markers (RNA)

Table 9: CosMx Segmentation and Visualization Markers (RNA)

Kit Contents (Store at -80°C) Each kit is sufficient for four (4) slides	
Kit Name	**Kit Component**
CosMx™ Human Universal Cell Segmentation Kit (RNA), Ch 1/2	CosMx Hs CD298/B2M CosMx Nuclear Stain
CosMx™ Human IO PanCK/CD45 Supplemental Segmentation Kit (RNA), Ch 3/4 If Applicable	CosMx Hs PanCK/CD45
CosMx™ Human CD68 A La Carte Marker (RNA), Ch 5 If Applicable	CosMx Hs CD68
CosMx™ Human Cytokeratin 8/18 A La Carte Marker (RNA), Ch 5 If Applicable, not pictured	CosMx Hs CK 8/18

Flow-Cell Assembly Kit

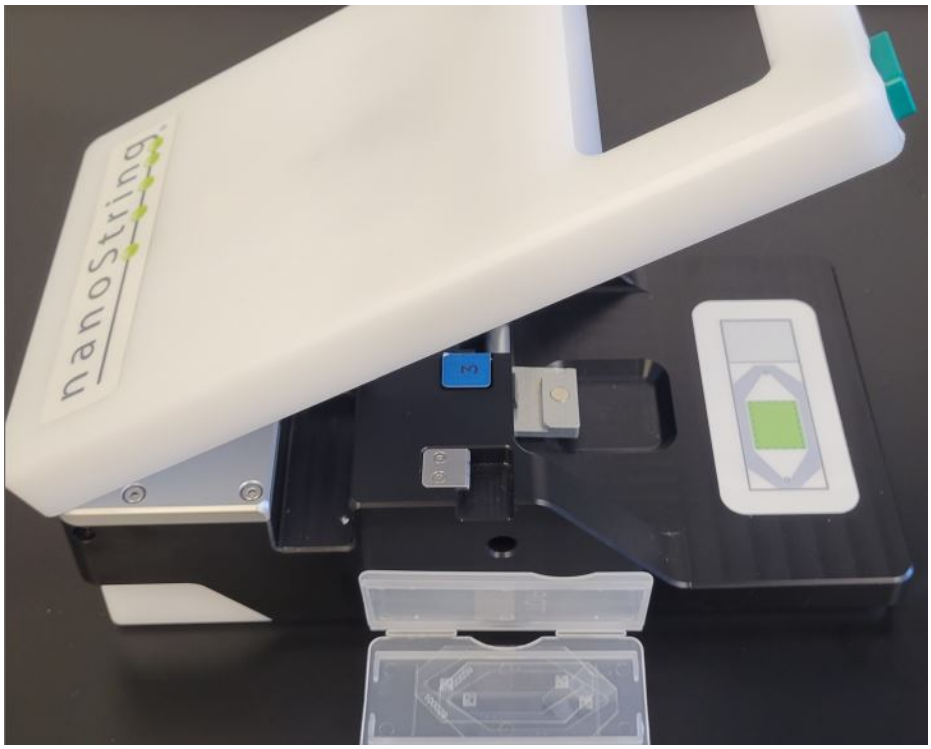

Figure 7: Flow Cell Assembly Kit

Table 10: Flow Cell Assembly Kit

Kit Contents (Store at Room Temperature)	
Flow Cell Assembly Tool	Flow Cell Coverslips

Prepare Tissue Samples

Appendix I: CosMx SMI Sample Preparation Guidelines on page 137 covers FFPE block selection and sectioning in detail. Review these guidelines as needed prior to beginning the FFPE Slide Preparation procedure.

NanoString has tested and validated 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.

Tissue Sectioning and Slide Preparation:

FFPE blocks should be sectioned at **4-6 µm thickness** and mounted on the label side of VWR Superfrost Plus Micro Slides or Leica BOND PLUS slides.

Tissue sections must be centered within the Scan Area (the green area) of the slide and be no larger than **20 mm Long by 15 mm Wide** (see Figure 8) (image not to scale). For best performance, ensure some glass is visible in all four corners and in the center of the scan area.

Label slides with pencil on the frosted label according to lab guidelines. If using an adhesive slide label, ensure the label is less than 295 µm thick and is not folded over on itself. Labels over the maximum thickness or labels that are not properly adhered may result is slide or flow cell damage during flow cell assembly and/or instrument loading.

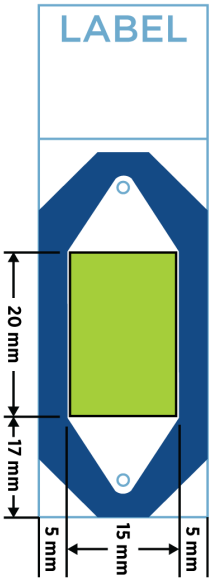

Figure 8: Tissue Scan Area (not to scale)

If sections are larger than the indicated size and/or placed off-center, continue with the slide preparation as outlined. Directly before applying the incubation frame, scrape off parts of the tissue exceeding the scan area. Scraping off tissue before target retrieval and ethanol drying could generate tissue folds that may result in staining/binding artifacts, while suboptimal scraping may result in poor incubation frame adherence. Applying the incubation frame over tissue could result in tissue damage when the frame is removed.

ⓘ IMPORTANT: The CosMx SMI instrument will only image the area inside the flow cell chamber, the tissue scan area. If the tissue section is outside of the scan area, it will not be imaged.

After sectioning and prior to use or storage**, to improve tissue adherence**, bake slides at 37°C overnight at an angle no greater than 45 degrees. Alternatively, slides can be baked at 37°C for two (2) hours and then dried overnight at room temperature. A polyethylene slide holder (VWR, 82024-524) can be used for overnight drying. Ensure sections are completely dry before storage.

A tissue section adhesive such as Epredia™ Tissue Section Adhesive (Fisher Scientific, 86014) can also be used to improve tissue adhesion. The use of an adhesive has not been validated but may improve tissue adherence for some tissue types. Follow the manufacturer's instructions for use guidelines.

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 in a desiccator at room temperature or at 4°C prior to processing.

Slide Preparation Day 0: Prepare Shelf Stable Reagents and Overnight Tissue Bake

Prepare shelf stable reagents. See **Prepare RNA Assay Reagents on page 26**.

Bake sections on slides overnight in a 60°C drying oven.

- Bake slides vertically in a slide rack overnight or in a slide holder at a 45 degree angle.

NOTE: the 60°C tissue baking step the day before slide preparation has shown to increase tissue adherence and stability during the slide preparation protocol. If overnight baking is not possible, bake slides for at least 2 hours on slide preparation day 1 before continuing to **Day 1: Deparaffinize FFPE Tissue Sections (20 minutes) on page 30**. The 2 hours bake has not been validated by NanoString but is preferable to skipping the second bake.

Prepare RNA Assay Reagents

IMPORTANT: Take care to maintain nuclease-free conditions. The greatest risk of contamination comes from CosMx SMI RNA probes and other oligos. NanoString recommends the use of RNase AWAY (Thermo Fisher 7003PK), as it will limit contamination from oligos, 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.

Label staining jars and prepare reagents using the instructions in the following table **(see Table 11)**

NOTE: Unless otherwise noted, reagents can be made up to two (2) weeks in advance and stored at room temperature.

Table 11: RNA Reagent Preparation

	Reagent	Dilution	Storage
☐	1X PBS (pH 7.4)	Prepare 1L of 1X PBS by combining 100 mL of 10X PBS and 900 mL of DEPC-treated water.	Room Temperature
☐	2X SSC	Prepare 1L of 2X SSC by combining 100 mL of 20X SSC and 900 mL of DEPC-treated water.	Room Temperature
☐	4X SSC	Prepare 500 mL of 4X SSC by combining 100 mL of 20X SSC and 400 mL of DEPC-treated water.	Room Temperature
☐	NBF stop buffer (Tris Glycine Buffer)	Combine 6.06g Tris base and 3.75g Glycine in 500 mL of DEPC-treated water. The final concentration of Tris and Glycine will be 0.1M each.	Room Temperature
☐	NHS-Acetate Mix	Individual aliquots can be prepared prior to slide preparation but should be kept at -20°C with a desiccant until use. **To prepare aliquots** (4-slide	-20°C in dessicant

	Reagent	Dilution	Storage
		preparation, see NOTE below): • Bring stock to room temperature for 1-2 hours prior to opening. • Prelabel four (4) 2.0 mL centrifuge tubes with the required information. • Using a weighing spatula, carefully weigh 25 mg of NHS-Acetate directly into the screw-top tube on an analytic scale. • Close the tube and label tube with final weight. Seal the tube with parafilm and **place into the -20°C with desiccant until use.** NOTE: If preparing only 2 slides, pre-weigh 15 mg aliquots into six (6) total tubes.	
☐	1X Target Retrieval Solution	Prepare 50 mL **fresh daily** by adding 5 mL of CosMx Target Retrieval Solution, 10X (provided by NanoString) to 45 mL DEPC-treated water. Adjust volume as needed. NOTE: NanoSting provides 20 mL of CosMx Target Retrieval Solution, 10X. A 50-100 mL capacity staining jar is recommended when preparing only 2 slides.	Make fresh daily
☐	Digestion Buffer	Dilute the 20 mg/mL Protease Solution (Proteinase K stock; provided by NanoString) to a working concentration of 3 µg/mL*	Make fresh daily Store on ice

	Reagent	Dilution	Storage
		in 1X PBS. **Prepare fresh daily.** A 2-step serial dilution is recommended. Accurate dilution of Proteinase K is critical for proper assay performance. • **Step 1**: Dilute 20 mg/mL stock to 200 µg/mL by adding 2 µL of Proteinase K stock to 198 µL of 1X PBS. • **Step 2**: Dilute the 200 µg/mL solution made in step 1 to the target concentration of 3 µg/mL by adding 30.0 µL of the 200 µg/mL solution to 1970 µL of 1X PBS. • Mix thoroughly by inverting tube or pipetting up and down using a clean pipetter tip. **Do not vortex.** NOTE: Protease Mix should be prepared fresh daily and stored on ice until ready to use. *NOTE: This concentration may differ for some tissue types, including CPA samples (see Appendix II: Tissue Specific Digestion on page 141).	
☐	Fiducials and Hybridizaton Mix	These reagents have additional steps that will be covered in detail in their respective sections. Remove ISH Probe Mix from storage and keep on ice until ready to use. NOTE: Fiducials are light sensitive and should be kept stored,	n/a

	Reagent	Dilution	Storage
		protected from light, until instructed to remove.	
☐	Day 2 Reagents	These reagents have additional steps that will be covered in detail in their respective steps. Follow Day 2 procedure for preparation of these reagents.	n/a

Day 1: Deparaffinize FFPE Tissue Sections (20 minutes)

You will need the following materials and reagents for this step: **staining jars, hybridization tray, pressure cooker, 1X Target Retrieval Solution, Xylene, 100% EtOH,** and **DEPC-treated water** (see Prepare RNA Assay Reagents on page 26).

Before beginning, slides should have already been baked overnight (see Slide Preparation Day 0: Prepare Shelf Stable Reagents and Overnight Tissue Bake on page 25). If slides were not baked overnight, slides can be baked for two (2) hours at 60°C; however, this is not optimal and may have a negative impact on tissue adherence.

Prepare Equipment and Washes

1. **Prepare hybridization tray** by lining the bottom of the tray with Kimwipes and carefully wet the Kimwipes with 2X SSC or DEPC-treated water. Kimwipes should be thoroughly damp but standing buffer should not be present.
2. **Preheat hybridization chamber and tray to 40°C** following manufacturer's instructions.
3. Ensure baking oven is still set to 60°C.
4. **Prepare staining jars with enough Xylene and Ethanol to cover tissue** (see Figure 11). Ensure you have sufficient buffer to cover all slides in container for the washes. Wash buffers need to completely cover the tissue on the slide but should be below the slide label. The washes may make the slide labels illegible if submerged.

⚠ WARNING : Xylene and Ethanol are Flammable chemicals and should be handled appropriately.

5. **Before turning on the pressure cooker**, ensure that the water is at the correct level per the manufacturer's instructions (4-8 cups depending on model used).

6. **Place the staining jar containing freshly prepared 1X Target Retrieval Solution into the pressure cooker to preheat** (see Figure 9). Ensure that water level is well below lid of jar, about halfway up jar will be sufficient. If needed, a trivet may be used to raise the jar.

Figure 9: Preheat Target Retrieval Buffer

⚠ WARNING: Nanostring does not recommend the use of glass staining jars in the pressure cooker.

7. Place a lid on the staining jar to prevent evaporation. To prevent pressure from building within the staining jar, **do not fully seal the lid to the jar**.
8. **Prime the pressure cooker** up to 100°C to prepare for step 4, Perform Target Retrieval (50 minutes) on page 33 (see Figure 10). **Ensure the pressure valve is closed during this preheating step.**

Figure 10: Prime Pressure Cooker

Deparaffinize FFPE tissue sections

1. Remove the slides from the backing oven and gently perform the following washes using staining jars (see Figure 11).

   NOTE: Ensure you have sufficient buffer to cover all slides in container for the washes. Wash buffers need to completely cover the tissue on the slide but should be below the slide label. The washes may make the slide labels illegible if submerged.

- Place slides into **first Xylene jar and wash for five (5) minutes**.
- **Repeat** the five (5) minute wash with a new staining jar containing Xylene.
- After second Xylene wash, **transfer slides to first 100% Ethanol** wash and wash slides for **two (2) minutes***.
- **Repeat** two (2) minute* wash with a new staining jar containing Ethanol.
- **If using Citrisolv** rather than Xylene, wash the slides two (2) times for 5 minutes each wash in 100% Ethanol.

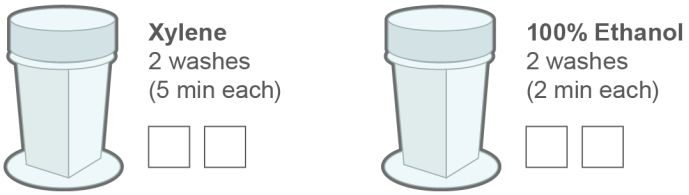

Figure 11: Deparaffinize FFPE Tissue Sections

⚠ WARNING: Xylene and Ethanol are Flammable chemicals and should be handled appropriately. Waste generated in these steps needs to be disposed of as flammable hazardous waste.

2. **Dry slides** in slide rack in 60°C oven for **5 minutes.** After 5 minutes, remove slides from oven and leave at room temperature until target retrieval solution has been pre-heated to 100°C.
3. **If not already aliquoted, remove stock NHS-Acetate powder from -20°C freezer** and leave at room temperature for 1-2 hours before use in NHS-Acetate Preparation and Application (25 minutes) on page 43. Pre-aliquoted NHS-Acetate powder can remain at -20°C until instructed to remove on on page 43.

Perform Target Retrieval (50 minutes)

You will need the following materials and reagents for this step: **pressure cooker, staining jars, 1X Target Retrieval Solution** (pre-heated in the previous step), **DEPC-treated water** and **1X PBS**.

1. Once Target Retrieval Solution has been preheated, press cancel on the pressure cooker, release the pressure valve, and wait for the pressure cooker to release pressure. Once pressure has released, remove the lid and **carefully but quickly remove the staining jar containing preheated 1X Target Retrieval Solution**. Once removed, the target retrieval solution will begin to rapidly cool, ensure the following steps are done as quickly and safely possible.

   ⚠ WARNING: When opening the pressure valve and removing the pressure cooker lid, hot steam will be released. The staining jar will also be VERY hot. Wear protective heat resistant gloves to open lid and remove staining jar.

2. **Place FFPE slides into the preheated solution** and replace lid on the staining jar to prevent evaporation. To prevent pressure from building within the container, **do not fully seal the lid to the jar** (see Figure 12).

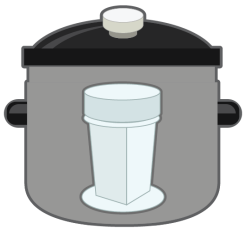

Figure 12: Perform Target Retrieval

3. Return the staining jar containing the slides **into the preheated pressure cooker.** Ensure that water level is well below lid of jar. About halfway up jar will be sufficient. If needed, a trivet may be used to raise the jar.
4. Reattach the pressure cooker lid, **open the pressure release valve to *Pressure Release* position** (see Figure 13) and return the pressure cooker to 100°C.

Figure 13: Set pressure valve to "Pressure Release"

5. **Once the pressure cooker temperature returns to 100°C**, start timer and run for **15 minutes** for FFPE tissue or 8 minutes for cell pellet arrays (CPA).
6. When the timer reaches zero, press cancel on pressure cooker to stop heating, **carefully remove the pressure cooker lid and remove the staining jar**.

⚠ WARNING: When starting to remove pressure cooker lid, hot steam will be released. Staining jar will also be VERY hot. Wear protective heat resistant gloves to open lid and remove staining jar.

7. **Immediately transfer all slides to staining jar containing fresh DEPC-treated water**. Move slides up and down for 15 seconds to wash slides (see Figure 14).

NOTE: Transfer slides from target retrieval buffer into DEPC-treated water carefully but quickly to ensure slides do not dry out. Drying can occur quickly while slides are hot.

8. **Transfer all slides to fresh 100% Ethanol** and incubate for **3 minutes**.
9. During Ethanol wash, clean bench space with RNase AWAY and lay out a fresh Kimwipe.
10. After 3 minutes, **remove slides from Ethanol and dry at room temperature for 30 minutes to one (1) hour**. Slides should be laid horizontally on clean Kimwipe to dry.

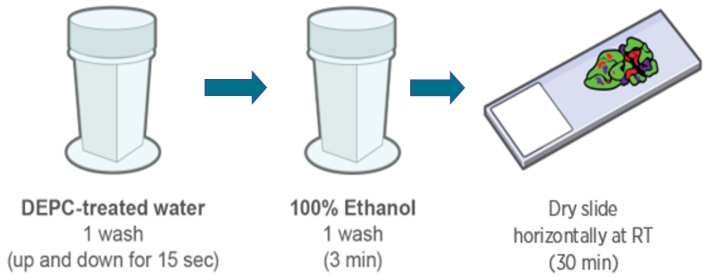

Figure 14: Ethanol Wash

11. While slide is drying, prepare digestion buffer (see Prepare RNA Assay Reagents on page 26).

Tissue Permeabilization (40 minutes)

You will need the following materials and reagents for this step: **staining jars, hybridization oven, hybridization tray, incubation frames** (CosMx FFPE Slide Prep Kit (RNA) Box 1/2, Stored at 4°C), **digestion buffer** (see Prepare RNA Assay Reagents on page 26), and **DEPC-treated water.**

1. Prepare the Incubation Frame:
   - Separate an individual frame from the strip by carefully tearing along the perforations.
   - Each frame is sandwiched between a thin solid polyester sheet and a thick polyester frame backing (with the center square removed).
2. If needed, trim the tissue following the template in Prepare Tissue Samples on page 24.
   - Use a clean razor blade to trim tissue and change blade as needed to ensure clean cuts and reduce the risk of cross-contamination between samples.
3. Using a clean Kimwipe, ensure that the surface of the slide that will come in contact with the incubation frame is **clean and dry**.
4. Apply the incubation frame (see Figure 15).
   - Carefully **remove the thin polyester sheet**, ensuring that the frame remains bound to the thick polyester frame backing (with the center square removed).
   - With the slide on a flat surface, careful not to touch the adhesive, **center the tissue within the incubation frame and carefully place the incubation frame** around each tissue section. Lightly press along the border of the incubation frame to ensure that it is well adhered to the slide.

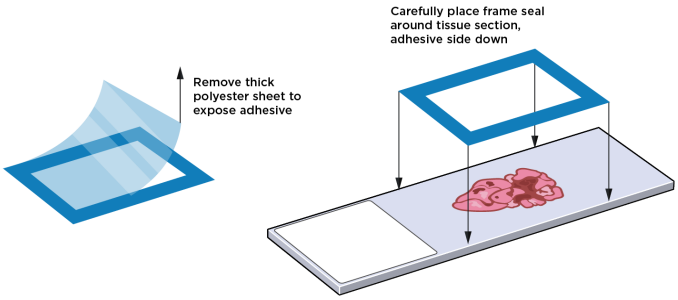

Figure 15: Apply Incubation Frame

5. With the slide still on a flat surface, use a clean razor blade to **carefully trim the long edges of the incubation frame to remove excess plastic** ensuring that there is no excess film extending over slide edges. Trim the short end of the incubation frame (opposite the slide label) as needed.
6. Remove digestion buffer from ice and **warm digestion buffer** by hand for about 3 minutes to bring the mixture to room temperature.
7. **Retrieve preheated hybridization tray** from hybridization oven.
8. Place slides into slide insert of hybridization tray and, using a P200 pipette, slowly **add 400 µL of digestion buffer to the tissue within incubation frame** (see Figure 16). Gently move tray side to side as needed to **ensure that digestion buffer covers the entire tissue.**

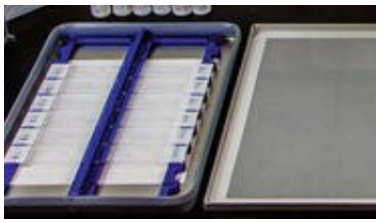

Figure 16: Hybridization Tray

NOTE: A pipette tip can also be used to carefully spread buffer over tissue within incubation frame. Carefully use a small volume pipette tip to spread buffer as needed by carefully laying the tip horizontally on top of the incubation frame and gently rolling the tip to spread the buffer until the tissue is completely covered. Be careful to avoid touching the tissue with the pipette.

9. Insert hybridization tray containing slides into hybridization oven and **incubate 40°C following the guidance in the table below** (see Table 12).

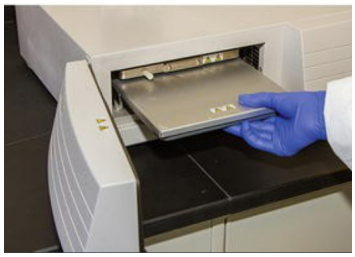

Figure 17: Hybridization Oven

Table 12: Digestion Times

Tissue Type	Digestion Time
Lymph node	15 minutes
Tonsil	30 minutes
All other tissue types*	30 minutes
Cell Pellet Array (CPA)	15 minutes

*NOTE: Incubation times and temperatures may differ by tissue and may need to be empirically determined. For this protocol, start with the default conditions: **30 minutes at 40°C** and adjust the time and concentration as needed. See Appendix II: Tissue Specific Digestion on page 141 for suggested concentration and incubation times based on R&D preliminary testing.

Decreasing digestion buffer concentration and/or incubation time may increase tissue stability for certain tissue types.

ⓘ **IMPORTANT**: After digestion buffer has been applied, avoid tissue drying in subsequent steps by working with only one (1) slide at a time.

10. During incubation, **remove fiducials and 2X SSC-T from 4°C and let come to room temperature, protected from light, for at least 10 minutes.**

11. Once fiducials have reached room temperature, prepare fiducial working solution following instructions for Fiducial Preparation and Application (20 minutes) on page 39.

12. After incubation, tap off excess digestion buffer **one slide at a time** and transfer slides to staining jar containing fresh DEPC-treated water.
13. Move slides up and down 3-5 times to wash and **repeat with a new jar of DEPC-treated water** (see Figure 18).

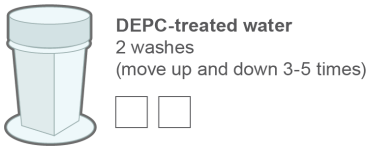

Figure 18: DEPC-treated water wash

14. Slides can be stored in DEPC-treated water while fiducials are prepared.

NOTE: Limit the time that the slides are kept in DEPC-treated water to minimize risk of target loss. Targets have been exposed and tissue has not yet undergone post-fixation.

Fiducial Preparation and Application (20 minutes)

You will need the following materials and reagents for this step: **ultrasonic bath, vortex, 2X SSC-T (at RT,** provided by NanoString**)**, **fiducials (at RT), staining jars**, **staining tray** (clean and dry), and **1X PBS.**

Volume for final working solution is sufficient for four (4) total slides. It is not recommended that less than 1 mL of fiducial working solution is made due to high risk of clumping. However, additional volume may be made as needed.

1. **Prepare fiducials for use following the below steps** (see Figure 19):

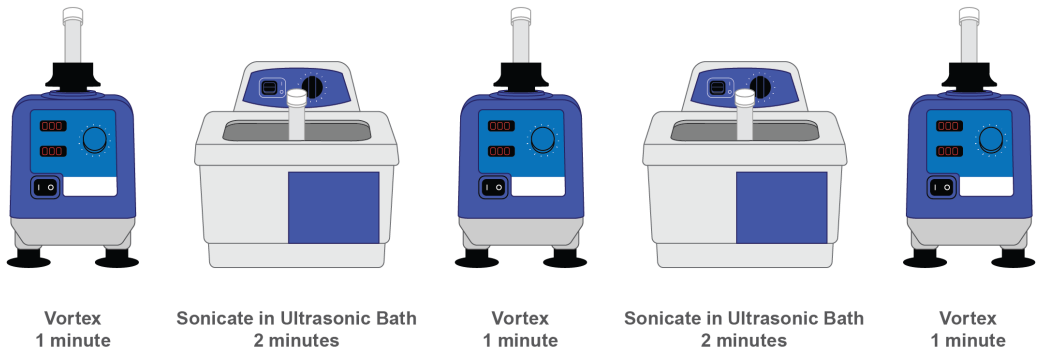

Figure 19: Fiducial preparation

NOTE: Failure to follow these steps will result in fiducial clumping and uneven distribution of fiducials within the tissue. This uneven distribution can result in a loss of readable area or loss of image registration.

2. Once fiducials are prepared, **dilute fiducial stock (0.1%) to the working concentration (0.001%) in 2X SSC-T**
   - Dilute stock 1:100 by adding **12 µL of the fiducial stock to 1188 µL of 2X SSC-T** (594 µL x 2).

   NOTE: The concentration may differ for some tissue types and need to be empirically determined. For this protocol, start with the default concentration of 0.001% and adjust the concentration as needed. See Appendix III: Tissue Specific Fiducial Concentrations on page 142 for suggested tissue specific concentrations based on R&D preliminary testing.

3. Remove slide from DEPC and gently tap tissue on a clean Kimwipe to remove excess water. Lay slide horizontally in staining tray.

4. **Immediately before applying fiducials to slides, vortex tube for 1 additional minute**. Vortex fiducials for 30 seconds between slides to keep fiducials in suspension and ensure consistent concentration across all slides.
5. **Apply up to 250 µL of the final fiducial solution, ensuring the solution covers glass and tissue within the incubation frame**. Fiducials must be present on the glass within the scan area for consistent focusing during the instrument run.

   Gently move tray side to side as needed to ensure that the fiducial solution covers the entire scan area, including glass.

   NOTE: A pipette tip can also be used to carefully spread buffer over tissue within the incubation frame. Carefully use a small volume pipette to spread buffer as needed by carefully laying the tip horizontally on top of the incubation frame and gently rolling the tip to spread the buffer until the tissue is completely covered. Be careful to avoid touching the tissue with the pipette.

6. **Incubate covered in staining tray for 5 minutes at room temperature.**

Figure 20: Incubate covered for 5 minutes

IMPORTANT: This step and all steps moving forward are light sensitive as fiducials contain fluorescent molecules that are sensitive to photo-bleaching.

7. After fiducial incubation, gently tap slides on a clean Kimwipe to remove excess solution and transfer slides to staining jar containing fresh 1X PBS.
8. Wash slides in staining jar with **1X PBS for 1 minute**.

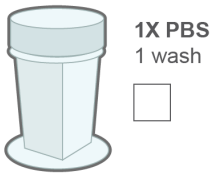

Figure 21: PBS Wash

Proceed to next steps immediately.

Post-Fixation (20 minutes)

You will need the following materials and reagents for this step: **staining jars, 10% NBF, NBF Stop Buffer (Tris-Glycine Buffer)** and **1X PBS** (see Prepare RNA Assay Reagents on page 26 for more information).

⚠ 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 the following washes (see Figure 22).

1. Transfer slides to **10% NBF and incubate for 1 minute** at room temperature.
2. **Immediately transfer the slides** to the first **NBF Stop Buffer and wash for 5 minutes**. **Repeat** wash with a second staining jar containing NBF Stop Buffer.
3. **Transfer slides to 1X PBS Wash** for **5 minutes**. Slides can sit in 1X PBS while NHS-acetate mix is prepared.

Figure 22: NBF Post Fix

4. During PBS wash, **remove RNase Inhibitor, CosMx RNA probe mix and add-on probes or stand-alone custom panel** from -20°C and thaw on ice.
5. **Remove Buffer R** from 4°C and bring to room temperature.

NHS-Acetate Preparation and Application (25 minutes)

You will need the following materials and reagents for this step: **analytical scale, 2.0 mL centrifuge tube, staining jar, NHS-Acetate powder, NHS-acetate buffer** (provided by NanoString, 4°C), and **2X SSC**.

1. **Prepare 100 mM NHS-acetate mixture** immediately before you are ready to apply the mixture onto the tissue (200 µL/sample). Ensure stock NHS-Acetate has reached room temperature before aliquoting.

   a. NHS-acetate powder is stored desiccated at -20°C. If not already done, prepare 25 mg aliquots (for a four slide preparation) of NHS-acetate powder by weighing out the powder directly into four (4) 2.0 mL centrifuge tubes. Label tubes with the exact weight, seal tubes with parafilm, and store tubes at -20°C in desiccant for later use. See **Prepare RNA Assay Reagents on page 26**.

   NOTE: If preparing only 2 slides, pre-weigh 15 mg aliquots into six (6) total tubes.

   b. **Add NHS-acetate buffer directly to aliquoted powder immediately before applying to the tissue**.

      i. Calculate the amount of NHS-acetate buffer to add to the NHS-acetate powder by multiplying the weight of NHS-acetate powder in mg by 38.5.

         - Example: for 25.0 mg of NHS-acetate powder: 25.0 * 38.5 = 962.5 µL of buffer to add.

   c. Slowly pipette up and down to mix. Bubbles may occur. Do not fully dispense liquid from pipette while mixing.

> ⓘ **IMPORTANT**: Reconstitute NHS-Acetate immediately before use. Do not prepare stock solutions for storage because the NHS ester moiety readily hydrolyzes and becomes non-reactive. Discard any unused reconstituted reagent.

2. Perform the following steps **one slide at a time** to prevent the tissue from drying out:

   - **Remove slide from 1X PBS,** gently tap slide on a clean kimwipe to remove excess buffer, and transfer to a clean staining tray.
   - **Apply 200-250 µL of NHS-Acetate mixture** onto the tissue within the incubation frame. Gently rock the tray side to side as needed to ensure that the NHS-Acetate solution covers

the entire tissue.

- Repeat with remaining slides and **incubate covered in staining tray for 15 minutes at room temperature** (see Figure 23).

Figure 23: Incubate 15 minutes

3. Following incubation, tap off excess liquid and wash slides in **2X SSC for at least 5 minutes**.

Figure 24: Two 5-minute 2X SSC Washes

4. **Repeat 2X SSC wash** for a total of two (2) washes. Slides will stay in second 2X SSC wash while hybridization mix is prepared.

In Situ Hybridization (overnight)

You will need the following materials and reagents for this step: **hybridization oven, hybridization tray, incubation frame covers** (CosMx FFPE Slide Prep Kit (RNA) Box 1/2, Stored at 4°C)), **thermal cycler, ice bucket with ice, Buffer R, CosMx RNA Probe Mix** (-20°C)**, RNase Inhibitor** (-20°C)**, custom probe add-in** (as needed, -20°C), and **DEPC-treated water**.

Do not begin in situ hybridization step until within 16-18 hours of day 2 start time. If not within that time frame, slides can be stored, protected from light, in 2X SSC wash for up to one (1) hour at room temperature or up to six (6) hours at 4°C.

IMPORTANT: Take care to maintain nuclease-free conditions. 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). Alternatively, mixes can be made in PCR workstations that have been decontaminated with UV light. Gloves should also be changed after handling any probe mixes to avoid cross-contamination.

Prepare buffers: Warm Buffer R to room temperature (RT) before opening.

Thaw RNA detection probes on ice. Before use, mix probes thoroughly by pipetting up and down 3-5 times.**Do not vortex probes**. Once thawed, probes can be refrozen at -20°C up to 5 times or refrigerated at 4°C for up to 6 months.

Set the hybridization oven temperature to 37°C according to product instructions. The hybridization 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. Pre-heat thermal cycler and lid to 95°C.
2. Remove an incubation frame cover and clean with Ethanol. Dry with a clean Kimwipe and visually inspect the cover for dust. Use a new Kimwipe as needed to remove any dust. Lay incubation frame cover on a clean Kimwipe until use.
3. Flick to mix probes and centrifuge before using. **Do not vortex probes**.
4. **Denature CosMx RNA probe mixes** (RNA Probe Mix and RNA Add-On), by transferring total

volumes needed for assay (see Table 13), from stock tubes into clean 0.2 mL PCR tubes **(probes and add-ons should be kept separate during denaturing**).

IMPORTANT: Ensure accurate pipetting. When preparing four (4) slides, there will be no excess RNA probe mix or add-on.

5. **Heat at 95°C for 2 minute**s on a thermal cycler with heated lid. **Immediately transfer to ice for at least 1 minute to crash cool.**
6. Make hybridization solution (see Table 13).

Table 13: Hybridization Solution (*n* = number of slides)

	Denatured Core Probe Mix	Denatured Add-on (off-the-shelf or Custom)	RNase Inhibitor	Buffer R	DEPC-treated water (or rRNA marker, see **note)	Total Volume
2-Slide	32 µL	16 µL	3.2 µL	256 µL	12.8 µL	320 µL
4-Slide	64 µL	32 µL	6.4 µL	512 µL	25.6 µL	640 µL
Custom Stand-alone Panel* (2 slide)	16 µL	-	3.2 µL	256 µL	44.8 µL	320 µL
Custom Stand-alone Panel* (4 slide)	32 µL	-	6.4 µL	512 µL	89.6 µL	640 µL

*Custom stand-alone panels are supplied by NanoString at 2X concentration compared to off-the-shelf RNA Probe Mix.

**A rRNA marker may be used in lieu of water for some RNA Assays, specifically when running the mouse neuro assay.

7. **Clean all equipment** and benchtop 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. Change gloves and clean workspace with RNase AWAY.
8. **Wet the Kimwipes with 2X SSC or DEPC-treated water**. Take care that the Kimwipes and 2X SSC do not contact the slides. Kimwipes should be thoroughly wet, but standing buffer should not be present.
9. To prevent the tissue from drying, perform the following steps **one slide at a time.**
   - Remove slides from 2X SSC, **gently tap slide to remove excess liquid.**
   - Carefully remove the thick polyester frame backing (with the center square removed) from the incubation frame to expose the top adhesive layer of the incubation frame. Ensure that the incubation frame does not lift from the slide when removing the polyester frame backing.

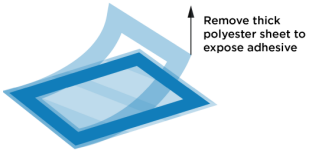

Figure 25: Remove Polyester Frame from Incubation Frame

   - Lay the slide flat on a clean surface and **add 150 µL of hybridization solution** directly to the tissue within the incubation frame.

     Start by adding the hybridization solution to the edge of the tissue opposite of the slide label within the frame. Applying the incubation frame cover will help move the hybridization solution across the tissue (see Figure 26).

     Avoid introducing bubbles by leaving a small residual volume in the pipette tip. In the event that a bubble is introduced, carefully aspirate bubble out using low volume pipette tip if possible. When removing air bubbles, removing small amounts of hybridization solution (as long as sufficient solution remains to cover the tissue) is preferable to having bubbles.

   - **Carefully apply incubation frame cover** (see Figure 26). Start by setting one edge of the cover down on the incubation frame edge, then gradually lay down the rest of the cover. The tab on the incubation frame cover should face the slide label. As it's lowered, the frame cover should naturally adhere to the incubation frame. **Do not press the center of the cover as it could damage the tissue.**

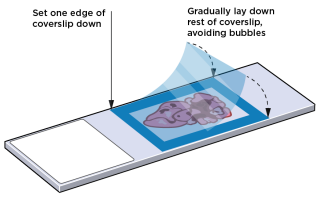

Figure 26: Apply Incubation Frame Cover

- Place the slide horizontally into the hybridization tray **(see Figure 27)**.

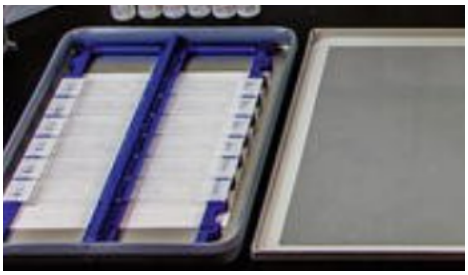

Figure 27: Hybridization Tray

- **Repeat step 9 for each slide.**

10. Close hybridization chamber, insert tray into oven, and clamp tray into place. **Incubate at 37°C overnight** (16 – 18 hours) **(see Figure 28)**.

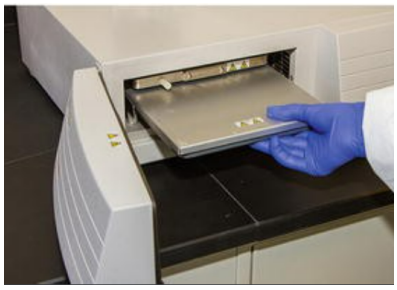

Figure 28: Incubate overnight at 37°C

If your oven does not seal (with a gasket) you may seal your hybridization chamber in a zip-lock bag to simulate a sealed chamber. Chambers sealed in this manner should be tested to ensure they maintain humidity for 24 hours (slides do not dry out) prior to use. Unsealed conditions lead to evaporation of the hybridization solution.

If not already done, prepare 40 mL formamide aliquots following the instructions in **Equipment, Materials, and Reagents on page 15** and store over night at 4°C.

Day 2: Perform Stringent Washes (90 minutes)

You will need the following materials and reagents for this step: **water bath, 4X SSC, 100% formamide,** and **2X SSC** (see Prepare RNA Assay Reagents on page 26).

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

1. Before you begin, **set water bath to 37°C**.
2. Remove nuclear stain and cell segmentation kits from the freezer and thaw on ice.
3. **Warm 100% formamide in the 37°C water bath for at least 30 minutes before opening**. Once formamide is at temperature, **prepare stringent wash directly in staining jars by mixing equal parts 4X SSC and 100% formamide**.

NOTE: Ensure you have sufficient buffer to cover all slides in container for the washes. Wash buffers need to completely cover the tissue on the slide but should be below the slide label. The washes may make the slide labels illegible if submerged.

4. **Preheat staining jars containing freshly prepared stringent wash in 37°C water bath**. It will take about 30 minutes to preheat wash.
5. If nearing the 18 hour maximum overnight incubation time, while jars are preheating, **transfer slides to 2X SSC.**
6. Perform the following steps **one slide at a time** to prevent the tissue from drying.
   - With a clean pair of forceps, carefully remove the incubation frame cover from the incubation frame. Dip slide back into 2X SSC as needed to avoid tissue drying. If cover will not come off without removing incubation frame, remove the frame and cover, the frame can be reapplied in a later step.
   - Repeat for all slides, cleaning the forceps with Ethanol between slides as needed.
7. Once both jars have pre-heated to 37°C and all incubation frame covers have been removed, perform the washes detailed below (see Figure 29). After the last wash, the slides can be stored in 2X SSC for up to one hour.
   - Gently tap each slide one at a time on a clean Kimwipe to remove excess 2X SSC and place slides in the **first stringent wash for 25 minutes**. **Repeat** wash with the second staining jar.

- During second stringent wash, begin preparing reagents for Nuclear and Cell Segmentation Staining (2 hours) on page 51.
- Following stringent washes, immediately **transfer slides to 2X SSC and wash for 2 minutes**. **Repeat** with second jar of 2X SSC. Leave slides in 2X SSC as needed until reagents have been prepared for nuclear and cell segmentation staining.

Figure 29: Perform stringent wash

IMPORTANT: Anything coming into contact with hybridization solution (which contains probes), such as containers for stringent wash solution and 2X SSC, needs to be exclusive for this purpose and thoroughly washed and cleaned with RNase AWAY, as probes may contaminate later runs. Use separate staining jars for different probe mixes. Staining jars should be cleaned with RNase AWAY before use.

Nuclear and Cell Segmentation Staining (2 hours)

You will need the following materials and reagents for this step: **incubation frames, staining jars, 1X PBS, Blocking Buffer** (4°C), **Nuclear Stain Stock** (-80°C), and **Segmentation Marker Kit** (-80°C) (see Prepare RNA Assay Reagents on page 26).

1. **Prepare the following reagents**:
   - Four (4) staining jars of 1X PBS
2. **Prepare 220 µL of Nuclear Stain Buffer per slide.**
   - **Vortex, then centrifuge thawed nuclear stain** 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*.
   - Dilute the nuclear stain stock 1:40 (where *n* equals the number of slides) (see Table 14).

Table 14: Prepare Nuclear Stain

Nuclear Stock	Blocking Buffer	Total Volume
5.5 µL x *n*	214.5 µL x *n*	220 µL x *n*

3. If a new incubation frame is needed, perform the following steps **one slide at a time**.
   - **Remove slide from 2X SSC**, gently tap slide on a clean Kimwipe. Using a clean Kimwipe, **dry the surface of the slide that will come into contact with the incubation frame**.

ⓘ **IMPORTANT**: Avoid wiping the slide within the scan area as this could remove the fiducials needed for imaging. See scan area template on the flow cell assembly tool or follow the guidelines in Prepare Tissue Samples on page 24.

   - Carefully apply a new incubation frame following the instructions in Tissue Permeabilization (40 minutes) on page 35. Ensure that the frame is well adhered to the slide by gently pressing around the frame with clean forceps.
   - Using a clean Kimwipe, carefully wick excess buffer from around the incubation frame as needed. Careful to not touch the area inside of the incubation frame.
   - Lay slide horizontally in staining tray and **slowly apply up to 200 µL of Nuclear Stain Buffer** directly to tissue. Gently move tray side to side as needed to ensure

that the Buffer covers the entire tissue.

- **Repeat** with remaining slides and cover tray.

4. **Incubate slides for 15 minutes at room temperature protected from light** (see Figure 30)**.**

Figure 30: Cover tray and incubate for 15 minutes

5. During nuclear stain incubation, **prepare segmentation and marker stain mix** using the following table where *n* = the number of slides (see Table 15). **Flick each tube to mix and centrifuge before use. Do not vortex mix.**

IMPORTANT: Ensure accurate pipetting. When preparing four (4) slides, there will be no excess segmentation and marker stain mix.

Table 15: Prepare Staining Mix

Cell Segmentation Mix 1 (CD298/B2M)	Marker Mix 1* (Optional PanCK/CD45)	Marker Mix 2* (Optional a la carte*)	Blocking Buffer	Total Volume
8 µL x *n*	8 µL x *n*	8 µL x *n*	176 µL x *n*	200 µL x *n*

*If not adding the optional PanCK/CD45 or à la carte markers, add Blocking Buffer in lieu of marker.

6. After nuclear stain incubation, **remove slides one at a time** from staining tray, gently tap slide on a clean Kimwipe to remove excess buffer, and **transfer slide to 1X PBS**.

7. Wash slide for **5 minutes** in 1X PBS.

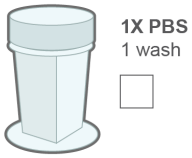

Figure 31: Wash for 5 minutes in 1X PBS

8. During PBS wash, add 2X SSC or DI water to the staining tray. Do not overfill. The water level should be well below the slides to avoid cross-contamination.
9. Following PBS wash, perform the following steps **one slide at a time** to prevent tissue drying:
   - **Remove slide from 1X PBS** and gently tap slide on a clean Kimwipe to remove excess PBS.
   - Lay slide horizontally in staining tray and **apply up to 200 µL of Staining Mix** directly to tissue. Gently move tray side to side as needed to ensure that the mix covers the entire tissue.
   - If needed, an incubation frame cover can be placed over the incubation frame to ensure that the mix completely covers the tissue.
   - Adjust volume to add as needed for tissues of varying sizes. The **segmentation and visualization stain mix needs to completely cover the tissue** but does not need to completely fill the incubation frame.
10. **Repeat** with remaining slides and cover tray (see Figure 32).

Figure 32: Cover tray and incubate for 1 hour

11. **Incubate slides for one (1) hour at room temperature** protected from light.
12. If new aliquots need to be made, **remove NHS-acetate from -20°C** and leave at room temperature during incubation.

13. Following segmentation and visualization incubation, **transfer slides to 1X PBS and wash for 5 minutes** (see Figure 33).

Figure 33: Wash 3X in 1X PBS

14. **Repeat wash two (2) times** for a total of three (3) PBS washes.

Continue to **Second NHS-Acetate Preparation and Application (25 minutes)**.

Second NHS-Acetate Preparation and Application (25 minutes)

You will need the following materials and reagents for this step: **analytical scale, 2.0 mL centrifuge tube, staining jar, NHS-Acetate powder, NHS-acetate buffer** (provided by NanoString, 4°C), and **2X SSC**.

1. **Prepare 100 mM NHS-acetate mixture** immediately before you are ready to apply the mixture onto the tissue (200 µL/sample). Ensure stock NHS-Acetate has reached room temperature before aliquoting.
   a. NHS-acetate powder is stored desiccated at -20°C. If not already done, prepare 25 mg aliquots of NHS-acetate powder by weighing out the powder directly into four (4) 2.0 mL centrifuge tubes. Label tubes with the exact weight, seal tubes with parafilm, and store tubes at -20°C in desiccant for later use. See **Prepare RNA Assay Reagents on page 26**.

      NOTE: If preparing only 2 slides, pre-weigh 15 mg aliquots into six (6) total tubes.

   b. **Add NHS-acetate buffer directly to the aliquoted powder immediately before applying to the tissue**.
      i. Calculate the amount of NHS-acetate buffer to add to the NHS-acetate powder by multiplying the weight of NHS-acetate powder in mg by 38.5.
         - Example: for 25.0 mg of NHS-acetate powder: 25.0 * 38.5 = 962.5 µL of buffer to add.

   c. Slowly pipette up and down to mix. Bubbles may occur. Do not fully aspirate liquid from pipette while mixing.

> ⓘ **IMPORTANT**: Reconstitute NHS-Acetate immediately before use. Do not prepare stock solutions for storage because the NHS ester moiety readily hydrolyzes and becomes non-reactive. Discard any unused reconstituted reagent.

2. Perform the following steps **one slide at a time** to prevent the tissue from drying out:
   - **Remove slide from 1X PBS** and transfer to a clean staining tray.
   - **Apply 200-250 µL of NHS-acetate mixture** onto tissue within the incubation frame. Gently move tray side to side as needed to ensure that the NHS-acetate solution covers the entire tissue.

- Repeat with remaining slides and **incubate covered in staining tray for 15 minutes at room temperature** (see Figure 34).

Figure 34: Incubate 15 minutes

3. Following incubation, tap off excess liquid and wash slides in **2X SSC for at least 5 minutes**.

Figure 35: Two 5-minute 2X SSC Washes

4. **Repeat 2X SSC wash** for a total of two (2) washes. Slides will stay in second 2X SSC wash while hybridization mix is prepared.
   - If samples need to be stored overnight and loaded onto the instrument the next day, remove the incubation frame following the guidelines from Day 2: Perform Stringent Washes (90 minutes). Ensure the entire incubation frame is removed then store according to Safe Storage Guidelines for RNA Slides.
   - If samples will be loaded onto the instrument the same day, continue to Flow Cell Assembly.

Safe Storage Guidelines for RNA Slides

After processing, slides must never be stored dry. Slides may be stored for up to six (6) hours protected from light and submerged in 2X SSC at room temperature.

Slides can be stored protected from light and submerged in 2X SSC at 4°C overnight, if needed. Slides may be stored longer than that, but RNA counts and staining efficiency will decrease as a function of days stored. For best results, minimize storage time between slide preparation and loading on the CosMx SMI instrument.

Slides must be stored in the dark (avoiding light is crucial as fiducials are sensitive to photobleaching).

RNA Fresh Frozen Manual Slide Preparation

The RNA Fresh Frozen Assay has been optimized and validated only for fresh frozen mouse brain tissue. The use of this assay with other fresh frozen tissue types will require additional testing. Incubation times, concentrations, and temperatures will need to be empirically determined.

CosMx SMI Slide Preparation Workflow

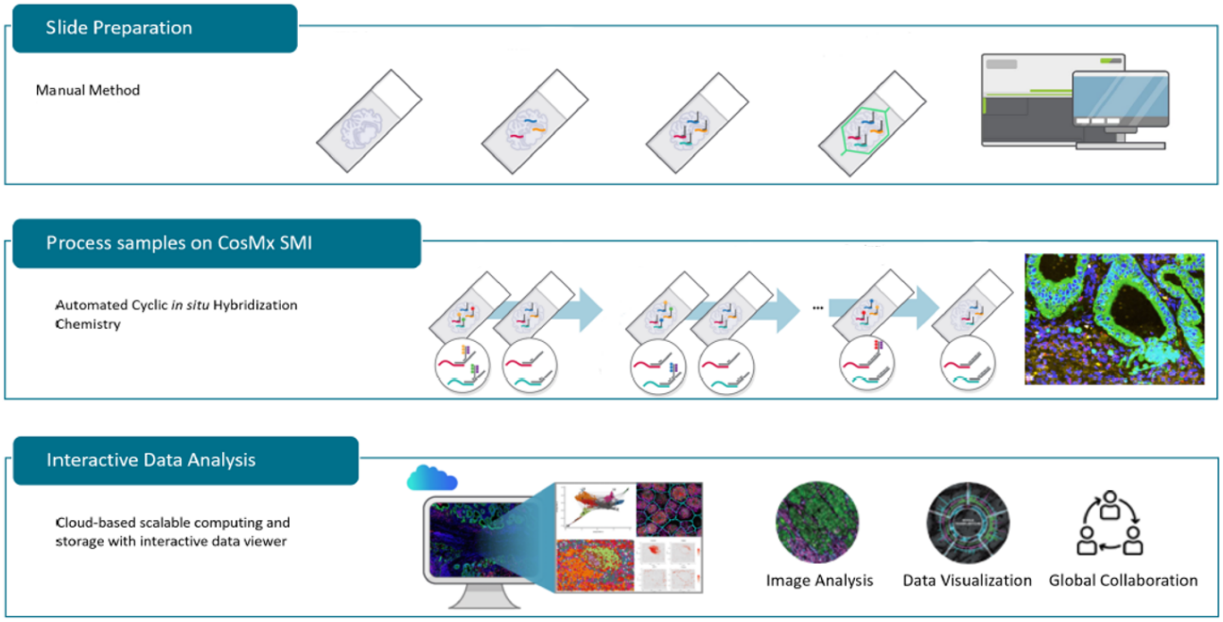

Figure 36: CosMx SMI Workflow Overview

Day 1: Slide Preparation. Prepare slides and incubate biological targets with ISH probes.

Day 2: Process Slides on CosMx SMI. Remove off-target probes and add cell segmentation markers to each slide. Load prepared flow cells into the CosMx SMI instrument and enter flow cell/study information. Tissue is scanned to capture RNA readout and morphology imaging within user-designated fields of view (FOVs).

After run completion: Create a **Data Analysis** study in the AtoMx Spatial Informatics Platform (SIP) and perform quality-control checks, data analysis, and generate analysis plots.

Equipment, Materials, and Reagents

The following equipment (see Table 16), materials (see Table 17), and reagents (see Table 18) are required for this protocol but are **not supplied by NanoString Technologies Inc.**

Equipment:

Table 16: Equipment not provided by Nanostring

	Equipment	Source	Part Number(s)
☐	**Baking Oven**	Quincy Lab, Inc (or comparable)	Various GC Models
☐	**Hybridization Oven including hybridization chamber** • RapidFISH Slide Hybridizer **or** • HybEZ oven • Humidity control tray NOTE: 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 CosMx SMI Sample Prep	Boekel Scientific or ACDBio	240200 for 120V or 240200-2 for 230V 321710/321720 310012
☐	**6-quart Pressure Cooker** NOTE: Pressure Cooker must have a built in temperature gauge for validation NOTE: Products from other vendors may require testing and optimization	BioSB® TintoRetriever	BSB-7087
☐	**Ultrasonic Bath** (500 mL capacity) NOTE: 40 kHz frequency with timer	General Lab Supplier	Example
☐	**Vortex mixer**	General Lab Supplier	Various

	Equipment	Source	Part Number(s)
☐	**Micro Centrifuge** for 1.5 mL microcentrifuge tubes	General Lab Supplier	Various
☐	**Water Bath** (temperature setting of 37°C)	General Lab Supplier	Various
☐	**Thermal Cycler** NOTE: must include 96-well 200 µL tube block	General Lab Supplier	Various
☐	**Analytic Scale with draft shield** NOTE: ensure scale can weigh in milligrams (mg) for accuracy.	Various	Example

Materials:

Table 17: Materials not provided by Nanostring

	Materials	Source	Part Number (s)
☐	**Pipettes for 2.0 – 1,000 µL**	General Lab Supplier	Various
☐	**Filter tips (RNase/DNase Free)**	General Lab Supplier	Various
☐	**2.0 mL Centrifuge Tubes (RNase/DNase Free)**	General Lab Supplier	Various
☐	**0.2 mL PCR tubes** or PCR strip tubes	General Lab Supplier	Various
☐	**VWR Superfrost Plus Micro Slide, Premium** or **Leica BOND PLUS slides**	VWR Leica Biosystems	48311-703 S21.2113.A

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

	Materials	Source	Part Number (s)
	NOTE: these slides have been validated by NanoString. Do not use other products.		
☐	**Slide Rack**	General Lab Supplier	Example
☐	**Polypropylene Slide Staining Jars** (24 required) or **Slide Staining Station** NOTE: due to the photo-sensitivity of this assay, the staining jars should be impermeable to light.	Ted Pella (or comparable) Amazon Fisher Scientific	21029 MH-SJ6302 NC1862866
☐	**Forceps** (for slide handling)	General Lab Supplier	Various
☐	**Razor Blades**	General Lab Supplier	Various
☐	**Timer**	General Lab Supplier	Various
☐	**RNase AWAY** NOTE: RNase ZAP and other alternatives cannot be used as substitutes as they do not adequately remove both nucleic acid and nuclease contaminants.	ThermoFisher	7003PK
☐	**Kimwipes (large and small)**	General Lab Supplier	Various
☐	**StainTray slide staining system with black lid**	Sigma Aldrich	Example

Reagents:

Table 18: Reagents not provided by Nanostring Technologies Inc.

	Reagent	Source / Part Number (s)	Storage Conditions
☐	**DEPC-Treated Water**	ThermoFisher, AM9922 (or comparable)	Room temperature
☐	**100% Ethanol (EtOH): ACS grade or Better**	General Lab Supplier	Flammable Storage Room temperature
☐	**10X Phosphate Buffered Saline pH 7.4 (PBS)**	ThermoFisher, AM9625 (or comparable)	Room temperature
☐	**SDS, 10% Solution, RNase-free**	ThermoFisher, AM9822	Room temperature
☐	**20X SSC (DNase, RNase free)**	Thermofisher, AM9763	Room temperature
☐	**Tris Base**	Sigma-Aldrich, 10708976001 (or comparable)	Room temperature
☐	**Glycine**	Sigma-Aldrich, G7126 (or comparable)	Room temperature
☐	**Sulfo NHS-acetate powder** NOTE: NHS-Acetate powder is shipped in a plastic bag with a desiccant and should be left in the bag and stored at -20°C until ready to use. NOTE: 100mg of NHS-Acetate is sufficient for 6-7 slides.	Fisher Scientific, 26777	-20°C
☐	**10% neutral buffered formalin (NBF)**	EMS Diasum, Cat # 15740-04 (or comparable)	Room temperature

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

	Reagent	Source / Part Number (s)	Storage Conditions
☐	**100% Deionized Formamide** NOTE: Deionized Formamide is optimal, however, formamide that is not deionized may also be used.	ThermoFisher , AM9342 or VWR, VWRV0606 (or comparable)	4°C (bring to RT for at least 30 before opening)
OPTIONAL: Large volume stock solutions (>500 mL) of Deionized Formamide can be aliquoted into 50 mL conical tubes and stored, protected from light, at 4°C. This will save time during day 2 slide preparation.			

NanoString Supplied Reagents

CosMx Fresh Frozen Slide Preparation Kit (RNA)

Figure 37: Fresh Frozen Slide Preparation Kit (RNA)

Table 19: CosMx Fresh Frozen Slide Prep Kit (RNA) Box 1/2

Kit Contents (Store at 4°C)	
CosMx Target Retrieval Solution (10X)	Buffer R
CosMx RNA Blocking Buffer	CosMx Fiducials
NHS-Acetate Buffer	2X SSC-T Media
Incubation Frames and covers	Protease A Buffer (not pictured)

Figure 38: CosMx Fresh Frozen Slide Prep Kit (RNA) Box 2/2

Table 20: CosMx Fresh Frozen Slide Prep Kit (RNA) Box 2/2

Kit Contents (Store at -20°C)	
CosMx RNase Inhibitor	CosMx Protease Solution (Proteinase K)
CosMx Protease A (2 vials - not pictured)	

CosMx RNA Probe Mix and/or Custom RNA Probe (see Panel and Cell Segmentation Marker Selection on page 11).

Figure 39: CosMx Mouse Neuro RNA Probe Mix

Table 21: CosMx Mouse Neuro RNA Probe Mix

Kit Contents (Store at -20°C)	
Includes: CosMx Mm Neuro RNA Probe Mix	Custom RNA Add-On Probes (if applicable) Custom RNA Add-On (if applicable)

CosMx Segmentation and Supplemental Markers

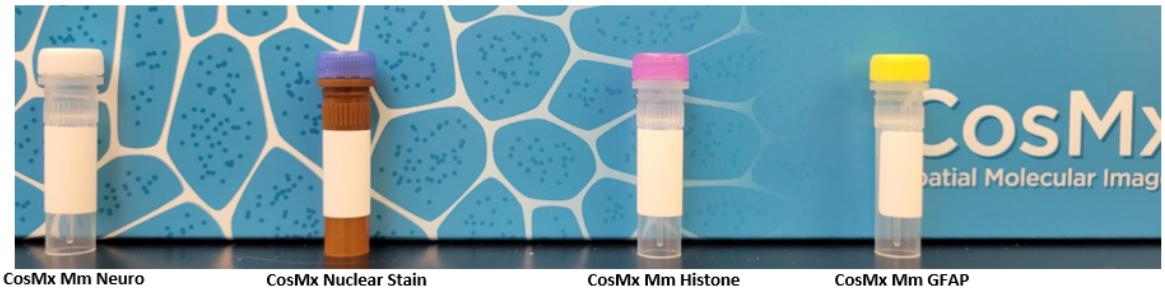

Figure 40: CosMx Segmentation and Supplemental Markers (Mouse Neuro FF RNA)

Table 22: CosMx Segmentation and Supplemental Markers (Mouse Neuro FF RNA)

Kit Contents (Store at -80°C) Each kit is sufficient for four (4) slides	
Kit Name	**Kit Component**
CosMx™ Mouse Neuroscience Cell Segmentation Kit (RNA), Ch 1/2	CosMx Nuclear Stain (x2) RNA Mm Neuro Segmentation Marker (rRNA)
CosMx™ Mouse Neuroscience Supplemental Segmentation Kit (RNA), Ch 3/4 Recommended	CosMx Mm Histone Marker Ch3 (RNA) CosMx Mm GFAP Marker Ch4 (RNA)

Flow-Cell Assembly Kit

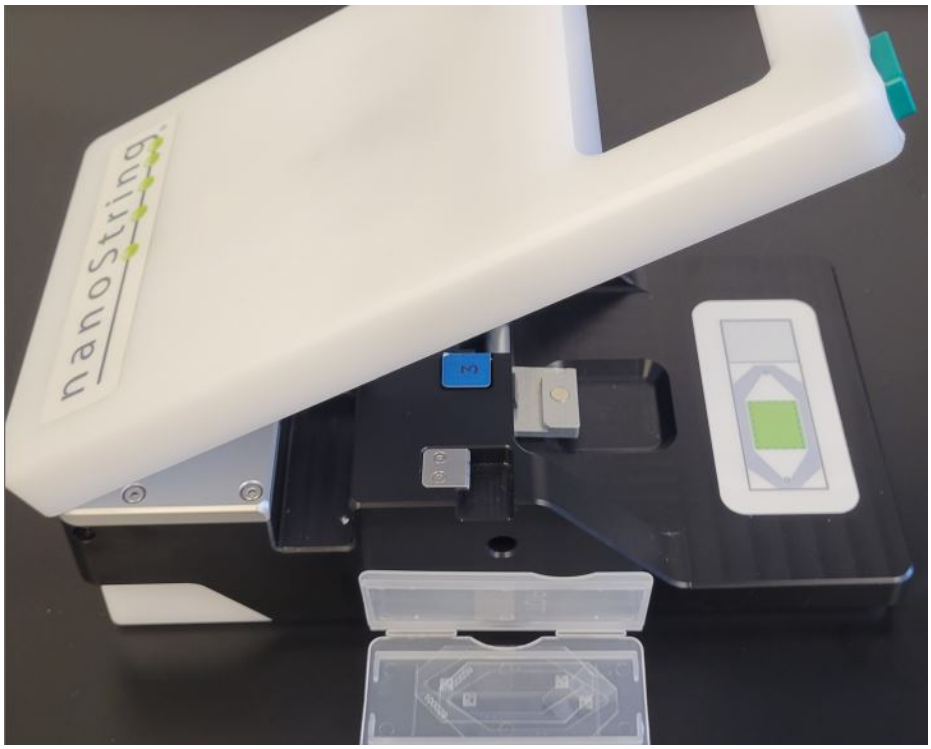

Figure 41: Flow Cell Assembly Kit

Table 23: Flow Cell Assembly Kit

Kit Contents (Store at Room Temperature)	
Flow Cell Assembly Tool	Flow Cell Coverslips

Prepare Fresh Frozen Tissue Samples

Appendix I: CosMx SMI Sample Preparation Guidelines on page 137 covers Fresh Frozen block selection and sectioning in detail. Please review sample preparation guidelines prior to beginning the RNA Fresh Frozen Sample Preparation procedure.

Sectioning Fresh Frozen Tissue

- Slides should be stored at -80°C prior to processing.
- Dry mount slides for 5-10 minutes at room temperature, or until dry. Once dry, return slide to dry ice store at -80°C.

Tissue Sectioning and Slide Preparation:

Fresh frozen blocks should be sectioned at **5- 10 µm thickness** and mounted on the label side of VWR Superfrost Plus Slides or Leica BOND PLUS slides.

Tissue sections must be placed within the Scan Area (the green area) of the slide and be no larger than **20 mm Long by 15 mm Wide** (see Figure 42) (image not to scale). For best performance, ensure some glass is visible in all four corners and in the center of the scan area.

Label slides with pencil on the frosted label according to lab guidelines. If using an adhesive slide label, ensure the label is less than 295 µm thick and is not folded over on itself. Labels over the maximum thickness or labels that are not properly adhered may result is slide or flow cell damage during flow cell assembly and/or instrument loading.

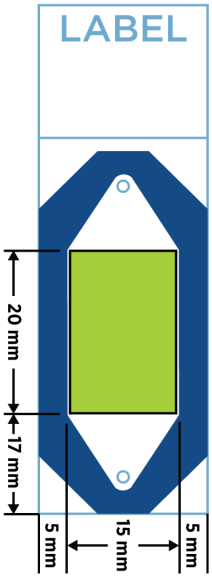

Figure 42: Tissue Scan Area (not to scale)

WARNING: If the tissue sections are less than 5 µm or the tissue is poor quality, the sample may degrade during the SDS wash step.

If sections are larger than the indicated size and/or placed off-center, continue with the slide preparation as outlined. Directly before applying the incubation frame, scrape off parts of the tissue exceeding the scan area. Scraping off tissue before target retrieval and ethanol drying could generate tissue folds that may result in staining/binding artifacts, while suboptimal scraping may result in poor incubation frame adherence. Applying the incubation frame over tissue could result in tissue damage when the incubation seal is removed.

ⓘ IMPORTANT: The CosMx SMI instrument will only image the area inside the flow cell chamber, the scannable area. If the tissue section is outside of the Scan Area, it will not be imaged.

Prepare RNA Fresh Frozen Assay Reagents

IMPORTANT: Take care to maintain nuclease- free conditions. The greatest risk of contamination comes from RNA probes and other oligos. NanoString recommends the use of RNase AWAY (Thermo Fisher 7003PK), as it will limit contamination from oligos, 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.

Prepare reagents using the instructions in the following table **(see Table 24)**.

NOTE: Unless otherwise noted, reagents can be made up to two (2) weeks in advance and stored at room temperature.

Table 24: RNA Fresh Frozen Reagent Preparation

	Reagent	Dilution	Storage
☐	1X PBS (pH 7.4)	Prepare 1L of 1X PBS by combining 100 mL of 10X PBS and 900 mL of DEPC-treated water.	Room Temperature
☐	2X SSC	Prepare 1L of 2X SSC by combining 100 mL of 20X SSC and 900 mL of DEPC-treated water.	Room Temperature
☐	4X SSC	Prepare 1L of 4X SSC by combining 200 mL of 20X SSC and 800 mL of DEPC-treated water.	Room Temperature
☐	NBF stop buffer (Tris Glycine Buffer)	Combine 6.06g Tris base and 3.75g Glycine in 500 mL of DEPC-treated water. The final concentration of Tris and Glycine will be 0.1M each.	Room Temperature
☐	4% SDS in 1X PBS	Before diluting: **warm 10% SDS for 10 minutes** in a 37°C water bath. After warming, vortex for one (1) minute. Prepare 50 mL of 4% SDS in 1X PBS by adding 5mL of 10X PBS	Make Fresh Daily

	Reagent	Dilution	Storage
		and 20 mL of 10% SDS to 25 mL of DEPC-treated water. **NOTE**: the concentration of SDS is critical. Carefully measure each volume to ensure the concentration does not exceed 4%.	
☐	70% Ethanol (EtOH)	Prepare 50 mL of 70% ethanol by adding 15 mL of DEPC-treated water to 35 mL 100% Ethanol.	Make Fresh Daily
☐	50% Ethanol (EtOH)	Prepare 50 mL of 50% ethanol by adding 25 mL of DEPC-treated water to 25 mL 100% Ethanol.	Make Fresh Daily
☐	1X Target Retrieval Buffer	Prepare 50 mL fresh daily by adding 5 mL of CosMx Target Retrieval Buffer, 10X (provided by NanoString) to 45 mL DEPC-treated water. NOTE: NanoSting provides 20 mL of CosMx Target Retrieval Solution, 10X. A 50-100 mL capacity staining jar is recommended when preparing only 2 slides.	Make Fresh Daily
☐	Digestion Buffer	Prepare immediately before use. See **Tissue Permeabilization (40 minutes) on page 78**.	Make immediately before use.
☐	NHS-Acetate Mix	Individual aliquots can be prepared prior to slide preparation but should be stored at -20°C with a desiccant until use. **To prepare aliquots** (4-slide preparation, see note below):	-20°C in desiccant

	Reagent	Dilution	Storage
		• Bring stock to room temperature for 1-2 hours prior to opening. • Prelabel four (4) screw top 1.5 mL centrifuge tubes with the required information. • Using a weighing spatula, carefully weigh 25 mg of NHS-Acetate directly into the screw-top tube on an analytic scale. • Close the tube and label tube with final weight. Seal the tube with parafilm, and **return to -20°C in desiccant until use.** NOTE: If preparing only 2 slides, prepare 15 mg aliquots into six (6) total tubes.	
☐	Fiducials and Hybridization Mix	These reagents have additional steps that will be covered in detail in their respective sections. Remove ISH Probe Mix from storage and keep on ice until ready to use. NOTE: Fiducials are light sensitive and should be kept stored, protected from light, until instructed to remove.	n/a
☐	Day 2 Reagents	These reagents have additional steps that will be covered in detail in their respective steps. Follow Day 2 procedure for preparation of these reagents.	n/a

Day 1: NBF Fixation and Bake

You will need the following materials and reagents for this step: **Staining jars** and **10% NBF.**

- **Pre-cool a staining jar** filled with 10% NBF to 4°C for a minimum of 15 minutes. Ensure you have sufficient buffer to cover all slides in container for the washes. Wash buffers need to completely cover the tissue on the slide but should be below the slide label. The washes may make the slide labels illegible if submerged.
- **Preheat oven** to 60°C.
- Remove sample slides from -80°C freezer and place onto dry ice to carry sample slides to slide preparation area.

NBF Fixation:

1. Remove slides from dry ice and immediately **transfer to pre-cooled 10% NBF**.

Figure 43: 2 Hour NBF Fixation

2. Incubate slides in **10% NBF for two (2) hours at 4°C.**
3. Following NBF fixation, **transfer slides to 1X PBS** and wash for 2 minutes.

Figure 44: Wash 3x with 1X PBS

4. **Repeat PBS wash twice**, using new staining jars for each wash, for a total of three (3) washes.
5. After final PBS wash, place slides into slide holder and **bake vertically at 60°C for 30 minutes.**

Wash and Rehydrate Fresh Frozen Tissue Sections (1 hour)

You will need the following materials and reagents for this step: **staining jars, 1X PBS, 4% SDS, 100% EtOH, 70% EtOH, 50% EtOH**, and **DEPC-treated water**. See **Prepare RNA Fresh Frozen Assay Reagents on page 70** for more details.

Wash and rehydrate Fresh Frozen tissue sections:

Time Critical Step: the following steps are time sensitive. Be sure to use a timer and transfer slides between washes carefully but quickly to avoid additional time in each wash. Exceeding the wash time may result in tissue degradation and loss of data.

1. After NBF fixation, **gently perform the following washes** using staining jars **(see Figure 45).**

NOTE: Ensure you have sufficient buffer to cover all slides in container for the washes. Wash buffers need to completely cover the tissue on the slide but should be below the slide label. The washes may make the slide labels illegible if submerged.

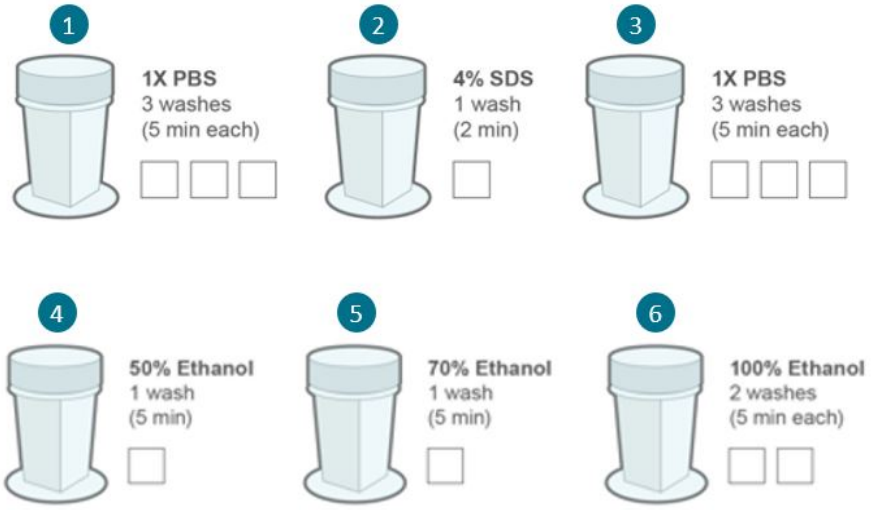

Figure 45: Rehydrate and Fix Tissue

2. During final Ethanol wash, clean workspace with RNase AWAY and lay down clean Kimwipes. Once final wash is complete, remove slides from Ethanol, lay horizontally on Kimwipes, and air dry for at least **10 minutes at room temperature.**

3. While slides are drying, preheat the pressure cooker and target retrieval solution.
4. **Before turning on the pressure cooker**, ensure that the water is at the correct level per the manufacturer's instructions (4-8 cups depending on model used). Refill with deionized water as needed.
5. **Place the staining jar containing freshly prepared 1X Target Retrieval solution into the pressure cooker to preheat** (see Figure 46). Ensure that water level is well below lid of jar. About halfway up jar will be sufficient. If needed, a trivet may be used to raise the jar.

Figure 46: Preheat Target Retrieval Solution

6. Place a lid on the staining jar to prevent evaporation. To prevent pressure from building within the container, **do not fully seal the lid to the jar**.
7. **Prime the pressure cooker** up to 100°C (see Figure 47) for step 5, Perform Target Retrieval (50 minutes) on page 76. **Ensure the pressure valve is closed**.

Figure 47: Prime Pressure Cooker

8. **If not already aliquoted, remove NHS-Acetate powder from -20°C freezer** and leave at room temperature for 1-2 hours before use in NHS-Acetate Preparation and Application (25 minutes) on page 85.

Perform Target Retrieval (50 minutes)

You will need the following materials and reagents for this step: **pressure cooker, staining jars, 1X Target Retrieval solution** (pre-heated in the previous step), **DEPC-treated water** and **1X PBS**.

1. Once Target Retrieval solution has been preheated, **press cancel to stop the program and carefully release the pressure valve.**

⚠ WARNING: When opening the pressure valve and removing the pressure cooker lid, hot steam will be released. The staining jar will also be VERY hot. Wear protective heat resistant gloves to open lid and remove staining jar.

2. Once the pressure has been released, **carefully but quickly remove the staining jar containing preheated 1X Target Retrieval solution**. Once removed, the target retrieval solution will begin to rapidly cool, ensure the following steps are done a quickly as safely possible.
3. **Place slides into the preheated solution** and replace lid on the staining jar to prevent evaporation. To prevent pressure from building within the container, **do not fully seal the lid to the jar** (see Figure 48).

Figure 48: Perform Target Retrieval

4. Return the staining jar containing the slides **into the preheated pressure cooker.** Ensure that water level is well below lid of jar. About halfway up jar will be sufficient. If needed, a trivet may be used to raise the jar.
5. Attach the pressure cooker lid, **open the pressure valve to *pressure release* position** and return the pressure cooker to 100°C (see Figure 49).

Figure 49: set pressure valve to pressure release

6. **Once the pressure cooker temperature returns to 100°C**, start timer and run for **15 minutes**.
7. When the timer reaches zero, press cancel on pressure cooker to stop heating. **Carefully remove the pressure cooker lid and remove the staining jar**.

   ⚠ WARNING: When starting to remove pressure cooker lid, hot steam will be released. The staining jar will also be VERY hot. Wear protective heat resistant gloves to open lid and remove staining jar.

8. **Immediately transfer all slides to staining jar containing fresh DEPC-treated water**. Move slides up and down for 15 seconds to wash slides (see Figure 50).

   NOTE: Transfer slides from target retrieval buffer into DEPC-treated water quickly to ensure slides do not dry out. Drying can occur quickly while slides are hot.

9. **Transfer all slides to fresh 100% Ethanol** and wash slides for **3 minutes**.
10. During Ethanol wash, clean bench space with RNase AWAY and lay out a fresh Kimwipe.
11. After 3 minutes, **remove slides from Ethanol and let dry at room temperature for 30 minutes to one (1) hour**. Slides should be laid horizontally on clean Kimwipe to dry.

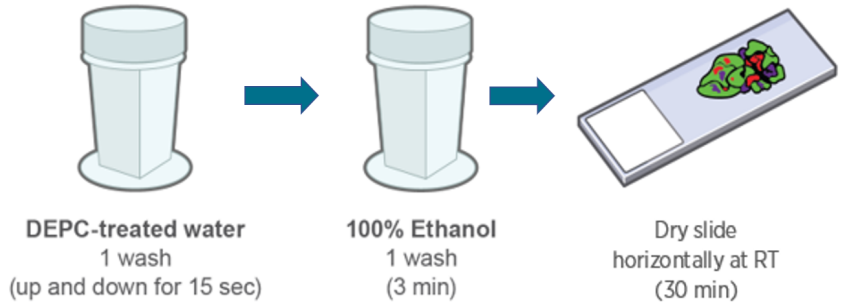

Figure 50: wash and dry sample

Tissue Permeabilization (40 minutes)

You will need the following materials and reagents for this step: **staining jars, hybridization oven, hybridization tray, incubation frames** (CosMx FF Slide Prep Kit (RNA) Box 1/2, Stored at 4°C)**, Proteinase K, Protease A, Protease A Buffer, 1X PBS** and **DEPC-treated water.**

1. Prepare the Incubation Frame:
   - Separate an individual frame from the strip by carefully tearing along the perforations.
   - Each frame is sandwiched between a thin solid polyester sheet and a thick polyester frame backing (with the center square removed).
2. Prepare Digestion Buffer:
   - **Resuspend Protease A** with 200 µL of Protease A Buffer.

NOTE: A 2-step serial dilution is recommended for both Protease A and Proteinase K. Accurate dilution of both proteases is critical for proper assay performance. After each dilution, mix thoroughly by inverting tube or pipetting up and down using a clean pipetter tip. **Do not vortex.**

   - **Create Proteinase K working stock:** dilute 20 mg/mL stock to 100 µg/mL by adding 2 µL of Proteinase K to 398 µL of 1X PBS.
   - **Create Protease A working stock**: dilute rehydrated Protease stock A 1:50 by adding 5 µL of Protease A to 245 µL of Protease A Buffer.
   - **Create Digestion Buffer**: add 82.5 µL of Proteinase K working stock and 16.5 µL of Protease A working stock into 1551 µL of Protease A Buffer.
   - Volume for final dilution is for **four (4) slides**. Adjust volume as needed.

NOTE: digestion buffer should be made within 10 minutes of use. This concentration is specific to fresh frozen mouse brain tissue and may differ for other tissue types.

3. If needed, trim the tissue following the template in **Prepare Fresh Frozen Tissue Samples on page 68**.
   - Use a clean razor blade to trim tissue and change blade as needed to ensure clean cuts and reduce the risk of cross-contamination between samples.

4. Using a clean Kimwipe, ensure that the surface of the slide that will come in contact with the incubation frame is **clean and dry**.
5. Apply the incubation frame (see Figure 51).
   - Carefully **remove the thin polyester sheet**, ensuring that the frame remains bound to the polyester frame (with the center square removed).
   - With the slide on a flat surface, careful not to touch the adhesive, **center the tissue within the incubation frame and carefully place the incubation frame** around each tissue section. Lightly press along the border of the incubation frame to ensure that it is well adhered to the slide.

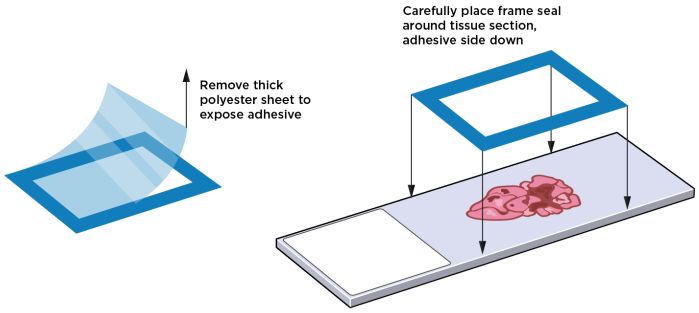

Figure 51: Apply Incubation Frame

6. With the slide still on a flat surface, use a clean razor blade to **carefully trim the long edges of the incubation frame to remove excess plastic** ensuring that there is no excess film extending over slide edges. If needed, trim the short end of the frame opposite of the slide label.
7. With the slide on a clean, flat service, **apply 400 µL of digestion buffer to completely cover tissue within incubation frame**. Gently move the slide side to side as needed to ensure that digestion buffer covers the entire tissue.
8. **Incubate for 30 minutes at room temperature.**

IMPORTANT: After digestion buffer has been applied, avoid tissue drying in subsequent steps by working with only one (1) slide at a time.

9. During incubation, **remove fiducials and 2X SSC-T from 4°C and let come to room temperature, protected from light, for at least 10 minutes.**

10. Once fiducials have reached room temperature, prepare fiducial working solution following instructions for **Fiducial Preparation and Application (20 minutes) on page 39**.

11. After incubation, tap off excess digestion buffer *one slide at a time* and **transfer slides to staining jar containing fresh 1X PBS**. Wash for 5 minutes.

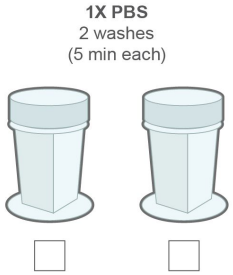

Figure 52: Wash 2X in 1X PBS

12. **Repeat with a new jar of 1X PBS.**

13. Slides can be stored in 1X PBS while fiducials are prepared.

NOTE: Limit the time that the slides are kept in 1X PBS to minimize risk of target loss. Targets have been exposed and tissue has not undergone post-fixation.

Fiducial Preparation and Application (20 minutes)

You will need the following materials and reagents for this step: **ultrasonic bath, vortex, 2X SSC-T (at RT,** provided by NanoString**)**, **fiducials (at RT), staining jars**, **staining tray** (clean and dry), and **1X PBS.**

Volume for final working solution is sufficient for four (4) total slides. It is not recommended that less than two (2) mL of fiducial working solution is made due to high risk of clumping. However, additional volume may be made as needed.

1. **Prepare fiducials for use following the below steps** (see Figure 53):

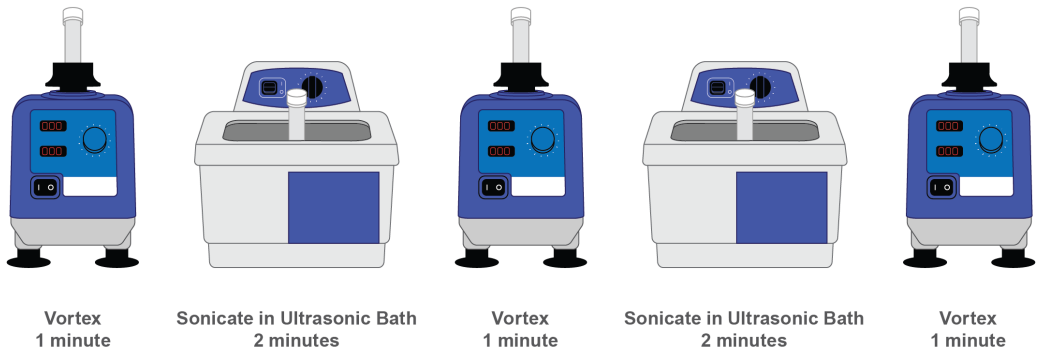

Figure 53: Fiducial preparation

NOTE: Failure to follow these steps will result in fiducial clumping and uneven distribution of fiducials within the tissue. This uneven distribution can result in a loss of readable area or loss of image registration.

2. Once fiducials are prepared, **dilute fiducial stock (0.1%) to the working concentration (0.00015%) in 2X SSC-T.** A 2-step serial dilution is recommended for fiducial preparation. Accurate dilution of fiducialsis critical for proper assay performance.
   - Dilute stock to 0.01% by adding **10 µL of the fiducial stock to 90 µL of 2X SSC-T**. Label tube as *Dilution 1 (D1)*.
   - Cover *Dilution 1* and **leave at room temperature for 10 minutes** protected from light.
   - After 10 minutes, vortex and quick spin *Dilution 1* and dilute to the final working concentration (0.00015%) using the following table (see Table 25).

Table 25: Fiducial Final Dilution

Number of Slides	Dilution 1 (D1) (0.01% Fiducials)	2X SSC-T	Final Volume
2-slides	7.5 µL	492.5 µL	500 µL
4-slides	15 µL	985 µL	1000 µL

NOTE: The concentration may differ for some tissue types and need to be empirically determined. For this protocol, start with the default concentration of 0.00015% and adjust the concentration as needed. See Appendix III: Tissue Specific Fiducial Concentrations on page 142 for suggested tissue specific concentrations based on R&D preliminary testing.

3. **Immediately before applying fiducials to slides, vortex tube for 1 additional minute**. Vortex fiducials for 30 seconds between slides to keep fiducials in suspension and ensure consistent concentration across all slides.
4. Remove slides from 1X PBS and gently tap tissue on a clean Kimwipe to remove excess buffer. Lay slide horizontally in staining tray.
5. **Apply up to 250 µL of the final fiducial solution, ensuring the solution covers glass and tissue within the incubation frame**. Fiducials must be present on the glass within the scan area for consistent focusing during the instrument run.
6. **Incubate covered in staining tray for 5 minutes at room temperature.**
   - Gently move tray side to side as needed to ensure that the fiducial solution covers the entire scan area, including glass.

Figure 54: Incubate covered for 5 minutes

NOTE: A pipette tip can also be used to carefully spread buffer over tissue within the incubation frame. Carefully use a small volume pipette to spread buffer as needed by carefully laying the tip horizontally on top of the incubation frame and gently rolling the tip to spread the buffer until the tissue is completely covered. Be careful to avoid touching the tissue with the pipette.

ⓘ **IMPORTANT**: This step and all steps moving forward are light sensitive as fiducials contain fluorescent molecules that are sensitive to photo-bleaching.

7. After fiducial incubation, *one slide at a time*, gently tap slides on a clean Kimwipe to remove excess solution and transfer slides to staining jar containing fresh 1X PBS.

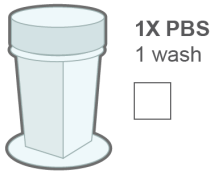

Figure 55: PBS Wash

8. Wash slides in staining jar with **1X PBS for 5 minutes**.

Proceed to next steps immediately.

Post-Fixation (20 minutes)

You will need the following materials and reagents for this step: **staining jars, 10% NBF, NBF Stop Buffer (Tris-Glycine Buffer)** and **1X PBS** (see Prepare RNA Fresh Frozen Assay Reagents on page 70 for more information).

⚠ 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 the following washes (see Figure 56).

1. Transfer slides to **10% NBF and incubate for 1 minute** at room temperature.
2. **Immediately transfer the slides** to the staining jar with the first **NBF Stop Buffer and wash for 5 minutes**. **Repeat** with a second staining jar containing NBF Stop Buffer.
3. **Transfer slides to 1X PBS Wash** for **5 minutes**.

Figure 56: NBF Post Fix

4. During PBS wash, remove **core panel** (or custom panel, if applicable), **segmentation markers, RNase Inhibitor, and add-on probes** (if applicable) from -20°C and thaw on ice.
5. **Remove Buffer R** from 4°C and bring to room temperature.

NHS-Acetate Preparation and Application (25 minutes)

You will need the following materials and reagents for this step: **analytical scale, 2.0 mL centrifuge tube, staining jar, NHS-Acetate powder, NHS-acetate buffer** (provided by NanoString, 4°C), and **2X SSC**.

1. **Prepare 100 mM NHS-acetate mixture** immediately before you are ready to apply the mixture onto the tissue (200 µL/sample). Ensure stock NHS-Acetate has reached room temperature before aliquoting.

   a. NHS-acetate powder is stored desiccated at -20°C. If not already done, prepare 25 mg aliquots (for a four slide preparation) of NHS-acetate powder by weighing out the powder directly into four (4) 2.0 mL centrifuge tubes. Label tubes with the exact weight, seal tubes with parafilm, and store tubes at -20°C in desiccant for later use. See **Prepare RNA Assay Reagents on page 26**.

   NOTE: If preparing only 2 slides, pre-weigh 15 mg aliquots into six (6) total tubes.

   b. **Add NHS-acetate buffer directly to aliquoted powder immediately before applying to the tissue**.

      i. Calculate the amount of NHS-acetate buffer to add to the NHS-acetate powder by multiplying the weight of NHS-acetate powder in mg by 38.5.

         - Example: for 25.0 mg of NHS-acetate powder: 25.0 * 38.5 = 962.5 µL of buffer to add.

   c. Slowly pipette up and down to mix. Bubbles may occur. Do not fully dispense liquid from pipette while mixing.

> ⓘ **IMPORTANT**: Reconstitute NHS-Acetate immediately before use. Do not prepare stock solutions for storage because the NHS ester moiety readily hydrolyzes and becomes non-reactive. Discard any unused reconstituted reagent.

2. Perform the following steps **one slide at a time** to prevent the tissue from drying out:

   - **Remove slide from 1X PBS,** gently tap slide on a clean kimwipe to remove excess buffer, and transfer to a clean staining tray.
   - **Apply 200-250 µL of NHS-Acetate mixture** onto the tissue within the incubation frame. Gently rock the tray side to side as needed to ensure that the NHS-Acetate solution covers

the entire tissue.

- Repeat with remaining slides and **incubate covered in staining tray for 15 minutes at room temperature** (see Figure 57).

Figure 57: Incubate 15 minutes

3. Following incubation, tap off excess liquid and wash slides in **2X SSC for at least 5 minutes**.

Figure 58: Two 5-minute 2X SSC Washes

4. **Repeat 2X SSC wash** for a total of two (2) washes. Slides will stay in second 2X SSC wash while hybridization mix is prepared.

In Situ Hybridization (overnight)

You will need the following materials and reagents for this step: **hybridization oven, hybridization tray, incubation frame covers** (CosMx FF Slide Prep Kit (RNA) Box 1/2, Stored at 4°C), **thermal cycler, ice bucket with ice, Buffer R, Core Probe Panel** (-20°C), **Segmentation Markers** (-20°C)**, RNase Inhibitor** (-20°C)**, custom stand-alone panel and probe add-on** (if applicable, -20°C), and **DEPC-treated water**.

STOP **Do not begin in situ hybridization step until within 16-18 hours of day 2 start time**. If not within that time frame, slides can be stored, protected from light, in 2X SSC wash for up to one (1) hour at room temperature or up to six (6) hours at 4°C.

IMPORTANT: Take care to maintain nuclease-free conditions. 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). Alternatively, mixes can be made in PCR workstations that have been decontaminated with UV light. Gloves should also be changed after handling any probe mixes to avoid cross-contamination.

Prepare buffers: Warm Buffer R to room temperature (RT) before opening.

Thaw RNA detection probes on ice. Before use, mix probes thoroughly by pipetting up and down 3-5 times. **Do not vortex probes**. Once thawed, probes can be refrozen at -20°C up to 5 times or refrigerated at 4°C for up to 6 months.

Set the hybridization oven temperature to 37°C according to product instructions. The hybridization 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. Pre-heat thermal cycler and lid to 95°C.
2. Remove the incubation frame cover and clean with Ethanol. Dry with a clean Kimwipe and visually inspect the cover for dust. Use a new Kimwipe or air blower as needed to remove any dust. Lay incubation frame cover on a clean Kimwipe until use.
3. Flick to mix probes and centrifuge before using. **Do not vortex probes**.
4. **Denature CosMx core probe mix and segmentation markers*** by transferring total

volumes needed for assay (see Table 26), from stock tubes into clean 0.2 mL PCR tubes **(probes and segmentation markers must be kept separate during denaturing**).

Stand-alone custom probe mix and any add-ons must also be denatured, if applicable.

*Segmentation markers are targeting ISH probes and used for upfront imaging. This probe is unique to the mouse neuro assay and is especially important for cell segmentation.

ⓘ **IMPORTANT**: Ensure accurate pipetting. When preparing four (4) slides, there will be no excess RNA probe mix or add-on.

5. **Heat at 95°C for 2 minute**s on a thermal cycler with heated lid. **Immediately transfer to ice for at least 1 minute to crash cool.**

6. Make hybridization solution (see Table 26). Prepare hybridization mix no more than 20 minutes before tissue application.

Table 26: Hybridization Solution (4-slide configuration)

	Denatured RNA Probe Mix	Denatured Add-on * (if applicable)	Segmentation Markers	RNase Inhibitor	Buffer R	DEPC-treated water	Total Volume
2-slide	30 µL	-	12 µL	3 µL	240 µL	15 µL	300 µL
4-slide	60 µL	-	24 µL	6 µL	480 µL	30 µL	600 µL
Custom Panel with custom add-on** (2-slide)	15 µL	15 µL	12 µL	3 µL	240 µL	15 µL	300 µL

	Denatured RNA Probe Mix	Denatured Add-on * (if applicable)	Segmentation Markers	RNase Inhibitor	Buffer R	DEPC-treated water	Total Volume
Custom Panel with add-on** (4-slide)	30 µL	30 µL	24 µL	6 µL	480 µL	30 µL	600 µL

***If an add-on is not being used, use 30 µL DEPC-treated water in leiu of add-on**

**Custom stand-alone panels are supplied by NanoString at 2X concentration compared to off-the-shelf RNA Probe Mix.

7. **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. Change gloves and clean workspace with RNase AWAY.
8. **Wet the Kimwipes with 2X SSC or Deionized (DI) water**. Take care that the Kimwipes and 2X SSC do not contact the slides. Kimwipes should be thoroughly wet, but standing buffer should not be present.
9. To prevent the tissue from drying, perform the following steps **one slide at a time.**
   - Remove slides from 2X SSC, **gently tap slide to remove excess liquid.**
   - Carefully remove the polyester frame (with the center square removed) from the incubation frame to expose the top adhesive layer of the incubation frame (see Figure 59) .

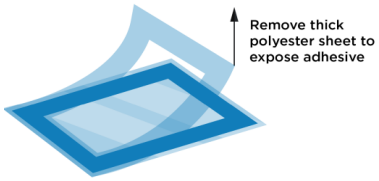

Figure 59: Remove polyester frame

- **Place the slide into the hybridization tray in a horizontal position** (see Figure 60).

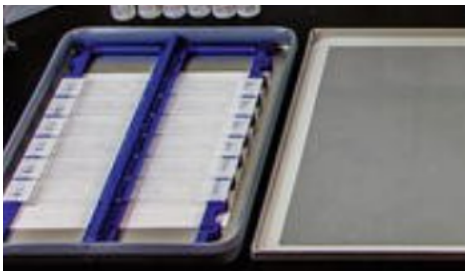

Figure 60: Hybridization Tray

- **Add 150 µL hybridization solution** directly to the tissue within the incubation frame to each slide.

  Start by adding the hybridization solution to bottom most part of the tissue within the frame. Applying the incubation frame cover will help move the hybridization solution across the tissue (see Figure 61).

  Avoid introducing bubbles by leaving a small residual volume in the pipette tip. In the event that a bubble is introduced, carefully aspirate bubble out using low volume pipette tip if possible. When removing air bubbles, removing small amounts of hybridization solution (as long as sufficient solution remains to cover the tissue) is preferable to having bubbles.

- **Carefully apply incubation frame cover** (see Figure 61). Start by setting one edge of the chamber down on the incubation frame edge, then gradually lay down the rest of the cover. Press around the edges of the cover (along the border of the incubation frame) to ensure good adherence. **Do not press the center of the cover as it could damage the tissue.**

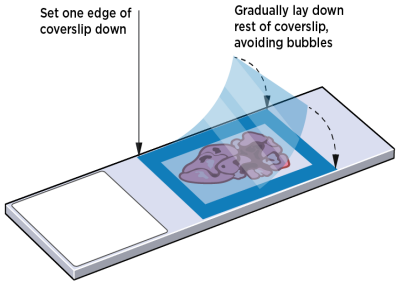

Figure 61: Apply Incubation Frame Cover

- **Repeat step 9 for each slide.**

10. Close hybridization chamber, insert tray into oven, and clamp tray into place. **Incubate at 37°C overnight** (16 – 18 hours) (see Figure 62).

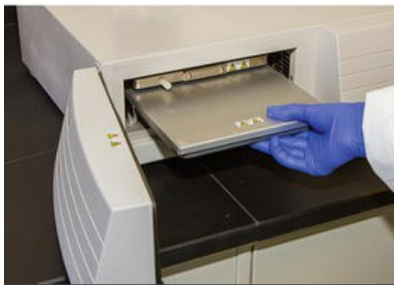

Figure 62: Incubate overnight at 37°C

If your oven does not seal (with a gasket) you may seal your hybridization chamber in a zip-lock bag to simulate a sealed chamber. Chambers sealed in this manner should be tested to ensure they maintain humidity for 24 hours (slides do not dry out) prior to use. Unsealed conditions lead to evaporation of the hybridization solution.

Day 2: Perform Stringent Washes (90 minutes)

You will need the following materials and reagents for this step: **water bath, 4X SSC, 100% formamide,** and **2X SSC** (see Prepare RNA Fresh Frozen Assay Reagents on page 70).

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

1. Before you begin, **set water bath to 37°C**.
2. Remove Nuclear Stain and Supplemental Cell Segmentation Kit (if applicable) from the freezer and thaw on ice.
3. **Warm 100% formamide to room temperature for at least 30 minutes before opening**. Once formamide is at room temperature, **prepare stringent wash directly in staining jars by mixing equal parts 4X SSC and 100% formamide**.

NOTE: Ensure you have sufficient buffer to cover all slides in container for the washes. Wash buffers need to completely cover the tissue on the slide but should be below the slide label. The washes may make the slide labels illegible if submerged.

4. **Preheat staining jars containing freshly prepared stringent wash in 37°C water bath**. It will take about 30 minutes to preheat wash.
5. While jars are preheating, **transfer slides to 2X SSC** and perform the following steps **one slide at a time** to prevent the tissue from drying. Dip slide back into 2X SSC as needed to avoid tissue drying.
   - With a clean pair of forceps, carefully remove the incubation frame cover from the incubation frame. Dip slide back into 2X SSC as needed to avoid tissue drying. If cover will not come off without removing incubation frame, remove the frame and cover, the frame can be reapplied in a later step.
   - Repeat for all slides, cleaning the forceps with Ethanol between slides as needed.
6. Once both jars have pre-heated to 37°C and all incubation frame covers have been removed, perform the washes detailed below (see Figure 63). After the last wash, the slides can be stored in 2X SSC for up to one hour.
   - Gently tap each slide, on at a time, on a clean Kimwipe to remove excess 2X SSC and place slides in the **first stringent wash and wash for 25 minutes**. **Repeat** with the second stringent wash.

- During the second stringent wash, begin preparing reagents for **Nuclear and Cell Segmentation Staining on page 94**.
- Following stringent washes, immediately **transfer slides to 2X SSC and wash for 2 minutes**. **Repeat** with second jar of 2X SSC. Leave slides in 2X SSC as needed until reagents have been prepared for nuclear and cell segmentation staining.

Figure 63: Perform stringent wash

IMPORTANT: Anything coming into contact with hybridization solution (which contains probes), such as containers for stringent wash solution and 2X SSC, needs to be exclusive for this purpose and thoroughly washed and cleaned with RNase AWAY, as probes may contaminate later runs. Use separate staining jars for different probe mixes. Staining jars should be cleaned with RNase AWAY before use.

Nuclear and Cell Segmentation Staining

You will need the following materials and reagents for this step: **incubation frames, staining jars, 1X PBS, Blocking Buffer** (4°C)**, DAPI Nuclear Stain Stock** (-80°C), and **supplemental markers** (if applicable, -80°C) (see Prepare RNA Assay Reagents on page 70).

1. **Prepare the following reagents**:
   - Four (4) staining jars of 1X PBS
2. **Prepare 220 µL of Nuclear Stain Buffer per slide.**
   - **Vortex, then centrifuge thawed nuclear stain** 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*.
   - Dilute the nuclear stain stock 1:40 (where *n* equals the number of slides) (see Table 27).

Table 27: Dilute nuclear stain

Nuclear Stock	Blocking Buffer	Total Volume
5.5 µL x *n*	214.5 µL x *n*	220 µL x *n*

3. To prevent the tissue from drying, perform the following steps **one slide at a time**.
   - **Remove slide from 2X SSC**, gently tap slide on a clean Kimwipe. Using a clean Kimwipe, **dry the surface of the slide that will come into contact with the incubation frame**.

   IMPORTANT: Avoid wiping the slide within the scan area as this could remove the fiducials needed for imaging. See scan area template on the flow cell assembly tool or follow the guidelines in Prepare Tissue Samples on page 68.

   - If needed, carefully reapply the incubation frame following the instructions in Tissue Permeabilization (40 minutes) on page 78. Ensure that the frame is well adhered to the slide by gently pressing around the frame with clean forceps.
   - Using a clean Kimwipe, carefully wick excess buffer from around the incubation frame as needed. Careful to not touch the area inside of the incubation frame.
   - Lay slide horizontally in staining tray and **slowly apply up to 200 µL of nuclear stain buffer** directly to tissue. Gently move tray side to side as needed to ensure that the DAPI Buffer covers the entire tissue.

- **Repeat** with remaining slides and cover tray.

4. **Incubate slides for 15 minutes at room temperature protected from light** (see Figure 64)**.**

Figure 64: Cover tray and incubate for 15 minutes

5. During nuclear stain incubation, **prepare supplemental marker mix** (if applicable) using the following table where *n* = the number of slides (see Table 28). **Flick each tube to mix and centrifuge before use**.

IMPORTANT: Ensure accurate pipetting. When preparing four (4) slides, there will be no excess supplemental marker mixes.

Table 28: Prepare Supplemental Marker Mix

Supplemental Marker Mix 1 (Mouse GFAP)	Supplemental Marker Mix 2 (Mm Neuro Histone)	Blocking Buffer	Total Volume
8 µL x *n*	8 µL x *n*	184 µL x *n*	200 µL x *n*

*If not adding the optional GFAP or Histone supplemental markers, add Blocking Buffer in lieu of marker.

6. After DAPI buffer incubation, **remove slides one at a time** from staining tray, gently tap slide on a clean Kimwipe to remove excess buffer, and **transfer slide to 1X PBS**.
7. Wash slide for **5 minutes** in 1X PBS.
8. During PBS wash, add 2X SSC or deionzied (DI) water to the staining tray. Do not overfill. The water level should be well below the slides to avoid cross-contamination.
9. Following PBS wash, perform the following steps **one slide at a time** to prevent tissue drying:
   - **Remove slide from 1X PBS** and gently tap slide on a clean Kimwipe to remove excess PBS.

- Lay slide horizontally in staining tray and **apply up to 200 µL of supplemental marker mix** directly to tissue. Gently move tray side to side as needed to ensure that the mix covers the entire tissue.
- If needed, an incubation frame cover can be placed over the incubation frame to ensure that the mix completely covers the tissue.
- Adjust volume to add as needed for tissues of varying sizes. The **supplemental marker mix needs to completely cover the tissue** but does not need to completely fill the incubation frame.

10. **Repeat** with remaining slides and cover tray (see Figure 65).

Figure 65: Cover tray and incubate for 1 hour

11. **Incubate slides for one (1) hour at room temperature** protected from light.
12. If new aliquots need to be made, **remove NHS-acetate from -20°C** and leave at room temperature during marker incubation.
13. Following incubation, **transfer slides to 1X PBS and wash for 5 minutes** (see Figure 66).

Figure 66: Wash 3X in 1X PBS

14. **Repeat two (2) times** for a total of three (3) PBS washes.

Continue to Second NHS-Acetate Preparation and Application (25 minutes).

Second NHS-Acetate Preparation and Application (25 minutes)

You will need the following materials and reagents for this step: **analytical scale, 2.0 mL centrifuge tube, staining jar, NHS-Acetate powder, NHS-acetate buffer** (provided by NanoString, 4°C), and **2X SSC**.

1. **Prepare 100 mM NHS-acetate mixture** immediately before you are ready to apply the mixture onto the tissue (200 µL/sample). Ensure stock NHS-Acetate has reached room temperature before aliquoting.
   a. NHS-acetate powder is stored desiccated at -20°C. If not already done, prepare 25 mg aliquots of NHS-acetate powder by weighing out the powder directly into four (4) 2.0 mL centrifuge tubes. Label tubes with the exact weight, seal tubes with parafilm, and store tubes at -20°C in desiccant for later use. See **Prepare RNA Assay Reagents on page 70**.
   NOTE: If preparing only 2 slides, pre-weigh 15 mg aliquots into six (6) total tubes.
   b. **Add NHS-acetate buffer directly to the aliquoted powder immediately before applying to the tissue**.
      i. Calculate the amount of NHS-acetate buffer to add to the NHS-acetate powder by multiplying the weight of NHS-acetate powder in mg by 38.5.
         - Example: for 25.0 mg of NHS-acetate powder: 25.0 * 38.5 = 962.5 µL of buffer to add.
   c. Slowly pipette up and down to mix. Bubbles may occur. Do not fully aspirate liquid from pipette while mixing.

> ⓘ **IMPORTANT**: Reconstitute NHS-Acetate immediately before use. Do not prepare stock solutions for storage because the NHS ester moiety readily hydrolyzes and becomes non-reactive. Discard any unused reconstituted reagent.

2. Perform the following steps **one slide at a time** to prevent the tissue from drying out:
   - **Remove slide from 1X PBS** and transfer to a clean staining tray.
   - **Apply 200-250 µL of NHS-acetate mixture** onto tissue within the incubation frame. Gently move tray side to side as needed to ensure that the NHS-acetate solution covers the entire tissue.

- Repeat with remaining slides and **incubate covered in staining tray for 15 minutes at room temperature** (see Figure 67).

Figure 67: Incubate 15 minutes

3. Following incubation, tap off excess liquid and wash slides in **2X SSC for at least 5 minutes**.

Figure 68: Two 5-minute 2X SSC Washes

4. **Repeat 2X SSC wash** for a total of two (2) washes. Slides will stay in second 2X SSC wash while hybridization mix is prepared.
   - If samples need to be stored overnight and loaded onto the instrument the next day, remove the incubation frame following the guidelines from Day 2: Perform Stringent Washes (90 minutes). Ensure the entire incubation frame is removed then store according to Safe Storage Guidelines for RNA Slides.
   - If samples will be loaded onto the instrument the same day, continue to Flow Cell Assembly.

Safe Storage Guidelines for RNA Slides

After processing, slides must never be stored dry. Slides may be stored for up to six (6) hours protected from light and submerged in 2X SSC at room temperature.

Slides can be stored protected from light and submerged in 2X SSC at 4°C overnight, if needed. Slides may be stored longer than that, but RNA counts and staining efficiency will decrease as a function of days stored. For best results, minimize storage time between slide preparation and loading on the CosMx SMI instrument.

Slides must be stored in the dark (avoiding light is crucial as fiducials are sensitive to photobleaching).

Protein FFPE Manual Slide Preparation

CosMx SMI Slide Preparation Workflow

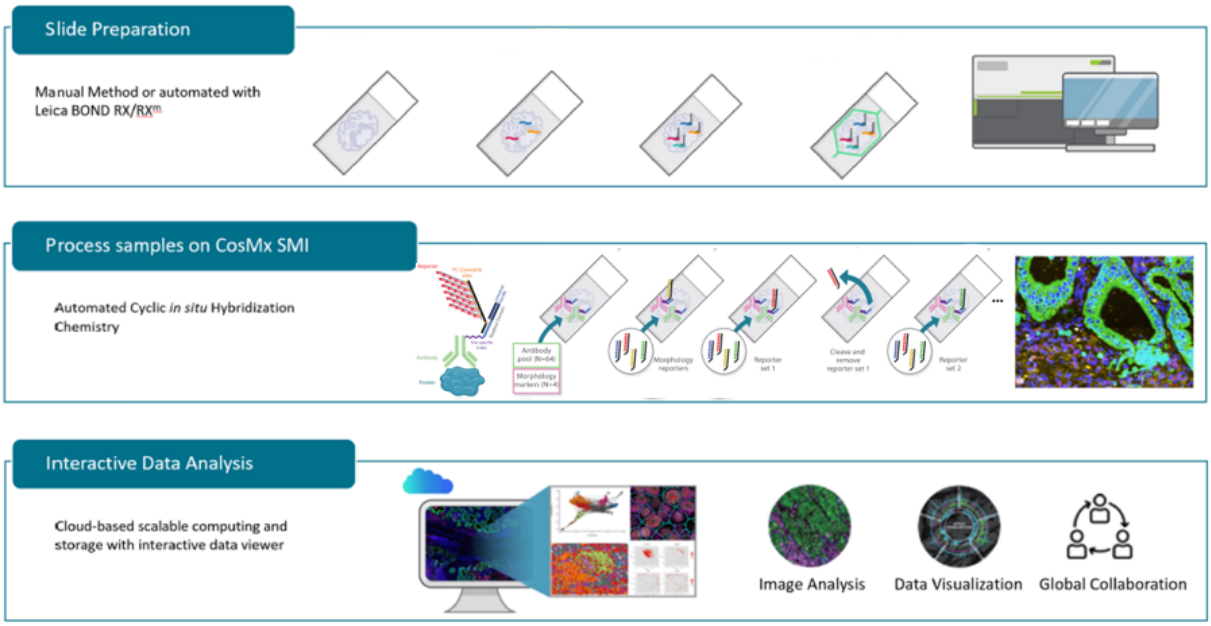

Figure 69: CosMx SMI Workflow Overview

Day 1: Slide Preparation. Deparaffinize, retrieve antigens, and block tissue sample. Slide preparation can be done manually or automatically with the BOND RX/RX^m IHC/ISH stainer (Leica Biosystems). Incubate samples with antibody mix and cell segmentation antibodies overnight.

Day 2: Process Slides on CosMx SMI. Apply fiducials and nuclear stain and assemble the flow cells. Load prepared flow cells into the CosMx SMI instrument and enter flow cell/study information. Tissue is scanned to capture protein expression and morphology imaging within user-designated fields of view (FOVs).

After run completion: Create a **Data Analysis** study in the AtoMx Spatial Informatics Platform (SIP). Perform quality-control checks, data analysis, and generate analysis plots.

Equipment, Materials, and Reagents

The following equipment (see Table 29), materials (see Table 30), and reagents (see Table 31) are required for this protocol but are not supplied by NanoString.

Equipment:

Table 29: Equipment not provided by Nanostring

	Equipment	Source	Part Number(s)
☐	**Baking Oven**	Quincy Lab, Inc (or comparable)	Various GC Models
☐	**6-quart Pressure Cooker** NOTE: Pressure Cooker must have a built in temperature gauge for validation NOTE: Products from other vendors may require testing and optimization	BioSB TintoRetriever	BSB-7087
☐	**Ultrasonic Bath** (500 mL capacity) NOTE: 400kHz frequency with timer	General Lab Supplier	Example
☐	**Vortex Mixer**	General Lab Supplier	Various
☐	**Micro Centrifuge** for 1.5 mL microcentrifuge tubes	General Lab Supplier	Various
☐	**Analytic Scale with draft shield** NOTE: ensure scale can weigh in milligrams (mg) for accuracy.	Various	Example

Materials:

Table 30: Materials not provided by Nanostring Technologies Inc.

	Materials	Source	Part Number (s)
☐	**Pipettes for 2 – 1,000 µL**	General Lab Supplier	Various
☐	**Filter Tips (RNase/DNase Free)**	General Lab Supplier	Various
☐	**2.0 mL Centrifuge Tubes (RNase/DNase Free)**	General Lab Supplier	Various
☐	**5.0 mL tube (RNase/DNase Free)**	General Lab Supplier	Various
☐	**VWR Superfrost Plus Micro Slide, Premium or** **Leica BOND PLUS slides** NOTE: these slides have been validated by NanoString. Do not use other products.	VWR Leica Biosystems	48311-703 S21.2113.A
☐	**Slide Rack**	General Lab Supplier	Example
☐	**Polypropylene Slide Staining Jars** (24 required) or **Slide Staining Station** **NOTE:** due to the photo-sensitivity of this assay following fiducial application, the staining jars should be impermeable to light.	Ted Pella (or comparable) Amazon Fisher Scientific	21029 MH-SJ6302 NC1862866
☐	**Humidity Chamber** (staining tray)	Simport	M920-2
☐	**Forceps (for slide handling)**	General Lab Supplier	Various
☐	**Razor Blades**	General Lab Supplier	Various

	Materials	Source	Part Number (s)
☐	**Timer**	General Lab Supplier	Various
☐	**KimWipes**	General Lab Supplier	Various

Reagents:

Table 31: Reagents not provided by Nanostring Technologies Inc.

	Reagent	Source/Part Number (s)	Storage Conditions
☐	**DEPC-treated Water**	ThermoFisher, AM9922 (or comparable)	Room temperature
☐	**100% Ethanol (EtOH): ACS grade or Better**	General Lab Supplier	Flammable Storage Room temperature
☐	**10X Phosphate Buffered Saline pH 7.4 (PBS)**	ThermoFisher, AM9625 (or comparable)	Room temperature
☐	**CitriSolv**	VWR, 1601/89426-268	Flammable Storage Room temperature
☐	**TBS with Tween (TBS-T), 20X**	ThermoFisher, J77500.K2	Room temperature
☐	**Sulfo NHS-acetate powder** NOTE: NHS-Acetate powder is shipped in a plastic bag with a desiccant and should be left in the bag and stored at -20°C until ready to use.	Fisher Scientific, 26777	-20°C

	Reagent	Source/Part Number (s)	Storage Conditions
☐	**Paraformaldehyde (PFA)** **16% Aqueous Solution** 	EMS,15710	-20°C

NanoString Supplied Reagents

CosMx SMI Protein Slide Preparation Kit

Figure 70: FFPE Protein Slide Preparation Kit

Table 32: CosMx FFPE Slide Prep Kit (Protein) Box 1/2

Kit Contents (Store at 4°C)	
NHS-Acetate Buffer	Buffer W (not pictured)
10X Target Retrieval Solution	CosMx Fiducials
Incubation Frames	Incubation Frame Covers

CosMx Protein Human Immuno-Oncology Panel (see Panel and Cell Segmentation Marker Selection on page 11).

Figure 71: CosMx Human Immuno-Oncology Panel (Protein)

Table 33: CosMx Human Immuno-Oncology Panel

Kit Contents (Store at -80°C)	
CosMx Human Immuno-Oncology Panel	Custom Protein Add-On Probes (if applicable)

CosMx Segmentation and Supplemental Markers

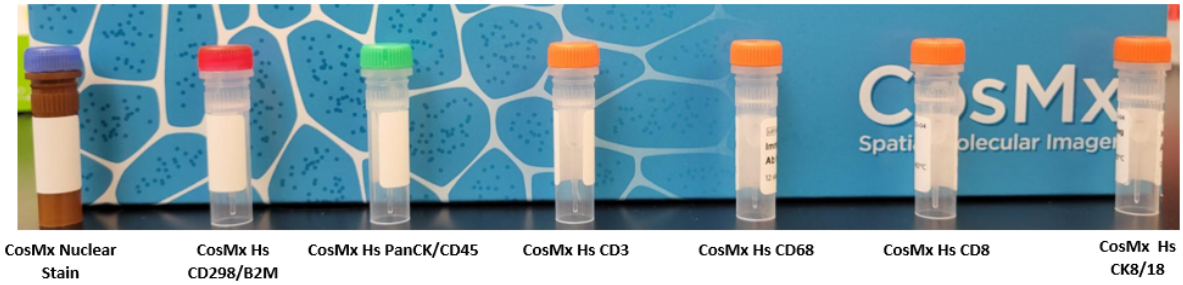

Figure 72: CosMx Segmentation and Suuplemental Markers (Protein FFPE)

Table 34:CosMx Segmentation and Suuplemental Markers (Protein)

Kit Contents (Store at -80°C) Each kit is sufficient for four (4) slides	
Kit Name	**Kit Component**
CosMx™ Human Universal Cell Segmentation Kit (Protein), Ch 1/2	CosMx DAPI Nuclear Stain CosMx Hs CD298/B2M Marker Mix Ch2 (Protein)
CosMx™ Human IO PanCK/CD45 Supplemental Segmentation Kit (Protein), Ch 3/4, if applicable	CosMx Hs PanCK/CD45 Marker Mix Ch3/4 (Protein)
CosMx™ Human CD3 A La Carte Marker (Protein), Ch 5, if applicable	CosMx Hs CD3 Marker Ch5 (Protein)
CosMx™ Human CD68 A La Carte Marker (Protein), Ch 5, if applicable	CosMx Hs CD68 Marker Ch5 (Protein)
CosMx™ Human CD8 A La Carte Marker (Protein), Ch 5, if applicable	CosMx Hs CD8 Marker Ch5 (Protein)
CosMx™ Human Cytokeratin 8/18 A La Carte Marker (Protein), Ch 5, if applicable	CosMx Hs CK 8/18 Marker Ch5 (Protein)

Flow-Cell Assembly Kit

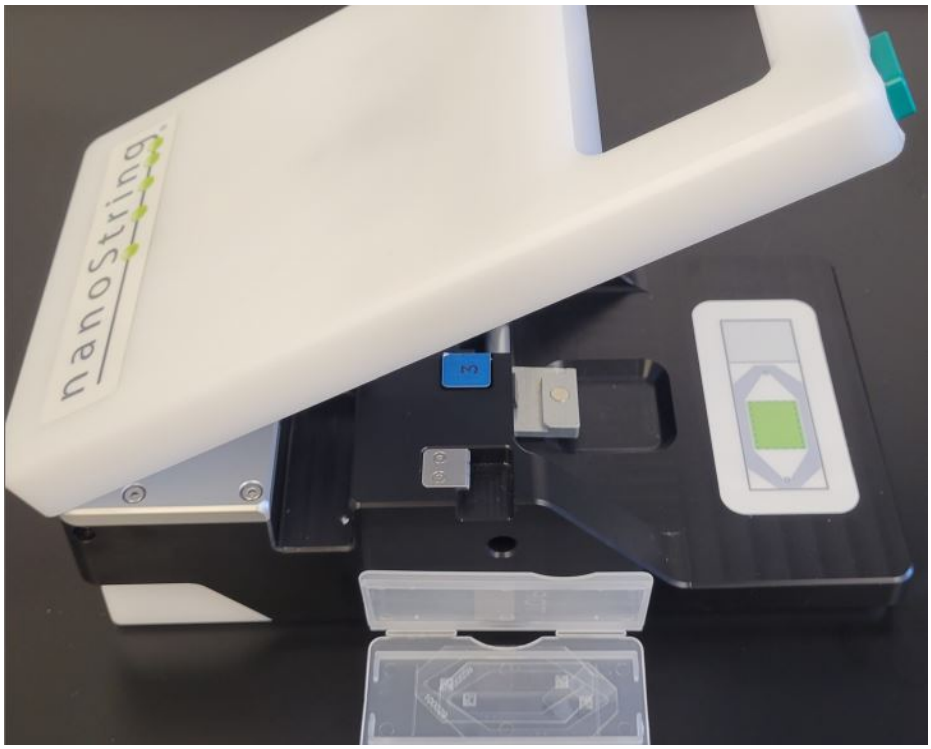

Figure 73: Flow Cell Assembly Kit

Table 35: Flow Cell Assembly Kit

Kit Contents (Store at Room Temperature)	
Flow Cell Assembly Tool	Flow Cell Coverslips

Prepare Tissue Samples

Appendix I: CosMx SMI Sample Preparation Guidelines on page 137 covers FFPE block selection and sectioning in detail. Review these guidelines as needed prior to beginning the FFPE Slide Preparation procedure.

Tissue Sectioning and Slide Preparation:

FFPE blocks should be sectioned at **5-10 µm thickness** and mounted on the label side of VWR Superfrost Plus Slides or Leica BOND PLUS slides.

Tissue sections must be placed within the Scan Area (the green area) of the slide and be no larger than **20 mm Long by 15 mm Wide** (see Figure 74) (image not to scale). For best performance, ensure some glass is visible within the scan area.

Label slides with pencil on the frosted label according to lab guidelines. If using an adhesive slide label, ensure the label is less than 295 µm thick and is not folded over on itself. Labels over the maximum thickness or labels that are not properly adhered may result is slide or flow cell damage during flow cell assembly and/or instrument loading.

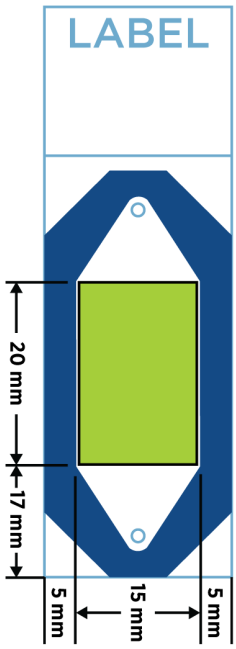

Figure 74: Tissue Scan Area

Dry tissue at room temperature overnight. Tissues can be baked overnight at 37°C to improved tissue adherence.

If sections are larger than the indicated size and/or placed off-center, continue with the slide preparation as outlined. Immediately before applying the incubation frame, scrape off parts of the tissue exceeding the scan area. Scraping off tissue before target retrieval and ethanol drying could generate tissue folds that may result in staining and/or binding artifacts, while suboptimal scraping may result in poor incubation frame adherence. Applying the incubation frame over tissue could result in tissue damage when the incubation frame is removed.

ⓘ IMPORTANT: The CosMx SMI instrument will only image the area inside the flow cell

chamber, the tissue scan area. If the tissue section is outside of the scan area, it will not be imaged.

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 in a desiccator at room temperature or at 4°C prior to processing.

Prepare Assay Reagents

Prepare the reagents using the instructions in the following table (see Table 36).

NOTE: Unless otherwise noted, reagents can be made up to two (2) weeks in advance and stored at room temperature.

Table 36: Prepare protein assay reagents

	Reagent	Dilution	Storage
☐	1X Tris-Buffered Saline with Tween 20 (TBS-T)	Dilute 100 mL of 10X TBS-T in 900mL of DEPC-treated water.	Room Temperature
☐	1X PBS	Prepare 1L of 1X PBS by diluting 100 mL of 10X PBS into 900 mL of DEPC-treated water.	Room Temperature
☐	NHS-Acetate Mix	Individual aliquots can be prepared prior to slide preparation but should be kept at -20°C with a desiccant until use. **To prepare aliquots:** • Bring stock to room temperature for 1-2 hours prior to opening. • Prelabel four (4) screw top 1.5 mL centrifuge tubes with the required information. • Using a weighing spatula, carefully weigh 20-25mg of NHS Acetate directly into the screw-top tube on an analytic scale. • Label tube with final weight, close tube, seal the tube with parafilm, and return to -	-20°C in desiccant

	Reagent	Dilution	Storage
		20°C until use.	
☐	1X Target Retrieval Solution	Dilute 25 mL of 10X Target Retrieval Solution into 225 mL of DEPC-treated water. Must be prepared on the day of slide preparation. Do not prepare ahead of time.	Make fresh daily
☐	95% Ethanol (EtOH)	Prepare 500 mL of 95% ethanol by adding 25 mL of DEPC-treated water to 475 mL 100% Ethanol. Prepare fresh on the day of slide preparation.	Make fresh daily
☐	70% Ethanol (EtOH)	Prepare 500 mL of 70% ethanol by adding 150 mL of DEPC-treated water to 350 mL 100% Ethanol. Prepare fresh on the day of slide preparation.	Make fresh daily
☐	Segmentation Markers and Target Antibodies	These reagents have additional steps that will be covered in detail in their respective sections. Remove these reagents from storage and keep on ice until ready to use.	n/a
☐	Day 2 Reagents	These reagents have additional steps that will be covered in detail in their prospective steps. See Day 2: Post-Fixation (50 minutes)	n/a

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

	Reagent	Dilution	Storage
		on page 123 for preparation of these reagents.	
☐	4% Paraformaldehyde (PFA)	Prepare 1 mL of 4% PFA by adding 250 µL of 16% PFA to 750 µL of DEPC-treated water.	Prepare Fresh

Day 1: Deparaffinize and Rehydrate FFPE Tissue Sections (45 minutes)

You will need the following materials and reagents for this step: **staining jars, humidity (staining) tray, pressure cooker, 1X Target Retrieval Solution, Citrisolv, 100% EtOH, 95% EtOH, 70% EtOH**, and **1X PBS**. See Prepare Assay Reagents on page 111 for more details.

Prepare Equipment

1. **Bake sections on slides** in a 65°C drying oven for **one (1) hour**.
   - Slides should be baked vertically in a slide rack.
   - Slides can be baked for up to three (3) hours to improve tissue adherence.
2. While slides are baking, **fill the pressure cooker with deionized water** to the correct level per the manufacturer's instructions (4-8 cups depending on model used).
3. **Place the staining jar containing freshly prepared 1X Target Retrieval Solution into the pressure cooker to preheat** (see Figure 75). Ensure that water level is well below lid of jar, about halfway up jar will be sufficient. If needed, a trivet may be used to raise the jar.

Figure 75: Preheat Target Retrieval Buffer

4. Place a lid on the staining jar to prevent evaporation. To prevent pressure from building within the staining jar, **do not fully seal the lid to the jar**.
5. **Prime the pressure cooker** up to 100° C (see Figure 76) for Perform Antigen Retrieval (1 hour) on page 116.

Figure 76: Prime the pressure cooker up to 100° C

6. **Prepare the humidity chamber (staining tray) according to product instructions**. If your chamber is light-permeable, minimize light exposure (e.g., by wrapping the lid in aluminum foil).

Deparaffinize and rehydrate FFPE tissue sections.

After slides have baked for at least 1 hour, gently perform the following washes (see Figure 77) using staining jars. Slides should be dipped up and down gently several times when placing in and before removing from staining jars.

NOTE: Ensure you have sufficient buffer to cover all slides in container for the washes. Wash buffers need to completely cover the tissue on the slide but should be below the slide label. The washes may make the slide labels illegible if submerged.

Figure 77: Deparaffinize and Rehydrate FFPE tissue sections

⚠ WARNING: Ethanol is a Flammable chemical and should be handled appropriately. Waste generated in these steps needs to be disposed of as Flammable Hazardous Waste.

Perform Antigen Retrieval (1 hour)

You will need the following materials and reagents for this step: **staining jars, pressure cooker,** and **1X PBS**. See Prepare Assay Reagents on page 111 for more details.

1. Following the second PBS wash, and once the pressure cooker and target retrieval solution have preheated, press cancel on the pressure cooker and release the pressure valve (if closed).
2. **Carefully remove the pre-heated target retrieval solution** and add the slides to the pre-heated solution. When closing the jar, **do not fully seal the lid**. This will prevent pressure from building within the jar and potentially damaging the slides.

⚠ WARNING: When starting to remove pressure cooker lid, hot steam will be released. Staining jar will also be VERY hot. Wear protective heat resistant gloves to open lid and when removing the staining jar.

3. Carefully place the staining jar containing the slides back into the preheated pressure cooker (see Figure 78). Ensure that water level is well below lid of jar. About halfway up jar will be sufficient. If needed, a trivet may be used to raise the jar.

Figure 78: Antigen Retrieval

4. Attach the pressure cooker lid and **close the pressure valve.** Return the pressure cooker to 100°C (see Figure 76).
5. **Once the pressure cooker temperature returns to 100°C**, start timer and run for **15 minutes**.
6. When the timer reaches zero, press cancel on the pressure cooker to stop heating, release the pressure valve to release pressure. **Carefully and slowly remove the pressure cooker lid and remove the staining jar.**

7. Leave the staining jar containing sample slides at **room temperature for 25 minutes** (max one hour) (see Figure 79).

Figure 79: Stand at Room Temperature.

8. Once the slides have equilibriated to room temperature, transfer the slides to 1X PBS and **wash the slides in 1X PBS** for **5 minutes** (see Figure 80).

Figure 80: Wash 3X in 1X PBS

9. **Repeat wash two (2) times** for a total of three (3) washes.
10. Immediately proceed to next step (Blocking (1 hour) on page 118).

Blocking (1 hour)

You will need the following materials and reagents for this step: **incubation frames, humidity chamber**, and **Buffer W**. See Prepare Assay Reagents on page 111 for more details.

1. **Fill the humidity chamber** with enough water to cover the bottom of the trough. Do not overfill as splashing while moving the tray chamber should be avoided.
2. Prepare the Incubation Frame
   - Separate an individual frame from the strip by tearing along the perforations. Each frame is sandwiched between a thin polyester sheet and a thick polyester sheet (with the center square removed).
3. Trim the tissue following the template in Prepare Tissue Samples on page 109.
4. Using an absorbent wipe, ensure that the surface of the slide that will come in contact with the incubation frame is **dry and clean.**
5. Apply the incubation frame (see Figure 81).
   - Carefully **remove the thin polyester sheet**, ensuring that the frame remains bound to the thick polyester sheet (with the center square removed).
   - With the slide on a flat surface, **carefully place the incubation frame** around each tissue section careful not to touch the adhesive surface. Lightly press along the border of the incubation frame to ensure that it is well adhered to the slide. Be careful to avoid applying any pressure in the center of the frame so as not to damage the tissue.

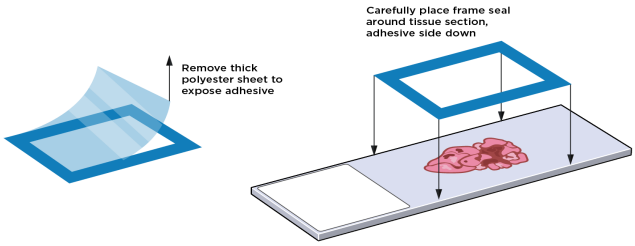

Figure 81: Apply Incubation Frame

6. Place the slides in the humidity chamber in a horizontal position. Add **200 µL Buffer W** directly to the tissue within the incubation frame and cover the humidity chamber (see Figure 82).

Figure 82: Incubate for 1 hour

7. **Incubate in Buffer W for 1 hour** at room temperature (RT) in a closed humidity chamber.
8. Remove Protein Panel, and cell segmentation kits, and any supplemental markers (if applicable) from freezer and **thaw mix on ice.**

Primary Antibody Incubation (overnight)

You will need the following materials and reagents for this step: **humidity chamber, protein antibody mix, Cell Segmentation Markers, Supplemental Markers** (if applicable), and **Buffer W**. See Prepare Assay Reagents on page 111 for more information.

Do not begin in situ hybridization step until within 16-18 hours of day 2 start time. If not within that time frame, slides can be stored, protected from light, in 2X SSC wash for up to one (1) hour at room temperature or up to six (6) hours at 4°C.

1. **Mix** the protein probe (antibody) mix by flicking the tube and spin down using the micro-centrifuge. **Do not vortex**.

   Each tube of protein probe mix contains sufficient material for four (4) slides. If you are using the entire mix in one week, store at 4°C. If not, **aliquot the protein antibody mix** and refreeze unused aliquots at -80°C. Do not exceed more than 2 freeze / thaw cycles with the protein antibody mix and do not freeze diluted mix.

2. Make a working antibody solution by diluting protein probe mix and Cell Segmentation Markers, and any supplemental or à la carte markers (if applicable) into Buffer W (n = number of slides) (see Table 37). Adjust volumes to cover the number of slides to be prepared (125 µL per slide).

   - Antibody mix: 1:2 dilution for each
   - Cell Segmentation and supplemental markers: 1:25 dilution for each marker.

Table 37: Antibody Solution Calculations

Protein Antibody Mix	CD298 / B2M Segmentation Marker	*PanCK / CD45 Marker (optional)*	*A la carte marker (optional)*	Buffer W (µL)	Total Volume (µL)
62.5 µL x *n*	5 µL x *n*	5 µL x *n*	5 µL x *n*	47.5 µL x *n*	125 µL x *n*

* If optional markers are not used, adjust Buffer W volume accordingly to bring total volume to 125 µL/slide.

To prevent the tissue from drying, perform the following steps **one slide at a time.**

3. Remove slides from the humidity chamber, **gently tap slide on a clean Kimwipe to remove excess Buffer W.**
4. Carefully remove the thick polyester frame (with the center square removed) from the incubation frame to expose the top adhesive layer of the incubation frame (see Figure 83).

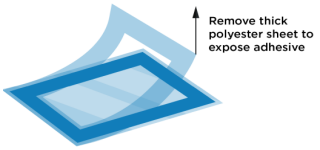

Figure 83: Remove polyester frame

5. **Place the slide into the humidity chamber in a horizontal position.**
6. **Add 125 µL of the diluted antibody solution** directly to the tissue within the incubation frame.

   Start by adding the antibody solution to the edge of the tissue within the frame. Applying the incubation frame cover will help move the antibody solution across the tissue (see Figure 84).

   Avoid introducing bubbles by leaving a small residual volume in the pipette tip. In the event that a bubble is introduced, carefully aspirate bubble out using low volume pipette tip if possible. When removing air bubbles, removing small amounts of antibody solution (as long as sufficient solution remains to cover the tissue) is preferable to having bubbles.

7. **Carefully apply incubation frame cover** (see Figure 84). Start by setting one edge of the cover down on the incubation frame edge, then gradually lay down the rest of the cover. Press around the edges of the cover (along the border of the incubation frame) to ensure good adherence. **Do not press the center of the cover as it could damage the tissue.**

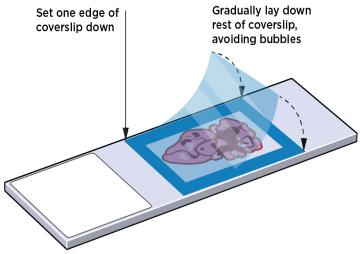

Figure 84: Apply Incubation Frame Cover

8. **Repeat step 3-7 for each slide.**

9. Transfer the humidity chamber to the 4°C refrigerator and incubate overnight (16-18 hours). Minimize exposure to light and ensure the humidity chamber stays level to avoid losing antibody solution **(see Figure 85)**.

Figure 85: Incubate overnight at 4°C

Day 2: Post-Fixation (50 minutes)

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

IMPORTANT: When tapping off slides, use a clean disposable surface such as a new Kimwipe to avoid contamination.

You will need the following materials and reagents for this step: **staining jars, 1X TBS-T, ultrasonic bath, vortex, fiducials** (at RT), **1X PBS** and **4% PFA**. See Prepare Assay Reagents on page 111 for more details.

1. **Gently tap off each slide** on a fresh, clean, disposable surface (e.g., Kimwipes) to remove excess solution.
2. Using clean forceps, **carefully remove the incubation frame cover from the from the incubation frame**. Dip slide into 1X TBS-T as needed to avoid tissue drying. If cover will not come off without removing incubation frame, remove the frame and cover, the frame can be reapplied in a later step.
3. Place the slides into the staining jar containing 1X TBS-T and wash for 15 minutes.

Figure 86: Wash 3X in TBS-T

4. During first wash, remove fiducials from 4°C storage and bring to room temperature for 10 minutes.
5. If not already done, remove the stock PFA from the -20°C and dilute to the 4% working concentration. See Prepare Assay Reagents on page 111.
6. **Repeat TBS-T wash two (2) times** for a total of three (3) TBS-T washes (see Figure 86).
7. During the final washing step, **prepare fiducials** (Fiducial Prep and Application (20 minutes) on page 124).

Fiducial Prep and Application (20 minutes)

You will need the following materials and reagents for this step: **ultrasonic bath, vortex, 1X TBS-T, fiducials** (at room temperature), **staining jars**, and **1X PBS.**

Prepare fiducials for use following the below steps **(see Figure 87)**:

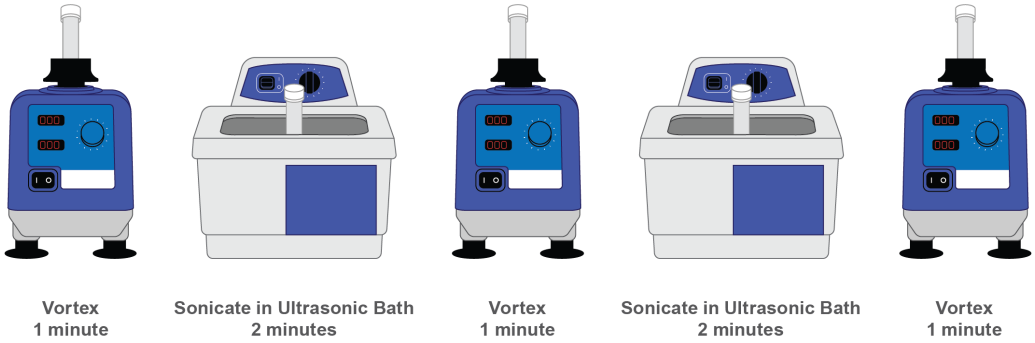

Figure 87: Prepare Fiducials

NOTE: Failure to follow these steps will result in fiducial clumping and uneven distribution of fiducials within the tissue. This uneven distribution can result in a loss of readable area or loss of image registration.

1. Once fiducials are prepared, **dilute fiducial stock (0.1%) to the working concentration (0.00005%) in 1X TBS-T.** A 2-step serial dilution is recommended for fiducial preparation. Accurate dilution of fiducialsis critical for proper assay performance.
   - Dilute stock to 0.01% by adding **10 µL of the fiducial stock to 90 µL of 1X TBS-T**. Label tube as *Dilution 1 (D1)*.
   - Cover *Dilution 1* and **leave at room temperature for 10 minutes** protected from light.
   - After 10 minutes, vortex and quick spin *Dilution 1* and dilute to the final working concentration (0.00005%) using the following table **(see Table 38)**.

Table 38: Fiducial Final Dilution

Number of Slides	Dilution 1 (D1) (0.01% Fiducials)	2X SSC-T	Final Volume
2-slides	2.5 µL	497.5 µL	500 µL

Number of Slides	Dilution 1 (D1) (0.01% Fiducials)	2X SSC-T	Final Volume
4-slides	5 µL	995 µL	1000 µL

2. After final 1X TBS-T wash, **transfer slides to 1X PBS and wash for two (2) minutes**.

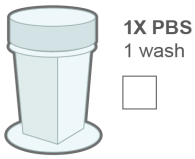

Figure 88: Wash for 2 minutes in 1X PBS

3. **Remove the slides one at a time** from 1X PBS and carefully **dry the slide around the tissue using a clean Kimwipe**. Ensure that the surface of the slide that will come into contact with the incubation frame is clean and dry while being careful to not touch the tissue.
4. If needed, carefully reapply the incubation frame following the instructions in **Blocking (1 hour) on page 118**. Ensure that the frame is well adhered to the slide by gently pressing around the frame with clean forceps.
5. Lay the slides horizontally in the staining tray.
6. **Immediately before applying fiducials to tissue, vortex for one (1) minute.** Vortex fiducials for 30 seconds between slides to keep fiducials in suspension and to ensure consistent concentration across all slides.
7. Apply up to 200 µL of **the final fiducial solution, ensuring the solution covers glass and tissue within the incubation frame**. Fiducials must be present on the glass within the scan area for consistent focusing during the instrument run.
8. **Incubate slides in humidity tray for 5 minutes at room temperature. Ensure that fiducial solutions completely covers the entire area within the incubation frame.**

ⓘ **IMPORTANT**: This step and all steps moving forward are light sensitive.

9. Following fiducial incubation, **transfer slides to fresh 1X PBS.** Wash slides for five (5) minutes in the 1X PBS.

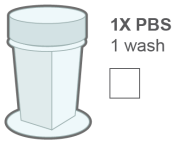

Figure 89: Wash 5 minutes in 1X PBS

10. After PBS wash, transfer slides into the humidity chamber and add **up to 200 µL 4% PFA directly to tissue. Incubate covered for 30 minutes** in the humidity chamber at room temperature **(see Figure 90)**.

Figure 90: Incubate 30 minutes

11. After PFA incubation, wash with **3 changes of 1X PBS for 5 minutes each** **(see Figure 91)****.**

Figure 91: Wash 3X with 1X PBS

12. During PBS washes, **remove DAPI stain and NHS Acetate from the freezer and bring to room temperature**.

Nuclear Staining (20 minutes)

You will need the following materials and reagents for this step: **humidity chamber, staining jars, Nuclear Stain,** and **1X PBS**. See Prepare Assay Reagents on page 111 for more details.

1. If you have not already, **remove Nuclear Stain** from the freezer (stored at -80°C) and **warm to room temperature on bench** for at least 10 minutes before use.
2. **Prepare 220 µL of Nuclear Stain Buffer per slide.**
   - **Vortex, then centrifuge thawed nuclear stain** 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*.
   - Dilute the nuclear stain stock 1:40 (where *n* equals the number of slides) (see Table 39).

Table 39: Prepare Nuclear Stain

Nuclear Stock	Blocking Buffer	Total Volume
5.5 µL x *n*	214.5 µL x *n*	220 µL x *n*

3. **Prepare 220 µL x *n* of DAPI Buffer** by diluting the nuclear stain stock 1:40 (where *n* equals the number of slides).
   - Add 5.5 µL x *n* of DAPI Nuclear Stain Stock into 214.5 µL x *n* of Blocking Buffer. Adjust volume as needed for more than one (1) slide.
   - Mix by pipetting up and down 3-5 times. Do not vortex.
4. **Place slides in humidity chamber** in a horizontal position and **slowly apply 200 µL of DAPI** directly to tissue within the incubation frame. Gently move the tray side to side as needed to ensure that the DAPI buffer covers the entire tissue.

NOTE: A pipette tip can also be used to carefully spread buffer over tissue within incubation frame. Carefully use a small volume pipette to spread buffer as needed by carefully laying the tip horizontally on top of the incubation frame and gently rolling the tip to spread the buffer until the tissue is completely covered. Be careful to avoid touching the tissue with the pipette.

5. **Incubate slide for 15 minutes** at room temperature in the covered humidity chamber (see Figure 92).

Figure 92: Incubate 15 minutes at RT

6. Following DAPI incubation, **wash slides for five (5) minutes in 1X PBS** (see Figure 93).

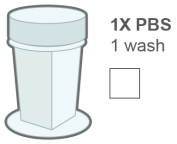

Figure 93: 1X PBS Wash

7. **Transfer** to a staining jar with **fresh 1X PBS and repeat wash** (see Figure 94).

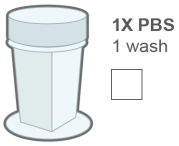

Figure 94: 1X PBS Wash

NHS-Acetate Preparation and Application (25 minutes)

You will need the following materials and reagents for this step: **scale, 2.0 mL centrifuge tube, staining jar, NHS-Acetate powder, NHS-acetate buffer** (provided by NanoString, 4°C), and **2X SSC**.

1. **Prepare 100 mM NHS-acetate mixture** immediately before you are ready to apply the mixture onto the tissue (200 µL/sample). Ensure stock NHS-Acetate has reached room temperature before opening stock bottle.

   a. NHS-acetate powder is stored desiccated at -20°C. If not already done, prepare 25 mg aliquots of NHS-acetate powder by weighing out the powder directly into 2.0 mL centrifuge tubes. Label tubes with the exact weight, seal tubes with parafilm, and store tubes at -20°C in desiccant for later use. See **Prepare Assay Reagents on page 111**.

   NOTE: If preparing only 2 slides, pre-weigh 15 mg aliquots into six (6) total tubes.

   b. **Add NHS-acetate buffer directly to aliquoted powder immediately before applying to the tissue**.

      i. Calculate the amount of NHS-acetate buffer to add to the NHS-acetate powder by multiplying the weight of NHS-acetate power in mg by 38.5.

         - Example: for 25.0 mg of NHS-acetate powder: 25.0 * 38.5 = 962.5 µL of buffer to add.

   ⓘ **IMPORTANT**: Reconstitute NHS-Acetate immediately before use. Do not prepare stock solutions for storage because the NHS ester moiety readily hydrolyzes and becomes non-reactive. Discard any unused reconstituted reagent.

2. Perform the following steps **one slide at a time** to prevent the tissue from drying out:

   - **Remove slide from 1X PBS** and transfer to a clean staining tray.

   - **Apply 200-250 µL of NHS-acetate mixture** onto tissue within the incubation frame in staining tray. Gently move tray side to side as needed to ensure that the NHS-acetate solution covers the entire tissue.

Figure 95: Incubate 15 minutes

- Repeat with remaining slides and **incubate covered in staining tray for 15 minutes at room temperature** (see Figure 95).

3. Carefully remove the incubation frame using clean forceps. Tap off excess liquid and submerge slides in **1X PBS** for a minimum of five (5) minutes.

- If samples need to be stored overnight and loaded onto the instrument the next day, remove the incubation frame following the guidelines from Day 2: Post-Fixation (50 minutes) on page 123 Day 2: Post-Fixation (50 minutes) on page 123. Ensure the entire incubation frame is removed then store according to Safe Storage Guidelines for Protein Slides on page 131.
- If samples will be loaded onto the instrument the same day, continue to Flow Cell Assembly.

Safe Storage Guidelines for Protein Slides

If not immediately loading prepared slides into a CosMx SMI Instrument, you must adhere to the following guidelines:

- Slides must never be stored dry; they may be submerged in 1X PBS if being loaded onto the CosMx SMI instrument within six (6) hours of slide preparation.
- If needed, slides can be stored overnight at 4°C.
- Slides must be stored in the dark.

Flow Cell Assembly

The CosMx SMI Flow Cell enables input of a tissue section sample into the CosMx SMI instrument for spatial profiling. It affixes to a 3 inch x 1 inch standard pathology grade slide with mounted tissue, creating an imageable fluidic channel. The CosMx SMI reagents required for the cycling chemistry are flowed across the tissue through the formed channel using the integrated fluidic input and output ports.

The provided flow cell assembly tool is a clamshell design that applies uniform force to adhere the flow cell coverslip onto the prepared slide (see Figure 96).

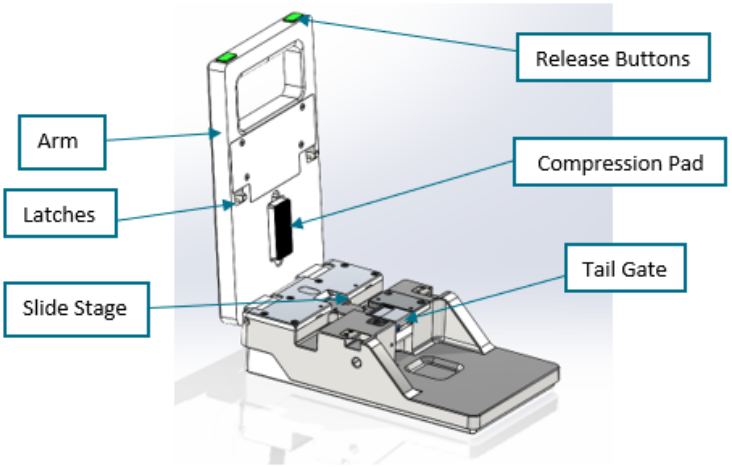

Figure 96: Flow Cell Assembly Tool

Before beginning flow cell assembly, verify tissue placement using the template provided on the flow cell assembly tool (see Figure 97), not to scale). The maximum tissue allowable area is 17mm x 20.8mm as represented by the solid bright green rectangle.

The imageable area is 15mm x 20mm as represented below by the dashed teal box. If needed, remove excess tissue using a clean razor blade.

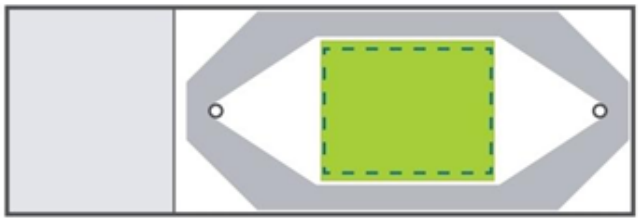

Figure 97: Tissue Allowable Area

Assemble the Flow Cell

1. **Clean the benchtop** with RNase AWAY or 70% Ethanol.
2. Prepare flow cell assembly tool by cleaning the stage with Ethanol or Isopropanol and then, blow any dust from the tool using an air blower. Glass fragments and slivers may be present, be careful when cleaning the flow cell assembly tool. Clean the top compression pad with DI Water. Do not clean the top compression pad with Ethanol or Isopropanol as this could damage the pad.

IMPORTANT: Do not use compressed air on the flow cell assembly tool or the CosMx SMI Instrument. NanoString recommends the following air blowers:

- Giottos AA1910 Medium Rocket Air Blaster (6.6)
- Camkix Keyboard Cleaning Kit

3. Inspect the flow cell coverslip for any damage such as cracks or chips and record the coverslip serial number. This will be the flow cell barcode needed when loading the instrument.

Assemble one (1) flow cell at a time to prevent the tissue from drying out.

1. Remove the sample slide from storage buffer using clean forceps.
2. Carefully **remove the incubation frame** if not already removed, and tap off excess buffer.
3. Dry the back of the slide and, using the template on the flow cell assembly tool, carefully dry the area around the tissue where the flow cell coverslip will adhere to the slide.
   - **Be careful to not wipe the slide within the imaging area** (shown in green) as this could remove fiducials required for on instrument imaging.
   - If using an adhesive slide label, ensure the label is less than 295 µm thick and is not folded over on itself. If label extends over frosted label area of the slide, carefully trim using a clean razor blade. Labels over the maximum thickness or labels that are not properly adhered may result in slide or flow cell damage during flow cell assembly and/or instrument loading.

4. **Lower the tail gate** (1) on the flow cell assembly tool (see Figure 98). Hold the labeled end of the slide and insert the slide, tissue side up, non-labeled end first, into the tool through the bottom opening. The slide is fully inserted once the non-labeled edge contacts the back of the slide stage (2).

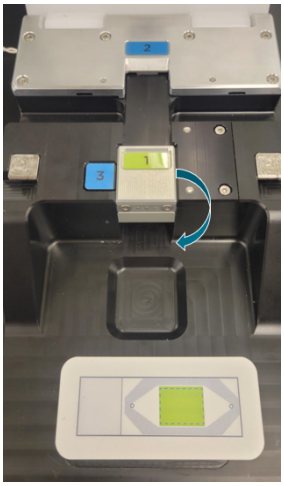

Figure 98: Lower Tailgate

5. **Raise the tailgate** to location 3 to secure the slide.
6. **Apply the flow cell coverslip:**
   - Use the air blower, if needed, to remove any dust from the coverslip immediately before applying.
   - Carefully **remove the adhesive backing** from the provided flow cell coverslip.
   - Hold the flow cell coverslip so that the serial number is readable. The backing will be on the opposite side.
   - Use clean forceps or gloved hands to gently hold the tab attached to the adhesive backing.
   - Slowly peel the adhesive backing away from the flow cell until it is completely removed.
7. **Place the flow cell coverslip onto the slide**, adhesive side down, within the slide stage area.
   - Hold the flow cell coverslip along the long sides and place the coverslip carefully onto the slide, adhesive side down, keeping the coverslip parallel to the slide.

- To confirm none of the edges of the flow cell coverslip are slightly lifted or are catching on a tool feature, lightly tap on the four corners of the coverslip (see Figure 99). The air gaps should be reduced and signs of adhesion (dark patches) should be present along the edges.

Figure 99: Flow Cell Placement

8. To complete assembly, **swing the arm of the flow cell assembly tool down until both latches on either side of the tool have engaged** (see Figure 100) Once engaged, both green release buttons will pop out

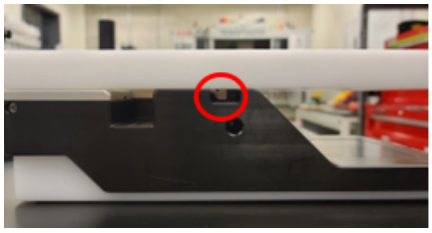

Figure 100: Fully Engaged Latch

9. After the latches have fully engaged, **release the arm** by pressing the two (2) buttons on the front of the assembly tool arm.
10. The newly assembled flow cell can be removed by reversing the steps of installing the slide.
    - Pull down the tail gate and then gently remove the flow cell.
11. **Check the slide and flow cell for any cracks** or damage and flow in 200 µL of storage buffer (2X SSC) into the flow cell port to ensure the tissue does not dry out.
    - Place the pipette tip directly over one of the fluidics ports on the flow cell, avoid putting pressure on the flow cell port as this could result in cracks around the flow cell port.
    - Slowly press the plunger and allow buffer to slowly fill the chamber and cover the tissue.

- Ensure there are no bubbles within the flow cell as this could result in imaging failure. Additional buffer may be flowed through the fluidics ports as needed to push out bubbles.
- Once the tissue has been covered and the flow cell chamber is full, remove the pipette tip without releasing the plunger and dispose of extra buffer.
- Use a clean Kimwipe to whisk away excess buffer from around the flow cell ports being careful to not touch the port with the Kimwipe.

12. Once assembled, place the flow cells into the clean staining tray, protected from light, until ready to load the instrument.

IMPORTANT: If the flow cell or slide is cracked, do not attempt to remove the flow cell coverslip as this could damage the tissue. Contact NanoString support for assistance and next steps.

Continue to the CosMx SMI Instrument Manual (MAN-10161) for instructions on loading the flow cell into the instrument and beginning data acquisition.

Appendix I: CosMx SMI Sample Preparation Guidelines

When preparing, sectioning, and storing FFPE blocks for use in the CosMx SMI instrument Protein and RNA assays, care should be taken to preserve sample integrity in all steps. The integrity of FFPE samples can be impacted by many factors, including time from excision to fixation, storage conditions, tissue type, and sample age. It is important to take such factors into consideration when selecting samples for the CosMx SMI assay. Samples with poor integrity are likely to give low signal, particularly in the RNA CosMx SMI assay.

CosMx SMI has been validated for samples up to 3 years old prepared from tissues with a cold ischemic time of less than 1 hour using 10% NBF or similar fixative. In general, for best results, do not use FFPE blocks greater than 10 years old. Assay performance, particularly for RNA, 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 the CosMx SMI assay.

1. Blocks should be fixed in 10% neutral-buffered formalin for 18 to 24 hours at room temperature. This applies to tissues up to 5 mm 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.

Sectioning FFPE Blocks

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

- For both the Protein and RNA assays, it is important to avoid any scratches and folds in the tissue section. These scratches and folds can be magnified by the subsequent slide washes on the CosMx SMI instrument resulting in tissue loss.
- Sections should be cut at 5 µm thickness on a calibrated microtome.
- Always discard the first few sections from the block face.
- Sections should be mounted in the center of the slide scannable area **(see Figure 101)** to allow adequate room for the flow cell and glass mapping.
- NanoString recommends the use of VWR® SuperFrost™ Plus Micro slides (for manual slide preparation) or BOND PLUS slides (for BOND RX/RXm semi-automated slide preparation). If mounting multiple sections per slide, ensure that all tissues are at least 2–3 mm apart and still contained within the scan area.
- 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.
- 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.

1. Unstained tissue sections should be sectioned with **5-10 µm thickness** and mounted on the label side of VWR Superfrost Plus Micro Slides or Leica BOND PLUS slides **(see Figure 101)**, figure not to scale).

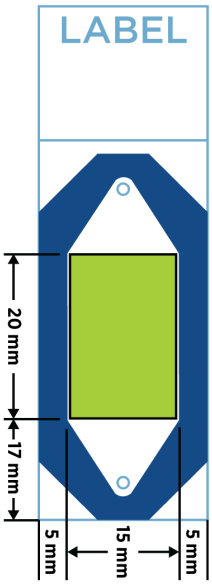

Figure 101: Tissue Scan Area

2. Tissue sections must be placed within the Scan Area (the green area) of the slide and be no larger than 20 mm Long by 15 mm Wide.

   If sections are larger than the indicated size and/or placed off-center, continue with the slide preparation as outlined. Directly before applying the incubation frame, scrape off parts of the tissue exceeding the scan area. Scraping off tissue before target retrieval and ethanol drying could generate tissue folds that may result in staining/binding artifacts, while suboptimal scraping may result in poor incubation frame adherence. Applying the incubation frame over tissue could result in tissue damage when the incubation frame is removed.

   ⓘ IMPORTANT: The CosMx SMI instrument will only image the area inside the flow cell chamber, the tissue scan area. If the tissue section is outside of the scan area, it will not be imaged.

3. **To improve tissue adherence**, bake slides at 37°C for two (2) hours after sectioning. After baking, dry at room temperature overnight prior to use or storage.

   A tissue section adhesive such as Epredia™ Tissue Section Adhesive (Fisher Scientific, 86014) can also be used to improve tissue adhesion. Follow the manufacturer's instructions for use guidelines.

Selecting Fresh Frozen Blocks

- Tissues should be selected that are known to have been snap frozen in liquid nitrogen as quickly as possible. Alternative freezing media may include isopentane pre-cooled with liquid nitrogen or isopentene cooled with dry ice.
- 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 media (OCT) before sectioning.
- Blocks embedded in OCT should be stored at -80°C.

Sectioning Fresh Frozen Blocks

- For both the Protein and RNA assays, it is critical to avoid any scratches and folds in the tissue section. These scratches and folds can be magnified by the subsequent slide washes on the CosMx SMI instrument resulting in tissue loss. Folds and wrinkles in fresh frozen tissues are highly susceptible to damage during washes and incubation frame removal.
- Sections should be cut at 5- 10 µm thickness on a calibrated cryostat and mounted immediately on a VWR Superfrost Plus Micro slide (for manual slide preparation) or BOND PLUS slide (for BOND/BOND RX^{m} semi-automated slide preparation). During sectioning, it is important to cut across the tissue with a smooth, consistent turn of the hand wheel.

  ⓘ **IMPORTANT**: Cryostat temperature should be set to -20°C. Place fresh frozen block inside of cryostat for a minimum of 30 minutes to equilibriate to temperature. Temperature may need to be adjusted +/- 5°C to optimize sample collection.

- Sections should be centered within the scan area as shown above **(see Figure 101)**.
- Always discard the first section from the block face.
- After sectioning, the exposed block face should be covered with OCT to avoid desiccation of the sample.
- Dry slides at room temperature for 5-10 mimutes then store at -80°C with a desiccant.
- Slides can be stored at -80°C for several weeks before use.

Appendix II: Tissue Specific Digestion

Optimal digestion concentration and incubation time may differ for some tissue types and needs to be empirically determined. For this protocol, start with the recommended default concentration and time and adjust as needed. The following tissue specific concentrations and incubation times are based on R&D preliminary testing and have not yet been validated.

Table 40: Proteinase Digestion

Tissue	Digestion Buffer Concentration	Digestion Time
Lymph Node	3 µg/mL	15 minutes
Tonsil	3 µg/mL	30 minutes
Liver	3 µg/mL	30 minutes
Pancreas	3 µg/mL	30 minutes
Kidney	3 µg/mL	30 minutes
Breast	3 µg/mL	30 minutes
Lung	3 µg/mL	30 minutes
Colon	3 µg/mL	30 minutes
Melanoma (Skin)	3 µg/mL	30 minutes
Tissue Microarray (TMA)	3 µg/mL	30 minutes*
Cell Pellet Array (CPA)	1 µg/mL	15 minutes

*TMA results are variable and depend on tissue type within TMA. Additional testing may be required to optimize.

Appendix III: Tissue Specific Fiducial Concentrations (RNA Assay Only)

Optimal fiducial concentration may differ for some tissue types and needs to be empirically determined. Start with the recommended default concentration and adjust the concentration as needed. The following tissue specific fiducial concentrations are based on R&D preliminary testing for RNA only and have not yet been validated.

Table 41: Tissue Specific Fiducial Concentrations

Tissue	Fiducial Concentration
Tonsil	0.001%
Melanoma	0.001%
Lung	0.001%
Breast	0.0015%
Liver	0.001%
Colon	0.0015%
Pancreas	0.0015%
Kidney	0.002%
Fresh Frozen Tissue	0.00015%
Cell Pellet Array (CPA)	0.001%

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