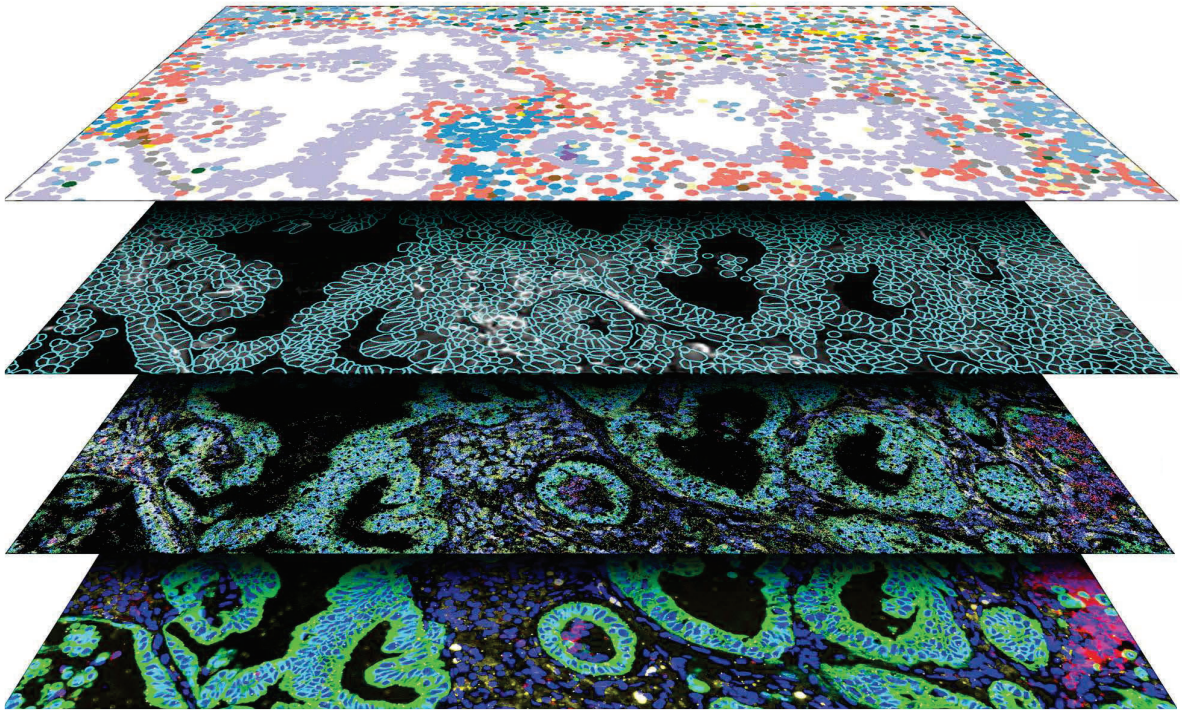
CosMx™ Spatial Molecular Imager

USER MANUAL

Manual Slide Preparation for RNA Assays



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nanoString

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Table of Contents

Changes in this Revision

Changes in this manual revision include:

- To improve run quality, the addition of enzymes to bottle 4 has been moved to the end of Day 1 slide preparation. See Add Enzymes to Buffer 4 on page 54 for FFPE Slide Preparation or page 101 for Fresh Frozen Slide Preparation.
- Updated texts and figures for clarity.

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 workflow.

⚠ **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 https://nanostring.com/resources/safety-data-sheets.

ⓘ **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 over 6,000 RNA and over 64 validated protein analytes. This flexible spatial single-cell solution drives deeper insights into the cell atlas, cell-cell interaction, cellular processes, and biomarker discovery.

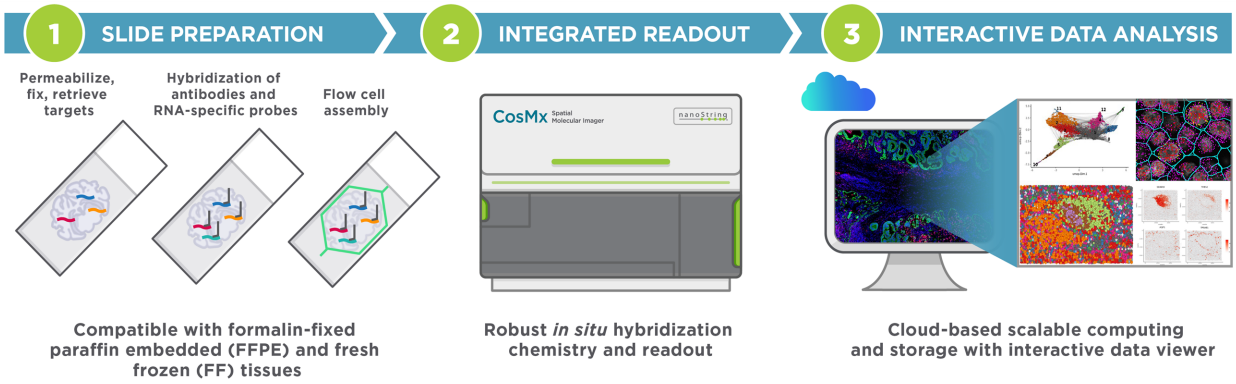


Figure 1: CosMx™ SMI is an integrated system that includes validated reagents and consumables, an instrument for chemistry and readout, and an interactive cloud-based software suite for data analysis.

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 (SIP) for analysis and visualization. Within AtoMx SIP, 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 over 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 for RNA Assays MAN-10184 CosMx SMI Manual Slide Preparation for Protein Assays MAN-10185	CosMx SMI Semi-Automated Slide Preparation for RNA Assays MAN-10186 CosMx SMI Semi-Automated Slide Preparation for Protein Assays MAN-10187
Workflow Step 2	CosMx SMI Instrument User Manual MAN-10161	
Workflow Step 3	CosMx SMI Data Analysis User Manual MAN-10162	

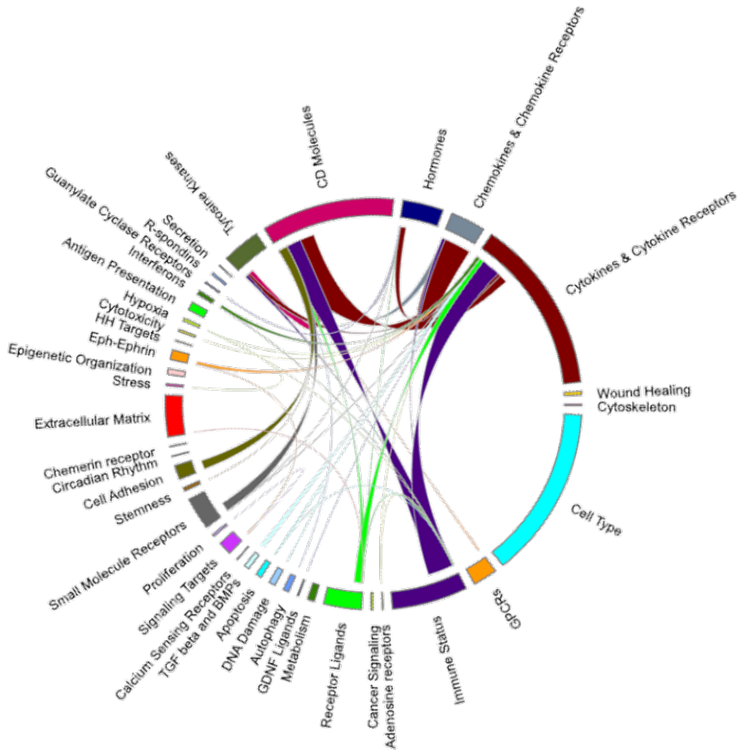
User manuals and other documents can be found online in the NanoString University Document Library at https://university.nanostring.com.

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

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

Additional data analysis support and resources can be found at https://github.com/Nanostring-Biostats.

Panel and Cell Segmentation Marker Selection



NanoString currently provides **7 pre-defined panels** for use with CosMx SMI:

- Human 6K Discovery Panel, 6K-plex, RNA
- Human Universal Cell Characterization Panel, 1000-plex, RNA
- Human Immuno-oncology Panel, 100-plex, RNA
- Mouse Neuroscience Panel, 1000-plex, RNA
- Mouse Universal Cell Characterization Panel, 1000-plex, RNA
- Human Immuno-oncology Panel, 64-plex, Protein
- Mouse Neuroscience Panel, 64-plex, Protein
    - Includes the CosMx Mouse Alzheimer's Pathology Module (must be run with the Mouse Neuroscience Core Panel)

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

- Add on up to 200 additional user-defined genes to the Human 6K-plex Discovery Panel.

  NOTE: The 6K compatible custom Add-On probes are not compatible with 100-plex or 1000-plex RNA assays.

- Swap in 7 to 10 user-defined genes to the Human Immuno-Oncology 100-plex RNA Panel or swap in 7 to 50 user-defined genes to any 1000-plex RNA Panel.
- Add on up to 8 user-defined protein targets to the 64-plex Protein Panels.

De novo RNA Custom Panels are also available and provide a ready-to-use assay for up to 300 targets.

- Collaborate with NanoString's Bioinformatics 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.

> ⓘ IMPORTANT: If using a custom panel, email AtoMxKitAdmin@nanostring.com **at least one business day prior to a run** so that the custom kit can be added into Control Center.

CosMx SMI Cell Segmentation Marker Selection

NanoString provides panel or tissue specific cell segmentation and supplemental marker kits for each analyte. See NanoString Supplied Reagents on page 22 for a detailed list of available kits.

Contact your NanoString Applications Scientist to discuss cell segmentation and supplemental marker selection.

FFPE Manual Slide Preparation

CosMx SMI Slide Preparation Workflow

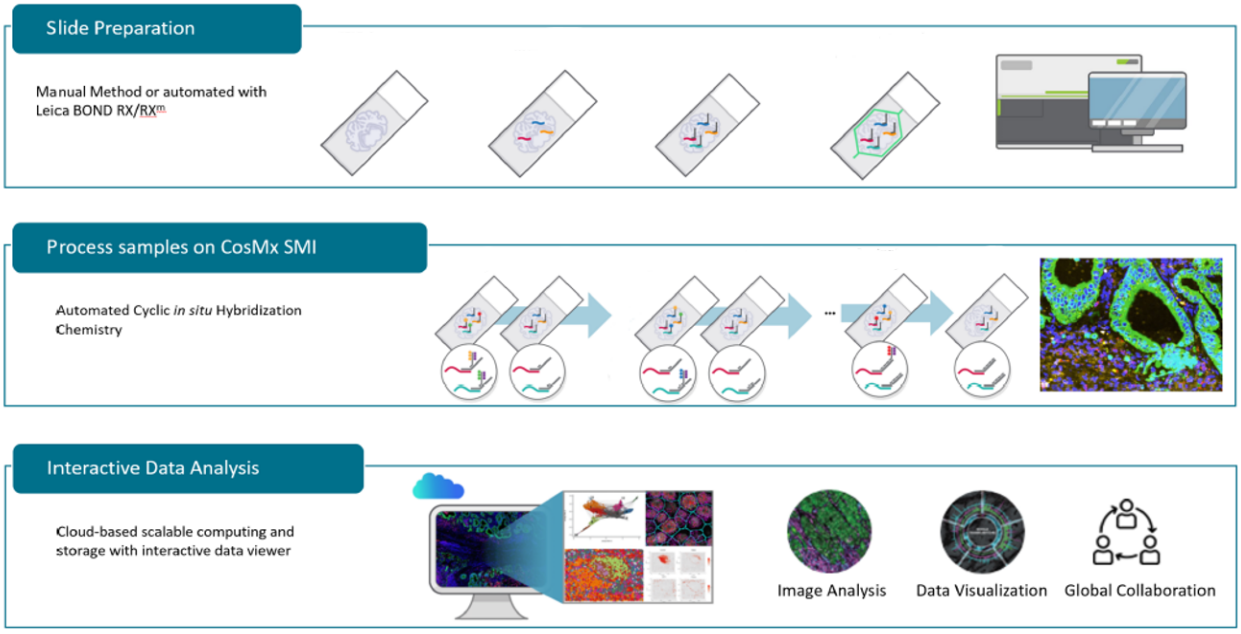


Figure 2: CosMx SMI Workflow Overview

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

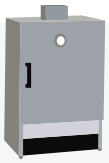
Day 2: Process Slides on CosMx SMI. Remove off-target probes and add cell segmentation markers to each slide. Load assembled flow cells into the CosMx SMI instrument and enter flow cell/study information. Tissue is scanned to capture RNA readout and morphology imaging within user-designated fields of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

RNA FFPE Manual Slide Preparation Overview

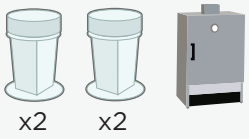
Day 0: Prepare Reagents and Bake Tissue Overnight

- Prepare shelf stable reagents

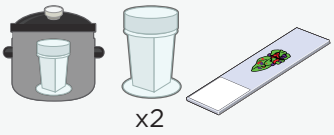


- Bake slides overnight at 60°C to improve tissue adherence

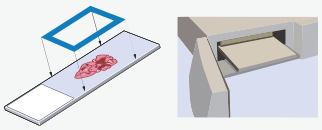
Day 1: Slide Preparation



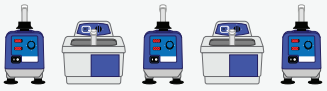
Remove Paraffin
- Xylene and EtOH washes
- Dry Slides 5 min at 60°C



Target Retrieval
- 15 mins at 100°C
- H2O rinse and 3 min EtOH wash
- Dry for 30 min-1 hour

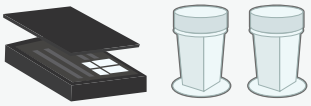


Protease Digestion
- Apply incubation frame
- Apply digestion buffer
- Incubate at 40°C for 30 mins
- Wash 2X in DEPC treated water

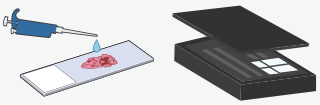


Apply Fiducials
- Prepare and apply fiducials
- Incubate for 5 minutes
- Wash with 1X PBS

Post-Fix Tissue
- Wash in 10% NBF for 1 min.
- 2 washes, 5 mins each, of NBF Stop Buffer
- Wash 5 min in 1X PBS



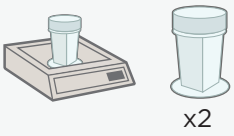
Blocking
- Prepare and apply NHS-Acetate
- Incubate for 15 mins.
- 2 washes, 5 mins each, in 2X SSC



Overnight Hybridization
- Prepare and apply assay specific probes
- Incubate at 37°C overnight

Add Buffer 4 Enzymes
- Add P2OX and Catalase to Buffer 4 and leave sealed on bench until instrument loading.

Day 2: Wash and Stain Slide

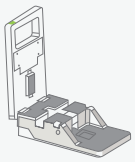


Stringent Washes
- 2 stringent washes, 25 mins each.
- 2 washes, 2 mins each, 2X SSC

Blocking & Nuclear Stain
- Prepare Nuclear Stain stock and apply to tissue
- Incubate 15 mins at RT
- Wash in 1X PBS for 5 mins

Segmentation Markers
- Prepare Segmentation mix and apply to tissue
- Incubate 1 hour at RT
- 3 washes, 5 mins each, 1X PBS

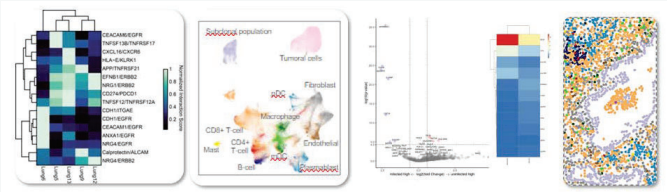
Prepare Flow Cells and Load Instrument



Prepare Flow Cells
- Use the flow cell assembly tool to assemble the flow cells.

Load Instrument
- Follow on-instrument prompts to load reagents and flow cells.
- Begin instrument run.

Analyze Data in AtoMx SIP



Equipment, Materials, and Reagents

The following equipment (Table 1), materials (Table 2), and reagents (Table 3) are required for this protocol but are **not supplied by NanoString.**

Equipment:

Table 1: Equipment not provided by NanoString

	Equipment	Source	Part Number
☐	**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: 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 for more fragile tissues. If a steamer is used, a thermometer is also needed.	BioSB® TintoRetriever Nesco® Hamilton Beach® Ovente® (not validated)	BSB 7015 ST-25F 3753OZ FS62S
☐	**Ultrasonic bath** 500 mL capacity, 40kHz frequency with timer	General Lab Supplier	Example (CPX-952-118R)
☐	**Vortex mixer**	General Lab Supplier	Various

	Equipment	Source	Part Number
☐	**Microcentrifuge** for 1.5 mL microcentrifuge tubes and 8-well PCR strip 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 2: Materials not provided by NanoString

	Materials	Source	Part Number
☐	**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
☐	**Leica BOND Plus slides or** **VWR Superfrost Plus Micro Slide, Premium** NOTE: These slides have been validated by NanoString. Do not use other products. NOTE: Leica BOND Plus slides are preferred for tissue sections prone to peeling.	Leica Biosystems VWR	S21.2113.A 48311-703
☐	**Slide rack**	General Lab Supplier	Example

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

Reagents:

Table 3: Reagents not provided by NanoString

	Reagent	Source/Part Number	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 32.	General Lab Supplier	Flammable Storage Room temperature
☐	**20X SSC** (DNase, RNase free)	ThermoFisher Scientific, 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: Sulfo-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	Storage Conditions
☐	**10% Neutral Buffered Formalin** (NBF) 	EMS Diasum®, 15740 (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)

Table 4: FFPE Slide Preparation Kit (Box 1 of 2)

Kit Contents (Box 1 of 2, Store at 4°C)	
	
10X Target Retrieval Buffer	Buffer R
CosMx RNA Blocking Buffer	NHS-Acetate Buffer
2X SSCT	CosMx Fiducials
Incubation Frames (not pictured)	Incubation Frame Covers (not pictured)

Table 5: FFPE Slide Preparation Kit (Box 2 of 2)

Kit Contents (Box 2 of 2, Store at -20°C)	
CosMx Proteinase K	

CosMx RNase Inhibitor

Table 6: CosMx RNase Inhibitor

Kit Contents (Store at -20°C)	
CosMx RNase Inhibitor IMPORTANT: CosMx RNase Inhibitor is required for the RNA Assay. RNase Inhibitor is **sold separately** and is used for both the RNA hybridization step and instrument loading.	

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

Table 7: CosMx RNA Panels

CosMx RNA Panels (Multiple Available) (Store at -20°C)	
Kit Name	**Kit Component**
CosMx Human 6K Discovery Panel, 6K-Plex, RNA	CosMx Hs 6K Discovery RNA Probe Mix
CosMx Human Universal Cell Characterization Panel, 1000-plex, RNA	CosMx Hs UCC RNA Probe Mix CosMx Hs UCC RNA Add-On Custom RNA Add-On replaces off-the-shelf RNA Add-On
CosMx Human Immuno-oncology Panel, 100-plex, RNA	CosMx Hs IO RNA Probe Mix CosMx Hs IO RNA Add-On Custom RNA Add-On replaces off-the-shelf RNA Add-On
CosMx Mouse Neuroscience Panel, 1000-plex, RNA	CosMx Mm Neuro RNA Probe Mix CosMx Mm Neuro RNA Add-On Custom RNA Add-On replaces off-the-shelf RNA Add-On
CosMx Mouse Universal (UCC) Panel, 1000-plex, RNA	CosMx Mm UCC RNA Probe Mix CosMx Mm UCC RNA Add-On Custom RNA Add-On replaces off-the-shelf RNA Add-On

CosMx Segmentation Markers

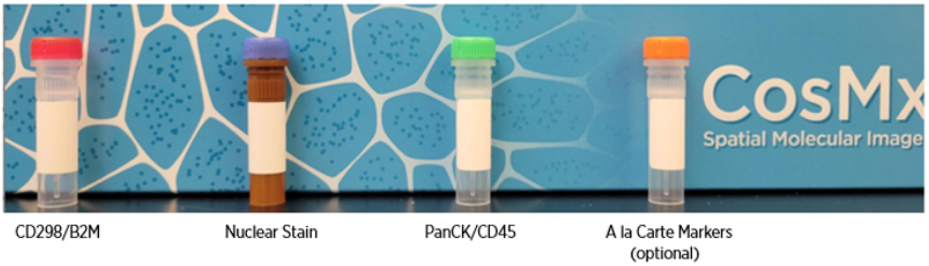


Table 8: CosMx SMI Cell Segmentation and Supplemental Marker Kits

Available Kits Each kit is sufficient for up to 4 slides (Store at -80°C)	
Kit Description	**Kit Components**
CosMx Human Universal Cell Segmentation Kit (RNA) Compatible with: Human 6K Discovery Panel, Human Immuno-Oncology 100-plex Panel, and any 1000-plex RNA Panel.	CosMx DAPI Nuclear Stain, Ch1
	CosMx Hs CD298/B2M Segmentation Marker Mix, Ch2 (RNA)
	CosMx Hs PanCK/CD45 Marker Mix Ch3/Ch4 (RNA)
CosMx Human Neuroscience Cell Segmentation Kit (RNA) Compatible with: Human 6K Discovery Panel	CosMx DAPI Nuclear Stain, Ch1
	CosMx Hs Neuro rRNA Neuro Marker, Ch2 (RNA)
	CosMx Mm/Hs Neuro Histone Marker, Ch3 (RNA)
	CosMx Mm/Hs GFAP Marker, Ch4 (RNA)

Available Kits Each kit is sufficient for up to 4 slides (Store at -80°C)	
Kit Description	**Kit Components**
CosMx Mouse Neuroscience Cell Segmentation Kit (RNA) Compatible with: Mouse Neuroscience 1000-plex Panel	CosMx DAPI Nuclear Stain, Ch1
	CosMx Mm Neuro rRNA Neuro Marker, Ch2 (RNA)
	CosMx Mm/Hs Neuro Histone Marker, Ch3 (RNA)
	CosMx Mm/Hs GFAP Marker, Ch4 (RNA)
CosMx Mouse Universal Cell Segmentation Kit (RNA) Compatible with: Mouse Universal Cell Characterization 1000-plex Panel.	CosMx DAPI Nuclear Stain, Ch1
	CosMx Mm CD298/B2M Marker Mix, Ch2 (RNA)
	CosMx Mm PanCK/CD45 Marker Mix Ch3/Ch4 (RNA)

The following markers are **optional and available to order à la carte:**

Table 9: A La Carte Markers

Compatible Cell Segmentation Kit	Item Description
Human Universal RNA Channel 5 (optional)	CosMx Hs CD68 A La Carte Marker, Ch5 (RNA)
	CosMx Hs Cytokeratin 8/18 A La Carte Marker, Ch5 (RNA)
	CosMx Hs/Mm CD3 A La Carte Marker, Ch5 (RNA)

Compatible Cell Segmentation Kit	Item Description
Mouse Universal RNA Channel 5 (optional)	CosMx Mm CD68 A La Carte Marker, Ch5 (RNA)
	CosMx Mm CD8 A La Carte Marker, Ch5 (RNA)
	CosMx Hs/Mm CD3 A La Carte Marker, Ch5 (RNA)

Flow Cell Assembly Tool and Kit

The Flow Cell Assembly tool is a one-time required purchase.

The Flow Cell Assembly Kit contains 4 single-use Flow Cell coverslips sufficient for a 4-slide experiment.

Prepare FFPE Tissue Samples

Appendix I: CosMx SMI Sample Preparation Guidelines on page 113 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.
>
> Tissues used for CosMx validation testing measure approximately 1.0 x 1.0 x 0.4 mm and are fixed for 24-36 hours before embedding.

Tissue Sectioning and Slide Preparation

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

Tissue sections must be centered within the Scan Area (the green area in Figure 3) of the slide and be no larger than **20 mm Long by 15 mm Wide** (image not to scale; see the Flow Cell Assembly Tool for a to-scale template). For best performance, ensure that some tissue-free glass is present in all four corners and within the scan area (the dashed teal line in Figure 3). For examples of tissue placement best practices, see Appendix I: CosMx SMI Sample Preparation Guidelines on page 113.

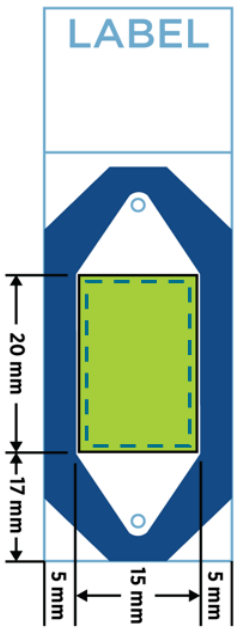


Figure 3: Tissue Scan Area (not to scale)

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 in slide or flow cell damage during flow cell assembly and/or instrument loading.

If sections are larger than the indicated size and/or placed off-center, continue with 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 or poor sealing of the incubation frame.

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 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.

It is recommended to use mounted sections within two weeks for best results. Older sections (1-2 months) may produce reasonable results, but results 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 Bake Tissue Overnight

- **Prepare shelf stable reagents**. See Prepare FFPE RNA Assay Reagents on page 29.
- In a slide rack, arrange slides vertically or in a slide holder at a 45 degree angle and **bake sections on slides** overnight in a 60°C drying oven.

Overnight tissue baking at 60°C has been shown to increase tissue adherence and stability during slide preparation. If slides cannot be baked overnight, then bake for at least 2 hours on Day 1 of Slide Prepearation prior to Day 1: Deparaffinize FFPE Tissue Sections (20 minutes) on page 32.

Please note that CosMx validation testing includes overnight baking.

Prepare FFPE 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 (ThermoFisher 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 (Table 10).

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

Table 10: RNA Reagent Preparation

	Reagent	Dilution	Storage
☐	1X PBS (pH 7.4)	Prepare 1 L of 1X PBS by combining 100 mL of 10X PBS and 900 mL of DEPC-treated water.	Room temperature
☐	2X SSC	Prepare 1 L 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 6.06 g Tris base and 3.75 g Glycine in 500 mL of DEPC-treated water. The final concentration of Tris and Glycine will be 0.1 M each.	Room temperature
☐	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 for 4 slides:**	-20°C in desiccant

	Reagent	Dilution	Storage
☐	NHS-Acetate Mix (cont.)	• Bring stock to room temperature for 1-2 hours prior to opening. • Prelabel four 2.0 mL screw top centrifuge tubes. • Using a weighing spatula, carefully weigh 25 mg of Sulfo-NHS-Acetate directly into one screw top tube on an analytic scale. • Close the tube and label 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 6 total tubes.	-20°C in desiccant
☐	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. NOTE: NanoString provides 20 mL of CosMx Target Retrieval Solution, 10X and recommends preparing 1X Target Retrieval Solution in a 60 mL staining jar.	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* 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	Make fresh daily Store on ice

	Reagent	Dilution	Storage
☐	Digestion Buffer (cont.)	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 tip. **Do not vortex.** NOTE: Digestion Buffer should be prepared fresh daily and stored on ice until ready to use. *NOTE: For CPA samples, prepare Digestion Buffer at 1 µg/mL. *Optimal concentration for other tissue types may need to be empirically determined. See Appendix II: Tissue Specific Slide Preparation Considerations on page 118).	Make fresh daily Store on ice
☐	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 later in this protocol.	n/a
☐	Day 2 Reagents	These reagents have additional steps that will be covered in detail in their respective sections. Follow Day 2 procedure for preparation of these reagents.	n/a

ⓘ IMPORTANT: If using a custom panel, email AtoMxKitAdmin@nanostring.com **at least one business day prior to a run** so that the custom kit is available in the CosMx SMI Control Center.

Day 1: Deparaffinize FFPE Tissue Sections (20 minutes)

You will need the following materials and reagents for this step: **staining jars, xylene and 100% ethanol. The hybridization tray, pressure cooker or steamer, 1X Target Retrieval Solution,** and **DEPC-treated water** are preheated here for their use in a later step (see Prepare FFPE RNA Assay Reagents on page 29).

Before beginning, slides should have already been baked overnight at 60°C (see Slide Preparation Day 0: Prepare Shelf Stable Reagents and Bake Tissue Overnight on page 28). If slides were not baked overnight, bake for 2 hours at 60°C on Day 1. Overnight baking is optimal.

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** (Figure 4). Ensure you have sufficient buffer in container to cover all slides. 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. Waste generated in these steps should also be disposed of properly.

5. Prepare the pressure cooker or steamer and preheat the target retrieval solution as follows. Content in purple boxes denotes steps or information specific to the pressure cooker. Content in orange boxes denotes steps or information specific to the steamer.

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

Pressure Cooker Method:

1. **Fill the pressure cooker** with water to the correct level per the manufacturer's instructions (4-8 cups depending on model used).
2. **Place the staining jar containing freshly prepared 1X Target Retrieval Solution into the pressure cooker to preheat**. Ensure that water level is well below lid of jar; about halfway up jar is sufficient. If needed, a trivet may be used to raise the jar.
3. 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**.
4. **Preheat the pressure cooker to 100°C** following the model-specific instructions below. Pressure cooker preheating takes about 1 hour.

BioSB Preheating Instructions:

Use the *TintoRetriever Pressure Cooker Preheating Cycle QuickStart Guide*, **Two Staining Dish Operation** provided with the BioSB pressure cooker to preheat the pressure cooker.

- With the pressure valve closed, press the **80°C button** on the face of the pressure cooker and press **Start** to run a cycle at 80°C with a 0-minute timer.

- Once the first cycle is complete, run a second cycle with a **45-minute timer at 100°C.**
- After the second cycle is complete, continue to Perform Target Retrieval (50 minutes) on page 36.

Steamer Method

1. **Fill the steamer reservoir up to the fill line** with water.
2. **Place two staining jars inside of the steamer**, one containing DEPC-treated water and one containing 1X Target Retrieval Solution. Ensure sufficient reagent volume to cover slides up to the label.
3. Loosely cover each jar with aluminum foil instead of the jar lid to allow for a thermometer reading in a later step.
4. **Preheat the steamer to 100°C.** More water may need to be added to the steamer during preheating.

The steamer may take up to 1 hour to heat the liquid in the jars to a stable maximum temperature near 100°C. Final temperature can be checked by inserting a digital thermometer through the hole in the lid of the steamer into the DEPC-water staining jar.

Deparaffinize FFPE tissue sections

1. Remove the slides from the baking oven and gently perform the following washes using staining jars (Figure 4).

   NOTE: Ensure you have sufficient buffer to completely cover the tissue on all slides without submerging the slide labels. Contact with buffer may make slide labels illegible.

   - Place slides into **first xylene jar and wash for 5 minutes**.
   - Transfer slides to new staining jar of xylene and wash for **5 minutes.**
   - **Transfer slides to 100% ethanol** and wash for **2 minutes***.
   - Transfer slides to new staining jar of ethanol and wash for **2 minutes***.

   ***If using Citrisolv** rather than xylene, wash the slides 2 times for 5 minutes each wash in 100% ethanol.

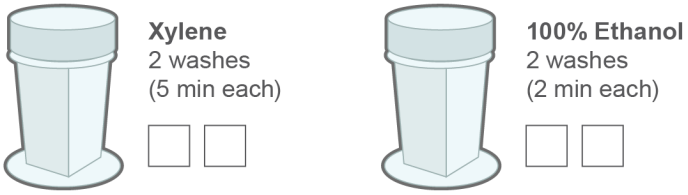


Figure 4: Deparaffinize FFPE Tissue Sections

⚠ **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 is 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 46. Pre-aliquoted NHS-Acetate powder can remain at -20°C until instructed to remove on page 46.

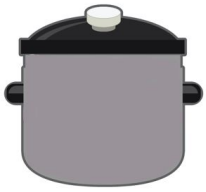
Perform Target Retrieval (50 minutes)

You will need the following materials and reagents for this step: **pressure cooker or steamer, staining jars, 1X Target Retrieval Solution** (pre-heated in the previous step), **DEPC-treated water** and **100% ethanol**.

> Target retrieval times were determined based on FFPE tissue blocks meeting the constraints outlined in the sample guidance section. These conditions may vary by sample, the amount of normal adjacent tissue, and other factors. See Appendix II: Tissue Specific Slide Preparation Considerations on page 118.

Content in purple box denotes steps or information specific to the pressure cooker. The orange box denotes steps or information specific to the steamer. Use the same target retrieval method (pressure cooker *or* steamer) throughout the study.

Pressure Cooker Method



1. Once Target Retrieval Solution is 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 as 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.

Steamer Method

1. Without removing the lid, place an instant- read digital thermometer through the vents in the steamer lid and pierce the aluminum foil covering the DEPC-treated water. Ensure the water has reached about 99°C. Add more water as needed.
2. Once the water has reached 99°C, carefully remove the steamer lid. Once removed, the Target Retrieval Solution will begin to rapidly cool. Ensure the following steps are done as quickly and safely as possible.

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.**
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 is 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** and return the pressure cooker to 100°C. For the BioSB, this can take up to 20 minutes.

NOTE: If after 10 minutes the temperature has not started to increase it may indicate that the float valve is not properly seated. Without removing the lid, turn the lid to the unlock position and then slowly back to lock.

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 removing the pressure cooker lid, hot steam will be released. Staining jar will also be VERY hot. Wear protective heat resistant gloves to remove lid and remove staining jar.

> ⚠ **WARNING**: Removing the steamer lid releases hot steam. Wear protective heat resistant gloves to open lid and remove the staining jar. Transfer slides using forceps or rack

3. Remove the foil from the Target Retrieval Solution jar and quickly transfer the slides to the Target Retrieval Solution. Replace the foil, then replace steamer lid.
4. Reinsert the thermometer into the DEPC- water jar and wait until the temperature returns to about 99°C.
5. **Once the steamer temperature returns to 99°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, **carefully remove the steamer lid and remove the staining jar.**

> ⚠ WARNING: When removing the steamer lid, hot steam will be released. Staining jar will also be VERY hot. Wear protective heat resistant gloves to remove 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 Figure 5.

> NOTE: Transfer slides from Target Retrieval Solution 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 lay horizontally on a clean Kimwipe. Dry at room temperature for 30 minutes to 1 hour**.

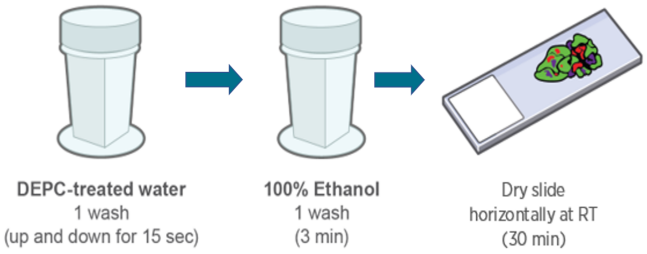


Figure 5: Water and Ethanol Wash

11. While slides are drying, continue to next page to prepare incubation frames and digestion buffer (see Tissue Permeabilization (40 minutes) on page 39).

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, Stored at 4°C), **digestion buffer** (see Prepare FFPE RNA Assay Reagents on page 29), and **DEPC-treated water.**

1. If needed, trim the tissue following the template in Prepare FFPE Tissue Samples on page 26.
    - 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.
2. 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).
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 (Figure 6).
    - 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.

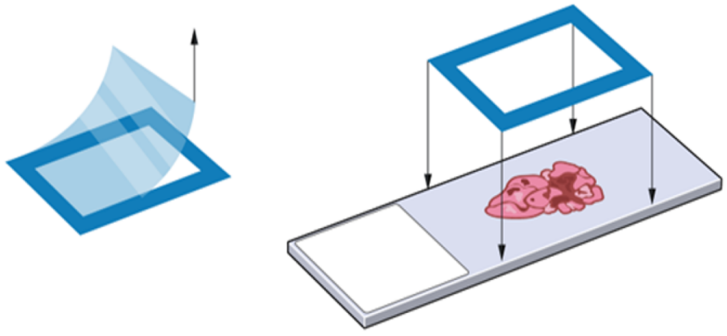


Figure 6: Apply Incubation Frame

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** (2 x 200 µL) **of digestion buffer to the tissue within incubation frame** (Figure 7). Gently move tray side to side as needed to **ensure that digestion buffer covers the entire tissue.**

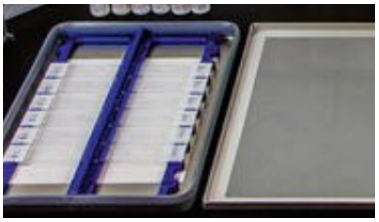


Figure 7: Hybridization Tray

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 tip.

9. Insert hybridization tray containing slides into hybridization oven and **incubate at 40°C following the guidance in the table** below (Table 11)(Figure 8).

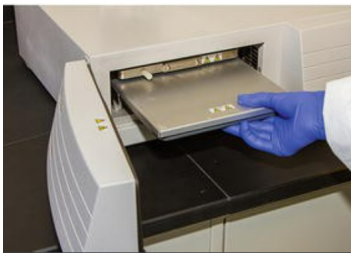


Figure 8: Hybridization Oven

Table 11: Digestion Times

Tissue Type	Digestion Time
FFPE Tissue (e.g., liver, tonsil, etc.)	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 Slide Preparation Considerations on page 118 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 slide at a time.

10. During slide 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 42.
12. After slide 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. Transfer slides to a new jar of DEPC-treated water and **repeat wash** (Figure 9).

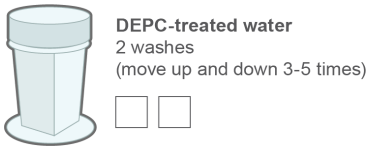


Figure 9: DEPC-treated water wash

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. At this point, 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.**

The volume of working solution prepared here is sufficient for 4 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. **Refer to the graphic below to vortex and sonicate the fiducials prior to use** (Figure 10):

> **IMPORTANT**: When sonicating the fiducial tube, be sure not to submerge the tube cap under the liquid level in the sonicator. Use a floating tube holder if needed to float the tubes in the ultrasonic bath.

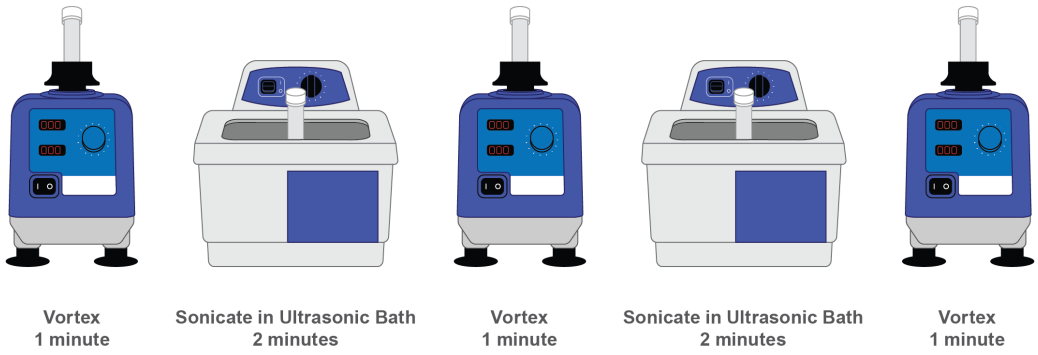


Figure 10: Fiducial preparation

> **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 following Figure 10, **make a 1:100 dilution** of fiducial stock (0.1%) to the working concentration (0.001%) **in 2X SSC-T** 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 Top 3 Tips for Successful CosMx™ SMI Single-cell Spatial Runs at 1000 plex for additional guidance.

3. Remove slide from DEPC-treated water and gently tap slide on a clean Kimwipe to remove excess water. Lay slide horizontally in staining tray.
4. **Immediately before applying fiducials to slides, vortex fiducials working solution for 1 additional minute**. Vortex fiducials for 30 seconds between applications to slides to keep fiducials in suspension and ensure consistent concentration across all slides.
5. **Apply up to 250 µL of the final fiducial working 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. 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 tip.

6. **Incubate covered in staining tray for 5 minutes at room temperature** (Figure 11). During fiducial incubation, prepare staining jars for next step.

Figure 11: 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, *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 12).

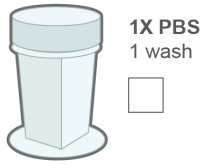


Figure 12: PBS Wash (1 minute)

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

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 FFPE RNA Assay Reagents on page 29 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 NBF.

Post-fix the tissue by performing the following washes (Figure 13).

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**. **Transfer slides** to a second staining jar containing NBF Stop Buffer and wash for **5 minutes**.
3. **Transfer slides to 1X PBS Wash** for **5 minutes**. Slides can sit in 1X PBS while NHS-Acetate mix is prepared.

Figure 13: 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, Sulfo-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 Sulfo- NHS- Acetate has reached room temperature before aliquoting to prevent condensation.
   a. Sulfo-NHS-Acetate powder is stored desiccated at -20°C. If not already done, prepare 25 mg aliquots (for a 4-slide preparation) of Sulfo-NHS-Acetate powder by weighing out the powder directly into four 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 FFPE RNA Assay Reagents on page 29.
      NOTE: If preparing only 2 slides, pre-weigh 15 mg aliquots into 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 Sulfo-NHS-Acetate powder by multiplying the weight of Sulfo-NHS-Acetate powder in mg by 38.5.
         - Example: For 25.0 mg of Sulfo-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 Sulfo-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** (Figure 14).

Figure 14: Incubate 15 minutes

3. Following incubation, tap off excess liquid and wash slides in **2X SSC for 5 minutes** (Figure 15).

Figure 15: Two 5-minute 2X SSC Washes

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

In Situ Hybridization (overnight)

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 1 hour at room temperature or up to 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.

You will need the following materials and reagents for this step: **hybridization oven, hybridization tray, incubation frame covers**, **thermal cycler, ice bucket with ice, Buffer R, CosMx RNA Probe Mix, custom OR off-the-shelf Add-On** (-20°C), **rRNA** (if applicable), **RNase Inhibitor** (-20°C), and **DEPC-treated water**.

> **IMPORTANT:** If preparing **human or mouse neural tissue**, rRNA is needed for the hybridization (Table 13). The rRNA marker is shipped and stored at -80°C with the Neuro Segmentation Marker kit. Only the rRNA marker should be removed from the kit for this step.

> **IMPORTANT:** If using a custom RNA Add-on, the custom Add-on will replace the standard probe Add-on for the 100- and 1000-plex assay. The Human 6K Discovery Panel does not include an off-the-shelf Add-on.

> **IMPORTANT**: If using a **custom panel**, email AtoMxKitAdmin@nanostring.com **at least one business day prior to a run** so that the custom kit is available in the CosMx Control Center.

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

Thaw probe mix, add-on probes (if applicable) and rRNA (if applicable) 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. 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. For each probe mix, flick to mix then centrifuge. **Do not vortex probes**.
4. **Denature CosMx RNA probe mixes** (RNA Probe Mix, RNA Add-On or Custom Add-On, and rRNA Segmentation Markers (Hu or Mm Neuro assay)), by transferring total volumes needed for assay from stock tubes into clean 0.2 mL PCR tubes **(probes, Add-ons, and rRNA should be kept separate during denaturing**).

   ⓘ IMPORTANT: Ensure accurate pipetting. When preparing 4 slides, there will be no excess RNA Probe Mix or Add-on.

5. **Heat at 95°C for 2 minutes** on a thermal cycler with heated lid. **Immediately transfer to ice for at least 1 minute to crash cool.**
6. Immediately before preparing hybridization mix, **flick to mix and centrifuge tubes.**
7. Make hybridization solution:

   Table 12 should be used if running **human or mouse non-neural tissue**.

   See Table 13 if using **human or mouse neural tissue** (note that the neural tissue workflow uses a rRNA marker in lieu of DEPC-treated water which should be thawed on ice and denatured just like the Probe Mix). **Prepare hybridization mix no more than 20 minutes before tissue application**.

   NOTE: The Human Neuroscience assay is run using the Human 6K Discovery Panel and the CosMx Human Neuroscience Cell Segmentation Kit on neural tissue. Only rRNA is needed from the Segmentation Kit for this overnight hybridization step. See Table 13.

Table 12: Hybridization Solution for **Non-Neural Tissue**

	Denatured RNA Probe Mix	Denatured Add-on* (if applicable)	RNase Inhibitor	Buffer R	DEPC-treated water	Total Volume
Off-the-shelf Panel (2-Slide)	32 µL	16 µL	3.2 µL	256 µL	up to final volume of 320 µL	320 µL
Off-the-shelf Panel (4-Slide)	64 µL	32 µL	6.4 µL	512 µL	up to final volume of 640 µL	640 µL
Custom Stand-alone Panel (2 slide)	16 µL**	-	3.2 µL	256 µL	up to final volume of 320 µL	320 µL
Custom Stand-alone Panel (4 slide)	32 µL**	-	6.4 µL	512 µL	up to final volume of 640 µL	640 µL

*If using a custom RNA Add-on, it **replaces the standard Panel Add-on for the 100- and 1000-plex assay**. The 6K Discovery Panel does not include an off-the-shelf Add-on. If an Add-on is not needed, use DEPC-water to reach the final volume.

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

Table 13: Hybridization Solution for Neural Tissue

	Denatured RNA Probe Mix	Denatured Add-on * (if applicable)	Denatured rRNA Segmentation Marker**	RNase Inhibitor	Buffer R	DEPC-treated water	Total Volume
Off-the-shelf Panel (2-Slide)	32 µL	16 µL	12.8 µL	3.2 µL	256 µL	up to final volume of 320 µL	320 µL
Off-the-shelf Panel (4-Slide)	64 µL	32 µL	25.6 µL	6.4 µL	512 µL	up to final volume of 640 µL	640 µL
Custom Panel with add-on (2-slide)	16 µL***	16 µL	12.8 µL	3.2 µL	256 µL	up to final volume of 320 µL	320 µL
Custom Panel with add-on (4-slide)	32 µL***	32 µL	25.6 µL	6.4 µL	512 µL	up to final volume of 640 µL	640 µL

*If using a custom RNA Add-on, **it replaces the standard panel Add-on for the Mm Neuro assay**. The 6K Discovery Panel does not include an off-the-shelf Add-on. If an Add-on is not needed, use DEPC-water to reach the final volume.

**Only the rRNA Segmentation marker is added during the overnight hybridization. All other segmentation markers will be added during Nuclear and Cell Segmentation Staining (2 hours) on page 57.

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

8. **Clean all equipment** and benchtop with RNase AWAY and allow to dry; or rinse with DEPC-treated water (see **IMPORTANT** note on page 48). **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.
9. **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.

10. 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 backing (with the center square removed) from the incubation frame to expose the top adhesive layer of the incubation frame (Figure 16). **Ensure that the incubation frame does not lift from the slide when removing the polyester frame backing.**

Figure 16: Remove Polyester Frame backing 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 (Figure 17).

      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** (Figure 17). 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; no additional pressure around the frame is needed. **Do not press the center of the cover as it could damage the tissue.**

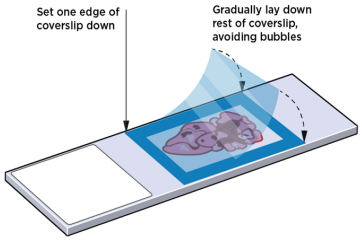


Figure 17: Apply Incubation Frame Cover

- Place the slide horizontally into the hybridization tray (Figure 18).

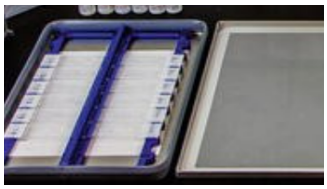


Figure 18: Hybridization Tray

- **Repeat step 10 for each slide.**
- Ensure that there is a good seal between the incubation frame and the slide, and the incubation frame cover and the frame by checking each slide for any leaks.

11. Close hybridization chamber, insert tray into oven, and clamp tray into place. **Incubate at 37°C overnight** (16 – 18 hours) (Figure 19).

    IMPORTANT! Continue to Add Enzymes to Buffer 4 on page 54 before ending Day 1 slide preparation.

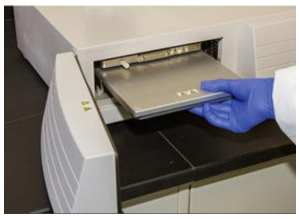


Figure 19: Incubate overnight at 37°C

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.

Add Enzymes to Buffer 4

To reduce oxidative damage to the tissue and probes and improve overall run quality, NanoString recommends adding the active enzymes Catalase and Pyranose Oxidase one day prior to starting a new instrument run. For additional guidance on which Instrument Buffer Kit to use, reference the CosMx SMI Instrument User Manual **Reagents and Consumables** section.

1. Remove lyophilized **Catalase** and **Pyranose Oxidase (P2OX)** from 4°C. Centrifuge the lyophilized vials and resuspend enzymes in DEPC-treated water:
   - For **RNA Buffer Kit, Small, add 250 µL of DEPC-water to each enzyme.** Vortex and centrifuge each tube.
   - For **RNA Buffer Kit, Medium, add 400 µL of DEPC-water to each enzyme.** Vortex and centrifuge each tube.
2. **Add Catalase** to Buffer 4:
   - **Pipette out 250 µL** (for an RNA Buffer Kit, Small) or **400 µL** (for an RNA Buffer Kit, Medium) from Catalase tube. Inspect the aliquot for any sign of precipitate and, if free of precipitate, add enzyme to **Buffer 4.**
3. **Add Pyranose Oxidase (P2OX)** to Buffer 4:
   - Due to the increased risk of precipitate in the P2OX enzyme, ensure the reconstituted enzyme has been vortexed and centrifuged, and any precipitate has been pelleted at the bottom of the tube.
   - Careful to avoid the pellet, **pipette out 125 µL** (for an RNA Buffer Kit, Small) or **200 µL** (for an RNA Buffer Kit, Small) from the P2OX tube. Inspect the aliquot for any sign of precipitate and, if free of precipitate, add enzyme to **Buffer 4.**

   ⓘ **IMPORTANT**: Presence of a precipitate in Buffer 4 could clog the instrument fluidics lines and cause run failure.
4. **Mark Buffer 4 bottle** after enzymes have been added, replace cap and dispose of excess P2OX according to laboratory guidelines.
5. Leave Buffer 4 sealed on the bench until ready to load onto instrument.

If not already done, prepare 40 mL formamide aliquots following the instructions in Equipment, Materials, and Reagents on page 15 and store overnight 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 FFPE RNA Assay Reagents on page 29).

> ⚠ **WARNING**: Use of appropriate personal protective equipment is advised as formamide is considered a hazardous material.

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 completely cover the tissue on all slides without submerging the slide labels. Contact with buffer may make slide labels illegible.

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 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.
   - Place slide into a 2X SSC wash and continue to the next slide.
   - 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 (Figure 20). 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**.
- Transfer slides to a second staining jar of Stringent Wash Solution and **wash for 25 minutes**.
- During second stringent wash, begin preparing reagents for Nuclear and Cell Segmentation Staining (2 hours) on page 57.
- Following stringent washes, immediately **transfer slides to 2X SSC and wash for 2 minutes**. **Transfer slides** to a second jar of 2X SSC and **wash for 2 minutes**. Leave slides in 2X SSC as needed until reagents have been prepared for nuclear and cell segmentation staining.

Figure 20: 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 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 FFPE RNA Assay Reagents on page 29).

1. **Prepare the following reagents**:
   - Four staining jars of 1X PBS
2. **Prepare 220 µL of Nuclear Stain Buffer per slide.**
   - **Vortex, then centrifuge thawed Nuclear Stain stock** 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) (Table 14).

Table 14: Prepare Nuclear Stain

Nuclear Stain 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 FFPE Tissue Samples on page 26.

   - Carefully apply a new incubation frame following the instructions in Tissue Permeabilization (40 minutes) on page 39. Ensure that the frame is well adhered to the slide by gently pressing around the frame with clean forceps.
4. Using a clean Kimwipe, carefully wick excess buffer from around the incubation frame as needed. Be careful to not touch the area inside of the incubation frame.
5. 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.

6. **Repeat** with remaining slides and cover tray.
7. **Incubate slides for 15 minutes at room temperature protected from light** (Figure 21).

Figure 21: Cover tray and incubate for 15 minutes

8. During nuclear stain incubation, prepare Segmentation Mix **(non-neural tissue: blue header** (Table 15)**, neural tissue: magenta header** (Table 16)) **Flick each tube to mix and centrifuge before use. Do not vortex mix.**

> ⓘ **IMPORTANT**: Ensure accurate pipetting. When preparing 4 slides, there will be no excess Segmentation Mix.

Table 15: Segmentation Mix for non-neural tissue (*n* = the number of slides)

Cell Segmentation Mix 1 (CD298/B2M)	Marker Mix 1* (Optional PanCK/CD45)	Marker Mix 2* (Optional a la carte)	Blocking Buffer	Total Volume
4 µL x *n*	8 µL x *n*	8 µL x *n*	180 µL x *n*	200 µL x *n*

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

Table 16: Segmentation Mix for neural tissue (*n* = the number of slides)

GFAP	Histone	Blocking Buffer	Total Volume
8 µL x *n*	8 µL x *n*	184 µL x *n*	200 µL x *n*

The rRNA Segmentation marker is added during the overnight hybridization. All other segmentation markers are added at this step.

9. 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** (Figure 22).

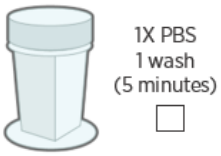


Figure 22: Wash for 5 minutes in 1X PBS

10. Wash slide for **5 minutes** in 1X PBS.
11. 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.
12. 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 Segmentation 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 Mix needs to completely cover the tissue** but does not need to completely fill the incubation frame.
13. **Repeat** with remaining slides and cover tray (Figure 23).

Figure 23: Cover tray and incubate for 1 hour

14. **Incubate slides for 1 hour at room temperature** protected from light.
15. Following Segmentation Mix incubation, **transfer slides to 1X PBS and wash for 5 minutes** (Figure 24).

Figure 24: Wash 3x in 1X PBS

16. **Repeat wash 2 times** for a total of 3 PBS washes.
17. If samples will be loaded onto the instrument the same day (**preferred**), remove the incubation frame following the guidelines from Day 2: Perform Stringent Washes (90 minutes) and then continue to Flow Cell Assembly on page 108.
18. 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 transfer slides to fresh 2X SSC and store according to **Safe Storage Guidelines for RNA Slides** below.

Safe Storage Guidelines for RNA Slides

After processing, slides must never be stored dry. Slides may be stored for up to 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).

Fresh Frozen Manual Slide Preparation

CosMx SMI Slide Preparation Workflow

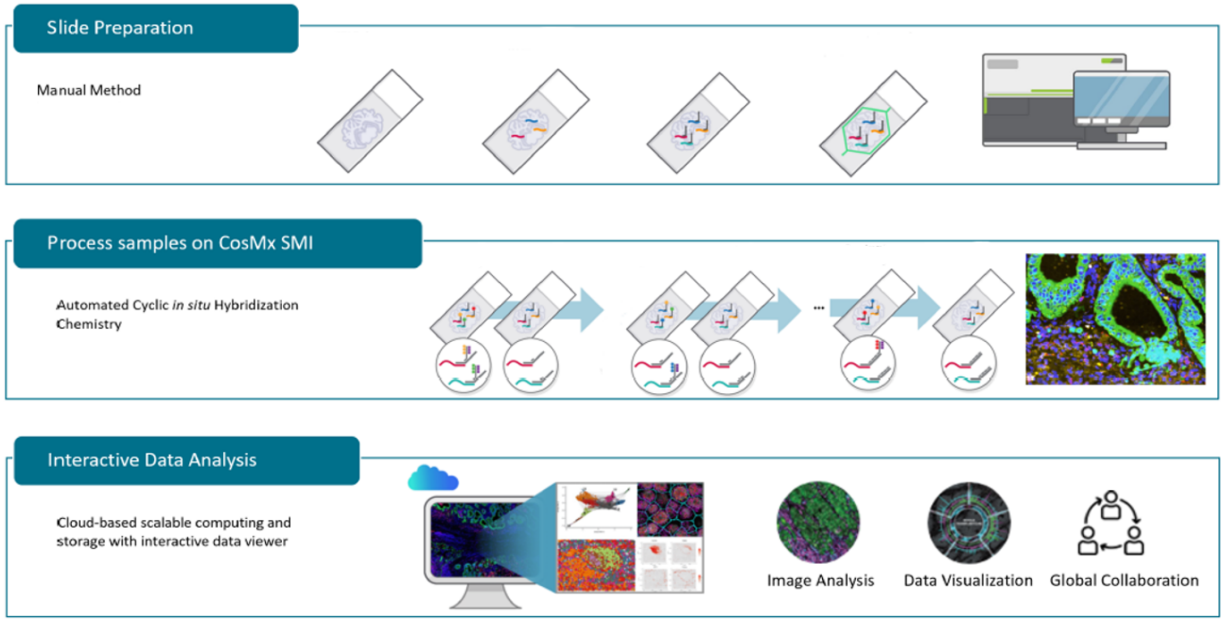


Figure 25: CosMx SMI Workflow Overview

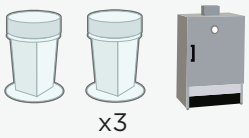
Day 1: Slide Preparation. Prepare slides and incubate biological targets with RNA specific probes.

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).

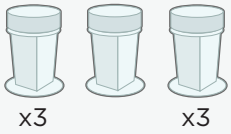
After run completion: Create a **Data Analysis** study in the AtoMx Spatial Informatics Platform (SIP) and perform quality-control checks, data analysis, and generate analysis plots.

RNA Fresh Frozen Slide Preparation Overview

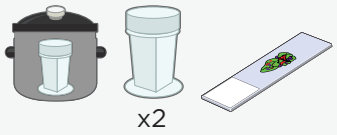
Day 1: Slide Preparation



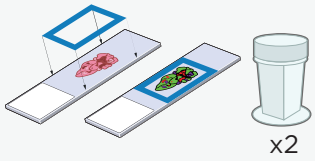
NBF Fixation and Bake
- 10% NBF Fixation
- 3 washes in 1X PBS
- 30-minute bake



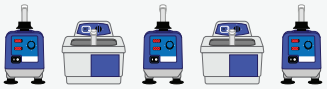
Wash and Rehydrate Tissue
- 3 washes in 1X PBS
- Wash in 4% SDS
- 3 washes in 1X PBS
- Wash in 50% EtOH
- Wash in 70% EtOH
- 3 washes in 100% EtOH



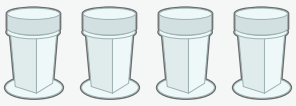
Target Retrieval
- 15 mins at 100°C
- H2O rinse and EtOH wash
- Dry for 30 min-1hour



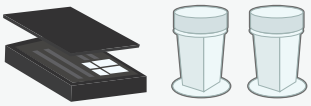
Protease Digestion
- Apply incubation frame
- Apply digestion buffer
- Incubate at RT for 30 mins
- 2 washes in 1X PBS



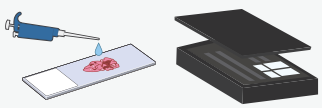
Apply Fiducials
- Prepare and apply fiducials
- Incubate for 5 minutes
- Wash with 1X PBS (not shown)



Post-Fix Tissue
- Wash in 10% NBF for 1 min.
- 2 washes, 5 mins each, of NBF Stop Buffer
- Wash 5 min in 1X PBS



Blocking
- Prepare and apply NHS-Acetate
- Incubate for 15 mins.
- 2 washes, 5 mins each, in 2X SSC



Overnight Hybridization
- Prepare and apply assay specific probes
- Incubate at 37°C overnight

Add Buffer 4 Enzymes
- Add P2OX and Catalase to Buffer 4 and leave sealed on bench until instrument loading.

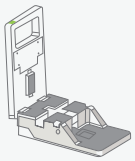
Day 2: Wash and Stain Slide

Stringent Washes
- 2 stringent washes, 25 mins each.
- 2 washes, 2 mins each, 2X SSC

Blocking & Nuclear Stain
- Prepare Nuclear Stain stock and apply to tissue
- Incubate 15 mins at RT
- Wash in 1X PBS for 5 mins

Segmentation Markers
- Prepare Segmentation mix and apply to tissue
- Incubate 1 hour at RT
- 3 washes, 5 mins each, 1X PBS

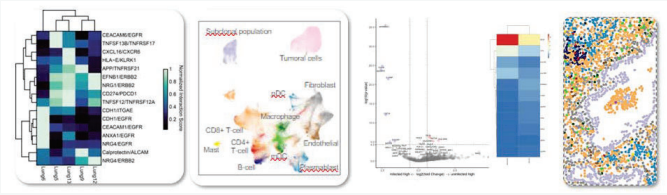
Prepare Flow Cells and Load Instrument



Prepare Flow Cells
- Use the flow cell assembly tool to assemble the flow cells.

Load Instrument
- Follow on-instrument prompts to load reagents and flow cells.
- Begin instrument run.

Analyze Data in AtoMx SIP



Equipment, Materials, and Reagents

The following equipment (Table 17), materials (Table 18), and reagents (Table 19) are required for this protocol but are **not supplied by NanoString.**

Equipment:

Table 17: Equipment not provided by NanoString

	Equipment	Source	Part Number
☐	**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**: 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 for more fragile tissues. If a steamer is used, a thermometer is also needed.	BioSB® TintoRetriever Nesco® Hamilton Beach® Ovente® (not validated)	BSB 7015 ST-25F 3753OZ FS62S
☐	**Ultrasonic Bath** 500 mL capacity, 40 kHz frequency with timer	General Lab Supplier	Example

	Equipment	Source	Part Number
☐	**Vortex mixer**	General Lab Supplier	Various
☐	**Microcentrifuge** for 1.5 mL microcentrifuge tubes and 8-well PCR strip 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 18: Materials not provided by NanoString

	Materials	Source	Part Number
☐	**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
☐	**Leica BOND Plus slides or** **VWR Superfrost Plus Micro Slide, Premium** NOTE: These slides have been validated by NanoString. Do not use other products. **Leica BOND Plus slides are preferred for tissue sections prone to peeling.**	Leica Biosystems VWR	S21.2113.A 48311-703

	Materials	Source	Part Number
☐	**Slide Rack**	General Lab	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 19: Reagents not provided by Nanostring Technologies Inc.

	Reagent	Source / Part Number	Storage Conditions
☐	**DEPC-Treated Water**	ThermoFisher, AM9922 (or comparable)	Room temperature
☐	**100% Ethanol (EtOH): ACS grade or Better** 	General Lab Supplier	Flammable Storage (RT)

	Reagent	Source / Part Number	Storage Conditions
☐	**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: Sulfo-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
☐	**10% Neutral Buffered Formalin (NBF)** 	EMS Diasum, 15740 (or comparable)	Room temperature
☐	**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)

Table 20a: CosMx Fresh Frozen Slide Preparation Kit (Box 1 of 2)

Kit Contents (Box 1 of 2, Store at 4°C)	
10X Target Retrieval Solution	Buffer R
CosMx RNA Blocking Buffer	NHS-Acetate Buffer
2X SSCT	CosMx Fiducials
Incubation Frames and covers (not pictured)	Protease A Buffer (not pictured)

Table 20b: CosMx Fresh Frozen Slide Preparation Kit (Box 2 of 2)

Kit Contents (Box 2 of 2, Store at -20°C)	
CosMx Proteinase K	

CosMx RNase Inhibitor

Table 21: CosMx RNase Inhibitor

Kit Contents (Store at -20°C)	
CosMx RNase Inhibitor IMPORTANT: CosMx RNase Inhibitor is required for the RNA Assay. RNase Inhibitor is **sold separately** and is used for both the RNA hybridization step and instrument loading.	

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

Table 22: CosMx RNA Panels

CosMx RNA Panels (Multiple Available) (Store at -20°C)	
Kit Name	**Kit Component**
CosMx Human 6K Discovery Panel 6K-Plex, RNA	CosMx Hs 6K Discovery RNA Probe Mix
CosMx Human Universal Cell Characterization Panel 1000-plex, RNA	CosMx Hs UCC RNA Probe Mix CosMx Hs UCC RNA Add-On Custom RNA Add-On replaces off-the-shelf RNA Add-On
CosMx Human Immuno-oncology Panel 100-plex, RNA	CosMx Hs IO RNA Probe Mix CosMx Hs IO RNA Add-On Custom RNA Add-On replaces off-the-shelf RNA Add-On
CosMx Mouse Neuroscience Panel 1000-plex, RNA	CosMx Mm Neuro RNA Probe Mix CosMx Mm Neuro RNA Add-On Custom RNA Add-On replaces off-the-shelf RNA Add-On
CosMx Mouse Universal (UCC) Panel 1000-plex, RNA	CosMx Mm UCC RNA Probe Mix CosMx Mm UCC RNA Add-On Custom RNA Add-On replaces off-the-shelf RNA Add-On

CosMx Segmentation Markers

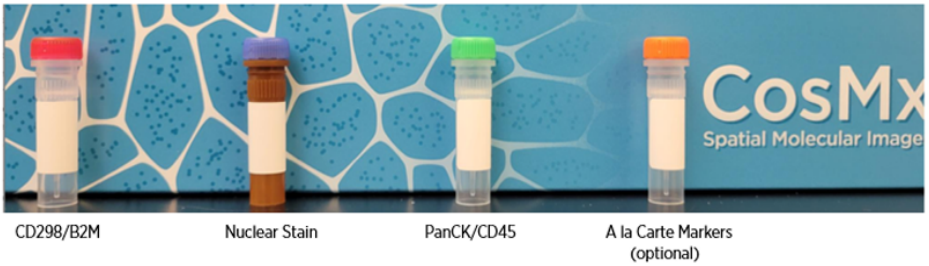


Table 23: CosMx SMI Cell Segmentation and Supplemental Marker Kits

Available Kits Each kit is sufficient for up to 4 slides (Store at -80°C)	
Kit Description	**Kit Components**
CosMx Human Universal Cell Segmentation Kit (RNA) Compatible with: Human 6K Discovery Panel, Human Immuno-Oncology 100-plex Panel, and any 1000-plex RNA Panel.	CosMx DAPI Nuclear Stain, Ch1
	CosMx Hs CD298/B2M Segmentation Marker Mix, Ch2 (RNA)
	CosMx Hs PanCK/CD45 Marker Mix Ch3/Ch4 (RNA)
CosMx Human Neuroscience Cell Segmentation Kit (RNA) Compatible with: Human 6K Discovery Panel	CosMx DAPI Nuclear Stain, Ch1
	CosMx Hs Neuro rRNA Neuro Marker, Ch2 (RNA)
	CosMx Mm/Hs Neuro Histone Marker, Ch3 (RNA)
	CosMx Mm/Hs GFAP Marker, Ch4 (RNA)

Available Kits Each kit is sufficient for up to 4 slides (Store at -80°C)	
Kit Description	**Kit Components**
CosMx Mouse Neuroscience Cell Segmentation Kit (RNA) Compatible with: Mouse Neuroscience 1000-plex Panel	CosMx DAPI Nuclear Stain, Ch1
	CosMx Mm Neuro rRNA Neuro Marker, Ch2 (RNA)
	CosMx Mm/Hs Neuro Histone Marker, Ch3 (RNA)
	CosMx Mm/Hs GFAP Marker, Ch4 (RNA)
CosMx Mouse Universal Cell Segmentation Kit (RNA) Compatible with: Mouse Universal Cell Characterization 1000-plex Panel.	CosMx DAPI Nuclear Stain, Ch1
	CosMx Mm CD298/B2M Marker Mix, Ch2 (RNA)
	CosMx Mm PanCK/CD45 Marker Mix Ch3/Ch4 (RNA)

The following markers are **optional and available to order à la carte:**

Table 24: A La Carte Markers

Compatible Cell Segmentation Kit	Item Description
Human Universal RNA Channel 5 (optional)	CosMx Hs CD68 A La Carte Marker, Ch5 (RNA)
	CosMx Hs Cytokeratin 8/18 A La Carte Marker, Ch5 (RNA)
	CosMx Hs/Mm CD3 A La Carte Marker, Ch5 (RNA)

Compatible Cell Segmentation Kit	Item Description
Mouse Universal RNA Channel 5 (optional)	CosMx Mm CD68 A La Carte Marker, Ch5 (RNA)
	CosMx Mm CD8 A La Carte Marker, Ch5 (RNA)
	CosMx Hs/Mm CD3 A La Carte Marker, Ch5 (RNA)

Flow Cell Assembly Tool and Kit

The Flow Cell Assembly tool is a one-time required purchase.

The Flow Cell Assembly Kit contains 4 single use Flow Cell coverslips sufficient for a four-slide experiment.

Prepare Fresh Frozen Tissue Samples

Selecting Fresh Frozen Blocks on page 116 covers Fresh Frozen block selection and sectioning in detail. Please review sample preparation guidelines prior to beginning the RNA Fresh Frozen Sample Preparation procedure.

Tissue Sectioning and Slide Preparation

Fresh frozen blocks should be sectioned at **5 µm thickness** and mounted on the label side of Leica BOND PLUS slides or VWR Superfrost Plus Micro Slides. Blocks may be sectioned up to 10 µm thickness; however, the instrument will only image the 5 µm closest to the slide.

Tissue sections must be centered within the Scan Area (the green area in Figure 26) of the slide and be no larger than **20 mm Long by 15 mm Wide** (image not to scale; see the Flow Cell Assembly Tool for a to-scale template). For best performance, ensure that some tissue-free glass is present in all four corners and within the scan area (the dashed teal line in Figure 26). For examples of tissue placement best practices, see Appendix I: CosMx SMI Sample Preparation Guidelines on page 113.

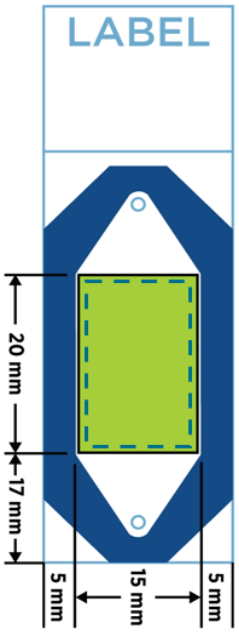


Figure 26: Tissue Scan Area (not to scale)

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 in slide or flow cell damage during flow cell assembly and/or instrument loading.

Dry mount slides for 5-10 minutes at room temperature, or until dry. Once dry, store slides at -80°C.

⚠ **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 or poor sealing of the incubation frame.

ⓘ **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.

Prepare RNA Fresh Frozen 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 (ThermoFisher 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 (Table 25).

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

Table 25: RNA Fresh Frozen Reagent Preparation

	Reagent	Dilution	Storage
☐	1X PBS (pH 7.4)	Prepare 1 L of 1X PBS by combining 100 mL of 10X PBS and 900 mL of DEPC-treated water.	Room temperature
☐	2X SSC	Prepare 1 L of 2X SSC by combining 100 mL of 20X SSC and 900 mL of DEPC-treated water.	Room temperature
☐	4X SSC	Prepare 1 L 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 6.06 g Tris base and 3.75 g Glycine in 500 mL of DEPC-treated water. The final concentration of Tris and Glycine will be 0.1 M each.	Room temperature
☐	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

	Reagent	Dilution	Storage
☐	50% Ethanol	Prepare 50 mL of 50% ethanol by adding 25 mL of DEPC-treated water to 25 mL 100% ethanol.	Make fresh daily
☐	4% SDS in 1X PBS	Before diluting: **warm 10% SDS for 10 minutes** in a 37°C water bath. After warming, vortex for 1 minute. Prepare 50 mL of 4% SDS in 1X PBS by adding 5 mL of 10X PBS 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%.	Make fresh daily
☐	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. NOTE: NanoString provides 20 mL of CosMx Target Retrieval Solution, 10X and recommends preparing 1X Target Retrieval Solution in a 60 mL staining jar.	Make fresh daily
☐	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 for 4 slides:** • Bring stock to room temperature for 1-2 hours prior to opening. • Prelabel four 2.0 mL screw top centrifuge tubes. • Using a weighing spatula, carefully weigh 25 mg of Sulfo-NHS-Acetate directly into one screw top tube on an analytic scale. • Close the tube and label with final weight. Seal the tube with parafilm and **place into**	-20°C in desiccant

	Reagent	Dilution	Storage
		the -20°C with desiccant until use. NOTE: If preparing only 2 slides, pre-weigh 15 mg aliquots into 6 total tubes	
☐	Digestion Buffer	Prepare immediately before use. See Tissue Permeabilization (40 minutes) on page 85.	Make immediately before use
☐	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 later in this protocol.	n/a
☐	Day 2 Reagents	These reagents have additional steps that will be covered in detail in their respective sections. Follow Day 2 procedure for preparation of these reagents.	n/a

ⓘ**IMPORTANT**: If using a custom panel, email AtoMxKitAdmin@nanostring.com **at least one business day prior to a run** so that the custom kit is available in the CosMx SMI Control Center.

Day 1: NBF Fixation and Bake

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

- **Pre-cool a staining jar** filled with 10% NBF to 4°C for a minimum of 15 minutes. Ensure you have sufficient buffer to completely cover the tissue on all slides without submerging the slide labels. Contact with buffer may make slide labels illegible.
- **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 27).
2. Incubate slides in **10% NBF for 2 hours at 4°C.**

Figure 27: 2 Hour NBF Fixation

3. Following NBF fixation, **transfer slides to 1X PBS** and wash for **2 minutes** (Figure 28).

Figure 28: Wash 3x with 1X PBS

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

Wash and Dehydrate Fresh Frozen Tissue Sections (1 hour)

You will need the following materials and reagents for this step: **pressure cooker** or **steamer, staining jars, 1X PBS, 4% SDS, 100% ethanol, 70% ethanol, 50% ethanol**, and **DEPC-treated water**. See Prepare RNA Fresh Frozen Assay Reagents on page 75 for more details.

Preheat Target Retrieval Solution

1. Prepare the pressure cooker or steamer and preheat the Target Retrieval Solution. Content in purple boxes denotes steps or information specific to the pressure cooker. Content in orange boxes denotes steps or information specific to the steamer.

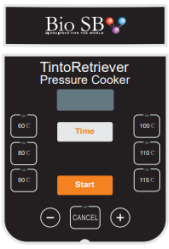
> ⚠ **WARNING**: NanoString does not recommend the use of glass staining jars in the pressure cooker.

Pressure Cooker Method:

1. **Fill the pressure cooker** with water to the correct level per the manufacturer's instructions (4-8 cups depending on model used).
2. **Place the staining jar containing freshly prepared 1X Target Retrieval Solution into the pressure cooker to preheat**. Ensure that water level is well below lid of jar; about halfway up jar is sufficient. If needed, a trivet may be used to raise the jar.
3. 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**.
4. **Preheat the pressure cooker to 100°C** following the model-specific instructions below. Pressure cooker preheating takes about 1 hour.

BioSB Preheating Instructions:

Use the *TintoRetriever Pressure Cooker Preheating Cycle QuickStart Guide*, **Two Staining Dish Operation** provided with the BioSB pressure cooker to preheat the pressure cooker.



- With the pressure valve closed, press the **80°C button** on the face of the pressure cooker and press **Start** to run a cycle at 80°C with a 0-minute timer.

- Once the first cycle is complete, run a second cycle with **a 45-minute timer at 100°C.** While the pressure cooker is preheating, continue to Wash and dehydrate Fresh Frozen tissue sections on page 81.
- After the second cycle is complete, continue to Perform Target Retrieval (50 minutes) on page 82.

Steamer Method

1. **Fill the steamer reservoir up to the fill line** with water.
2. **Place two staining jars inside of the steamer**, one containing DEPC-treated water and one containing 1X Target Retrieval Solution. Ensure sufficient reagent volume to cover slides up to the label.
3. Loosely cover each jar with aluminum foil instead of the jar lid to allow for a thermometer reading in a later step.
4. **Preheat the steamer to 100°C.** More water may need to be added to the steamer during preheating.

The steamer may take up to 1 hour to heat the liquid in the jars to a stable maximum temperature near 100°C. Final temperature can be checked by inserting a digital thermometer through the hole in the lid of the steamer into the DEPC-water staining jar.

Wash and Dehydrate 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 (Figure 29).

   NOTE: Ensure you have sufficient buffer to completely cover the tissue on all slides without submerging the slide labels. Contact with buffer may make slide labels illegible.

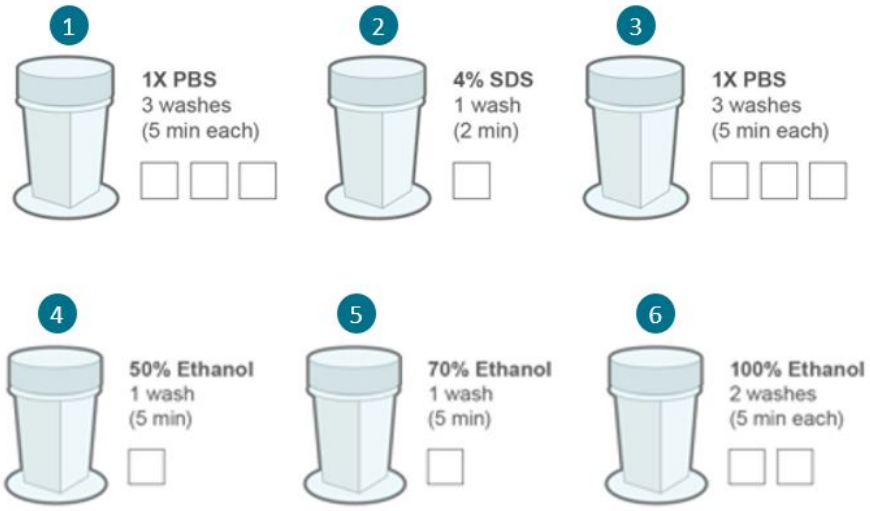


Figure 29: Rehydrate and Fix Tissue

2. During the 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. **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 93. Pre-aliquoted NHS-Acetate powder can remain at -20°C until instructed to remove on page 93.

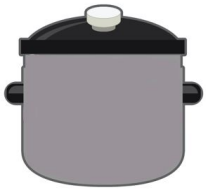
Perform Target Retrieval (50 minutes)

You will need the following materials and reagents for this step: **pressure cooker or steamer, staining jars, 1X Target Retrieval Solution** (pre-heated in the previous step), **DEPC-treated water** and **100% ethanol**.

> Target retrieval times were determined based on FF tissue blocks meeting the constraints outlined in the sample guidance section. These conditions may vary by sample, the amount of normal adjacent tissue, and other factors. See Appendix II: Tissue Specific Slide Preparation Considerations on page 118.

Content in purple box denotes steps or information specific to the pressure cooker. The orange box denotes steps or information specific to the steamer. Use the same target retrieval method (pressure cooker *or* steamer) throughout the study.

Pressure Cooker Method



1. Once Target Retrieval Solution is 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 as 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.

Steamer Method

1. Without removing the lid, place an instant- read digital thermometer through the vents in the steamer lid and pierce the aluminum foil covering the DEPC-treated water. Ensure the water has reached about 99°C. Add more water as needed.
2. Once the water has reached 99°C, carefully remove the steamer lid. Once removed, the Target Retrieval Solution will begin to rapidly cool. Ensure the following steps are done as quickly and safely as possible.

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.**
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 is 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** and return the pressure cooker to 100°C. For the BioSB, this can take up to 20 minutes.

NOTE: If after 10 minutes the temperature has not started to increase it may indicate that the float valve is not properly seated. Without removing the lid, turn the lid to the unlock position and then slowly back to lock.

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:** Removing the pressure cooker lid releases hot steam. Staining jar will also be very hot. Wear protective heat resistant gloves to remove lid and remove the staining jar.

⚠ **WARNING:** Removing the steamer lid releases hot steam. Wear protective heat resistant gloves to open lid and remove the staining jar. Transfer slides using forceps or rack.

3. Remove the foil from the Target Retrieval Solution jar and quickly transfer the slides to the Target Retrieval Solution. Replace the foil, then replace steamer lid.
4. Reinsert the thermometer into the DEPC- water jar and wait until the temperature returns to about 99°C.
5. **Once the steamer temperature returns to 99°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, **carefully remove the steamer lid and remove the staining jar.**

⚠ **WARNING:** Removing the steamer lid releases hot steam. Staining jar will also be very hot. Wear protective heat resistant gloves to remove lid and remove the 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 Figure 30.

> NOTE: Transfer slides from Target Retrieval Solution 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 lay horizontally on a clean Kimwipe. Dry at room temperature for 30 minutes to 1 hour**.

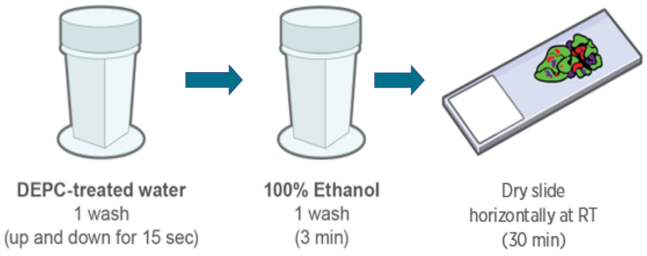


Figure 30: Water and Ethanol Wash

11. While slides are drying, continue to next page to prepare incubation frames and digestion buffer (see Tissue Permeabilization (40 minutes) on page 85).

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, Stored at 4°C)**, Proteinase K, Protease A, Protease A Buffer, 1X PBS** and **DEPC-treated water.**

1. If needed, trim the tissue following the template in Prepare Fresh Frozen Tissue Samples on page 73.
    - 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.
2. 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).
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 (Figure 31).
    - 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.

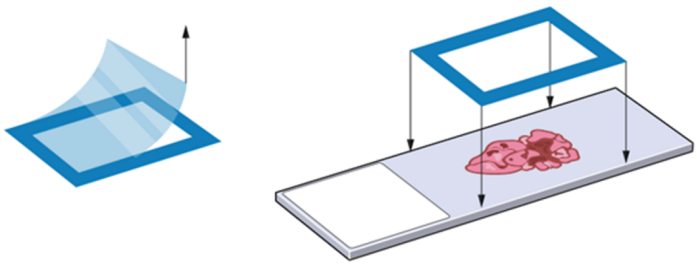


Figure 31: Apply Incubation Frame

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. If needed, trim the short end of the frame opposite of the slide label.

6. Prepare **Digestion Buffer**:

For **all tissue types except fresh frozen mouse neural tissue**, see the blue box. For **mouse neural tissue**, follow the guidance in the magenta box.

Digestion Buffer for all Fresh Frozen tissue types except mouse neural tissue:

Dilute the 20 mg/mL Protease Solution (Proteinase K stock; provided by NanoString) to a working concentration of 3 µg/mL* in 1X PBS. **Prepare fresh daily and stored on ice until ready to use.**

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: This concentration may differ for some tissue types, including CPA samples (see Appendix II: Tissue Specific Slide Preparation Considerations on page 118).

Digestion Buffer for Fresh Frozen mouse neural tissue:

NOTE: Digestion buffer for fresh frozen mouse neural tissue should be **made within 10 minutes of use**.

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 (Table 26). After each dilution, mix thoroughly by inverting tube or pipetting up and down using a clean pipetter tip. **Do not vortex.**

- **Resuspend Protease A** with 200 µL of Protease A Buffer.
- **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 A stock 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 **4 slides**. Adjust volume as needed.

Table 26: Digestion Buffer Preparation

Working Stocks		
Stock	2 µL Proteinase K (20 mg/mL)	5µL rehydrated Protease A
Diluent	398 µL of 1X PBS	245 µL of Protease A Buffer
Total Volume	400 µL ProK working stock	250 µL ProA working stock
Final Digestion Buffer		
Working Stock	82.5 µL ProK working stock	16.5 µL ProA working stock
Diluent	1551 µL Protease A Buffer	
Total Volume	1650 µL Digestion Buffer	

7. With the slide on a clean, flat service, **apply 400 µL** (2 x 200 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 1 slide at a time.

9. During slide 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 89.
11. After slide 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 32).

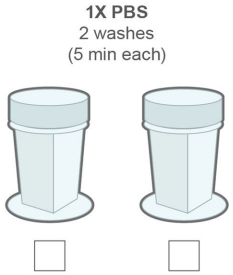


Figure 32: Wash 2X in 1X PBS

12. Transfer slides to a new jar of 1X PBS and **wash for 5 minutes.**
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. At this point, 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.**

The volume of working solution prepared here is sufficient for 4 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. **Refer to the graphic below to vortex and sonicate the fiducials prior to use** (Figure 33):

> **IMPORTANT**: When sonicating the fiducial tube, be sure not to submerge the tube cap under the liquid level in the sonicator. Use a floating tube holder if needed to float the tubes in the ultrasonic bath.

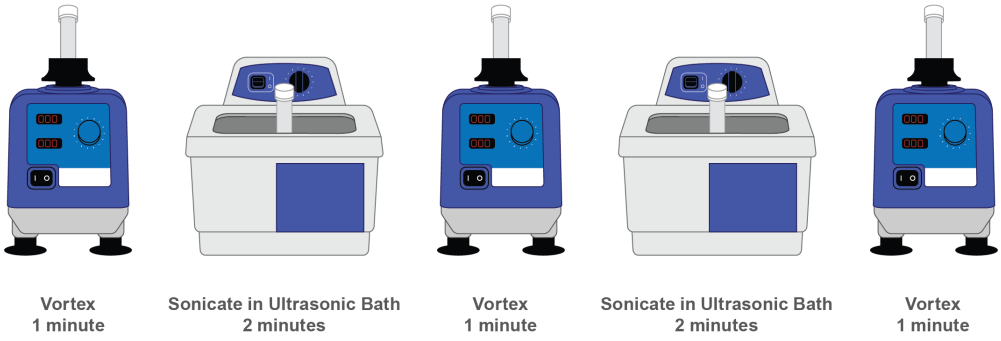


Figure 33: Fiducial preparation

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.

1. Once fiducials are prepared following Figure 33, **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 fiducials is 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 (Table 27).

Table 27: 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 Top 3 Tips for Successful CosMx™ SMI Single-cell Spatial Runs at 1000 plex for additional guidance.

2. Remove slides from 1X PBS and gently tap slide on a clean Kimwipe to remove excess buffer. Lay slide horizontally in staining tray.
3. **Immediately before applying fiducials to slides, vortex tube for 1 additional minute**. Vortex fiducial working solution for 30 seconds between applications to slides to keep fiducials in suspension and ensure consistent concentration across all slides.
4. **Apply up to 250 µL of the fiducial working 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. 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 tip.

5. **Incubate covered in staining tray for 5 minutes at room temperature** (Figure 34).

Figure 34: Incubate covered for 5 minutes

6. 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.

7. Wash slides in staining jar with **1X PBS for 5 minutes** (Figure 35). During PBS wash, prepare staining jars for next step.

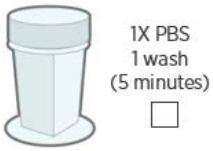


Figure 35: PBS Wash

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 Fresh Frozen Assay Reagents on page 75 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 NBF.

Post-fix the tissue by performing the following washes (Figure 36).

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**. **Transfer slides** to a second staining jar containing NBF Stop Buffer and wash for **5 minutes**.
3. **Transfer slides to 1X PBS Wash** for **5 minutes**. Slides can sit in 1X PBS while NHS-Acetate mix is prepared.

Figure 36: 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, Sulfo-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 Sulfo- NHS- Acetate has reached room temperature before aliquoting to prevent condensation.
   a. Sulfo-NHS-Acetate powder is stored desiccated at -20°C. If not already done, prepare 25 mg aliquots (for a 4-slide preparation) of Sulfo-NHS-Acetate powder by weighing out the powder directly into four 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 Fresh Frozen Assay Reagents on page 75.
      NOTE: If preparing only 2 slides, pre-weigh 15 mg aliquots into 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 Sulfo-NHS-Acetate powder by multiplying the weight of Sulfo-NHS-Acetate powder in mg by 38.5.
         - Example: For 25.0 mg of Sulfo-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 Sulfo-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** (Figure 37).

Figure 37: Incubate 15 minutes

3. Following incubation, tap off excess liquid and wash slides in **2X SSC for 5 minutes** (Figure 38).

Figure 38: Two 5-minute 2X SSC Washes

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

In Situ Hybridization (overnight)

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 1 hour at room temperature or up to 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.

You will need the following materials and reagents for this step: **hybridization oven, hybridization tray, incubation frame covers**, **thermal cycler, ice bucket with ice, Buffer R, CosMx RNA Probe Mix, custom OR off-the-shelf Add-On** (-20°C), **rRNA** (if applicable), **RNase Inhibitor** (-20°C), and **DEPC-treated water**.

> **IMPORTANT:** If preparing **human or mouse neural tissue**, rRNA is needed for the hybridization (Table 29). The rRNA marker is shipped and stored at -80°C with the Neuro Segmentation Marker kit. Only the rRNA marker should be removed from the kit for this step.

> **IMPORTANT:** If using a custom RNA Add-on, the custom Add-on will replace the standard probe Add-on for the 100- and 1000-plex assay. The Human 6K Discovery Panel does not include an off-the-shelf Add-on.

> **IMPORTANT**: If using a **custom panel**, email AtoMxKitAdmin@nanostring.com **at least one business day prior to a run** so that the custom kit is available in the CosMx Control Center.

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

Thaw probe mix, add-on probes (if applicable) and rRNA (if applicable) 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. 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. For each probe mix, flick to mix then centrifuge. **Do not vortex probes**.
4. **Denature CosMx RNA probe mixes** (RNA Probe Mix, RNA Add-On or custom Add-On, and rRNA Segmentation Markers (Hu or Mm Neuro assay)), by transferring total volumes needed for assay from stock tubes into clean 0.2 mL PCR tubes **(probes, Add-ons, and rRNA should be kept separate during denaturing**).

   ⓘ **IMPORTANT**: Ensure accurate pipetting. When preparing 4 slides, there will be no excess RNA Probe Mix or Add-On.

5. **Heat at 95°C for 2 minutes** on a thermal cycler with heated lid. **Immediately transfer to ice for at least 1 minute to crash cool.**
6. Immediately before preparing hybridization mix, **flick to mix and centrifuge tubes.**
7. Make hybridization solution:

   Table 28 should be used if running **human or mouse non-neural tissue**.

   See Table 29 if using **human or mouse neural tissue** (note that the neural tissue workflow uses a rRNA marker in lieu of DEPC-treated water which should be thawed on ice and denatured just like the Probe Mix). **Prepare hybridization mix no more than 20 minutes before tissue application**.

   NOTE: The Human Neuroscience assay is run using the Human 6K Discovery Panel and the CosMx Human Neuroscience Cell Segmentation Kit on neural tissue. Only rRNA is needed from the Segmentation Kit for this overnight hybridization step. See Table 29.

Table 28: Hybridization Solution for **Non-Neural Tissue**

	Denatured RNA Probe Mix	Denatured Add-on* (if applicable)	RNase Inhibitor	Buffer R	DEPC-treated water	Total Volume
Off-the-shelf Panel (2-Slide)	32 µL	16 µL	3.2 µL	256 µL	up to final volume of 320 µL	320 µL
Off-the-shelf Panel (4-Slide)	64 µL	32 µL	6.4 µL	512 µL	up to final volume of 640 µL	640 µL
Custom Stand-alone Panel (2 slide)	16 µL**	-	3.2 µL	256 µL	up to final volume of 320 µL	320 µL
Custom Stand-alone Panel (4 slide)	32 µL**	-	6.4 µL	512 µL	up to final volume of 640 µL	640 µL

*If using a custom RNA Add-on, it **replaces the standard Panel Add-on for the 100- and 1000-plex assay**. The 6K Discovery Panel does not include an off-the-shelf Add-on. If an Add-on is not needed, use DEPC-water to reach the final volume.

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

Table 29: Hybridization Solution for Neural Tissue

	Denatured RNA Probe Mix	Denatured Add-on * (if applicable)	Denatured rRNA Segmentation Marker**	RNase Inhibitor	Buffer R	DEPC-treated water	Total Volume
Off-the-shelf Panel (2-Slide)	32 µL	16 µL	12.8 µL	3.2 µL	256 µL	up to final volume of 320 µL	320 µL
Off-the-shelf Panel (4-Slide)	64 µL	32 µL	25.6 µL	6.4 µL	512 µL	up to final volume of 640 µL	640 µL
Custom Panel with add-on (2-slide)	16 µL***	16 µL	12.8 µL	3.2 µL	256 µL	up to final volume of 320 µL	320 µL
Custom Panel with add-on (4-slide)	32 µL***	32 µL	25.6 µL	6.4 µL	512 µL	up to final volume of 640 µL	640 µL

*If using a custom RNA Add-on, **it replaces the standard panel Add-on for the Mm Neuro assay**. The 6K Discovery Panel does not include an off-the-shelf Add-on. If an Add-on is not needed, use DEPC-water to reach the final volume.

**Only the rRNA Segmentation marker is added during the overnight hybridization. All other segmentation markers will be added during Nuclear and Cell Segmentation Staining (2 hours) on page 104.

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

8. **Clean all equipment** and benchtop with RNase AWAY and allow to dry; or rinse with DEPC-treated water (see **IMPORTANT** note on page 95). **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.
9. **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.

10. 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 backing (with the center square removed) from the incubation frame to expose the top adhesive layer of the incubation frame (Figure 39). **Ensure that the incubation frame does not lift from the slide when removing the polyester frame backing.**

Figure 39: Remove Polyester Frame backing 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 (Figure 40).

      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** (Figure 40). 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; no additional pressure around the frame is needed. **Do not press the center of the cover as it could damage the tissue.**

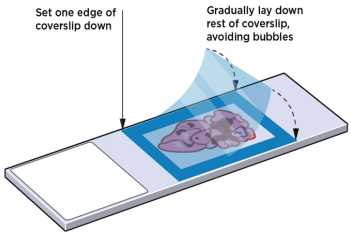


Figure 40: Apply Incubation Frame Cover

- Place the slide horizontally into the hybridization tray (Figure 41).

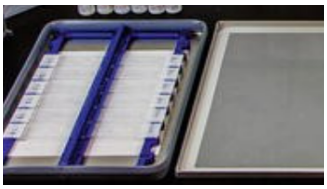


Figure 41: Hybridization Tray

- **Repeat step 10 for each slide.**
- Ensure that there is a good seal between the incubation frame and the slide, and the incubation frame cover and the frame by checking each slide for any leaks.

11. Close hybridization chamber, insert tray into oven, and clamp tray into place. **Incubate at 37°C overnight** (16 – 18 hours) (Figure 42).

    IMPORTANT! Continue to Add Enzymes to Buffer 4 on page 101 before ending Day 1 slide preparation.

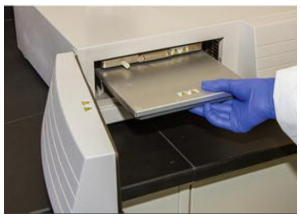


Figure 42: Incubate overnight at 37°C

> 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.

Add Enzymes to Buffer 4

To reduce oxidative damage to the tissue and probes and improve overall run quality, NanoString recommends adding the active enzymes Catalase and Pyranose Oxidase one day prior to starting a new instrument run. For additional guidance on which Instrument Buffer Kit to use, reference the CosMx SMI Instrument User Manual **Reagents and Consumables** section.

1. Remove lyophilized **Catalase** and **Pyranose Oxidase (P2OX)** from 4°C. Centrifuge the lyophilized vials and resuspend enzymes in DEPC-treated water:
   - For **RNA Buffer Kit, Small, add 250 µL of DEPC-water to each enzyme.** Vortex and centrifuge each tube.
   - For **RNA Buffer Kit, Medium, add 400 µL of DEPC-water to each enzyme.** Vortex and centrifuge each tube.
2. **Add Catalase** to Buffer 4**:**
   - **Pipette out 250 µL** (for an RNA Buffer Kit, Small) or **400 µL** (for an RNA Buffer Kit, Medium) from Catalase tube. Inspect the aliquot for any sign of precipitate and, if free of precipitate, add enzyme to **Buffer 4.**
3. **Add Pyranose Oxidase (P2OX)** to Buffer 4:
   - Due to the increased risk of precipitate in the P2OX enzyme, ensure the reconstituted enzyme has been vortexed and centrifuged, and any precipitate has been pelleted at the bottom of the tube.
   - Careful to avoid the pellet, **pipette out 125 µL** (for an RNA Buffer Kit, Small) or **200 µL** (for an RNA Buffer Kit, Small) from the P2OX tube. Inspect the aliquot for any sign of precipitate and, if free of precipitate, add enzyme to **Buffer 4.**

> ⓘ**IMPORTANT**: Presence of a precipitate in Buffer 4 could clog the instrument fluidic lines and cause run failure.

4. **Mark Buffer 4 bottle** after enzymes have been added, replace cap and dispose of excess P2OX according to laboratory guidelines.
5. Leave Buffer 4 sealed on the bench until ready to load onto instrument.

If not already done, prepare 40 mL formamide aliquots following the instructions in Equipment, Materials, and Reagents on page 63 and store overnight 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 Fresh Frozen Assay Reagents on page 75).

> ⚠ WARNING: Use of appropriate personal protective equipment is advised as formamide is considered a hazardous material.

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 completely cover the tissue on all slides without submerging the slide labels. Contact with buffer may make slide labels illegible.

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 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.
   - Place slide into a 2X SSC wash and continue to the next slide.
   - 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 (Figure 43). 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**.
- Transfer slides to a second staining jar of Stringent Wash Solution and **wash for 25 minutes**.
- During second stringent wash, begin preparing reagents for Nuclear and Cell Segmentation Staining (2 hours) on page 104.
- Following stringent washes, immediately **transfer slides to 2X SSC and wash for 2 minutes**. **Transfer slides** to a second jar of 2X SSC and **wash for 2 minutes**. Leave slides in 2X SSC as needed until reagents have been prepared for nuclear and cell segmentation staining.

Figure 43: 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 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 FFPE RNA Assay Reagents on page 75).

1. **Prepare the following reagents**:
   - Four staining jars of 1X PBS
2. **Prepare 220 µL of Nuclear Stain Buffer per slide.**
   - **Vortex, then centrifuge thawed Nuclear Stain stock** 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) (Table 30).

Table 30: Prepare Nuclear Stain

Nuclear Stain 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 FFPE Tissue Samples on page 73.

   - Carefully apply a new incubation frame following the instructions in Tissue Permeabilization (40 minutes) on page 85. Ensure that the frame is well adhered to the slide by gently pressing around the frame with clean forceps.
4. Using a clean Kimwipe, carefully wick excess buffer from around the incubation frame as needed. Be careful to not touch the area inside of the incubation frame.
5. 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.

6. **Repeat** with remaining slides and cover tray.
7. **Incubate slides for 15 minutes at room temperature protected from light** (Figure 44).

Figure 44: Cover tray and incubate for 15 minutes

8. During nuclear stain incubation, prepare Segmentation Mix **(non-neural tissue: blue header** (Table 31)**, neural tissue: magenta header** (Table 32)**). Flick each tube to mix and centrifuge before use. Do not vortex mix.**

> ⓘ **IMPORTANT**: Ensure accurate pipetting. When preparing 4 slides, there will be no excess Segmentation Mix.

Table 31: Segmentation Mix for non-neural tissue (*n* = the number of slides)

Cell Segmentation Mix 1 (CD298/B2M)	Marker Mix 1* (Optional PanCK/CD45)	Marker Mix 2* (Optional a la carte)	Blocking Buffer	Total Volume
4 µL x *n*	8 µL x *n*	8 µL x *n*	180 µL x *n*	200 µL x *n*

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

Table 32: Segmentation Mix for neural tissue (*n* = the number of slides)

GFAP	Histone	Blocking Buffer	Total Volume
8 µL x *n*	8 µL x *n*	184 µL x *n*	200 µL x *n*

The rRNA Segmentation marker is added during the overnight hybridization. All other segmentation markers are added at this step.

9. 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** (Figure 45).

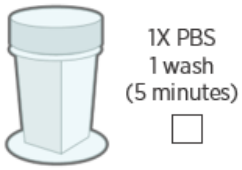


Figure 45: Wash for 5 minutes in 1X PBS

10. Wash slide for **5 minutes** in 1X PBS.
11. 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.
12. 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 Segmentation 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 Mix needs to completely cover the tissue** but does not need to completely fill the incubation frame.
13. **Repeat** with remaining slides and cover tray (Figure 46).

Figure 46: Cover tray and incubate for 1 hour

14. **Incubate slides for 1 hour at room temperature** protected from light.
15. Following Segmentation Mix incubation, **transfer slides to 1X PBS and wash for 5 minutes** (Figure 47).

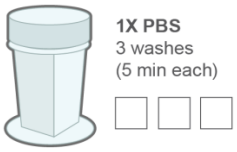


Figure 47: Wash 3x in 1X PBS

16. **Repeat wash 2 times** for a total of 3 PBS washes.
17. If samples will be loaded onto the instrument the same day (**preferred**), remove the incubation frame following the guidelines from Day 2: Perform Stringent Washes (90 minutes) and then continue to Flow Cell Assembly on page 108.
18. 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 transfer slides to fresh 2X SSC and store according to **Safe Storage Guidelines for RNA Slides** below.

Safe Storage Guidelines for RNA Slides

After processing, slides must never be stored dry. Slides may be stored for up to 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).

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 (Figure 48).

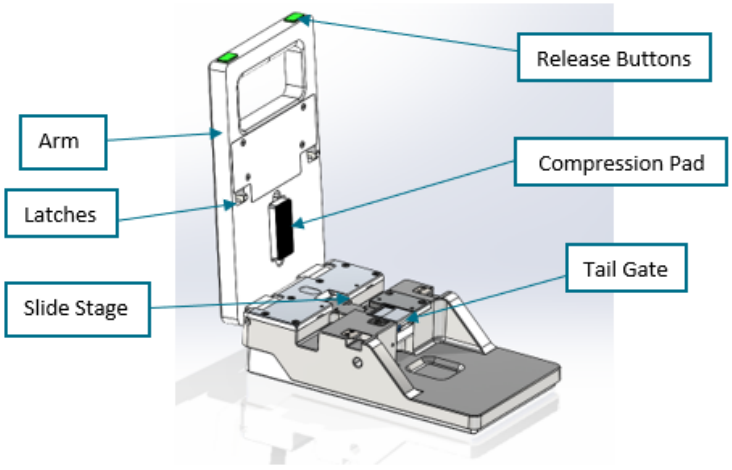


Figure 48: Flow Cell Assembly Tool

Before beginning flow cell assembly, verify tissue placement using the template provided on the flow cell assembly tool (Figure 49, not to scale). The maximum tissue allowable area is 17 mm x 20.8 mm as represented by the solid bright green rectangle.

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

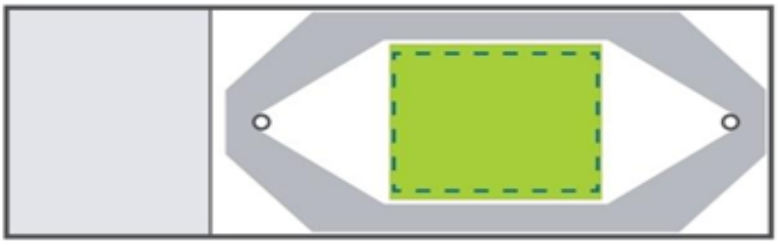


Figure 49: Tissue Allowable Area in green

Assemble the Flow Cell

1. **Clean the benchtop** with RNase AWAY or 70% ethanol.

> ⓘ **IMPORTANT**: RNase AWAY must be used for the RNA Assay as 70% ethanol does not adequately remove both nucleic acid and nuclease contaminants.

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 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 not to 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 tailgate** (tool marker 1) on the flow cell assembly tool (Figure 50). 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 (tool marker 2).

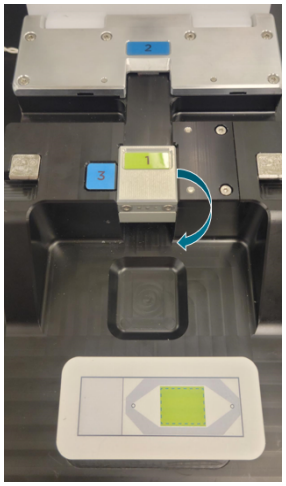


Figure 50: Lower Tailgate

5. **Raise the tailgate** to location 3 (tool marker 3) to secure the slide.
6. **Apply the flow cell coverslip:**
   - Use the air blower, if needed, to remove any dust from both sides of 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 (marked with stars in Figure 51). The air gaps should be reduced and signs of adhesion (dark patches) should be present along the edges.

Figure 51: 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** (Figure 52). Once engaged, both green release buttons will pop out

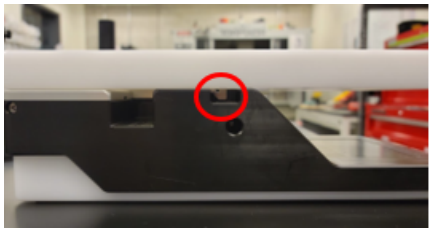


Figure 52: Fully Engaged Latch

9. After the latches have fully engaged, **release the arm** by pressing the 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 tailgate 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 wick 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 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 CosMx SMI RNA 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.

For additional guidance sample sectioning, see Sample Sectioning Tips and Tricks for CosMx SMI and GeoMx DSP Experiments (MAN-10175) available in the NanoString University Document Library.

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 5 mm in thickness. Thicker 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 exhaustive 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 scan area (Figure 53) while allowing adequate room for flow cell coverslip adhesives at the edges of the scan area.
- NanoString recommends the use of Leica BOND Plus slides or VWR Superfrost Plus Micro slides (for manual slide preparation). Leica BOND Plus slides are required for BOND RX/RX^{m} semi-automated slide preparation and are preferable in manual slide preparation for tissues with poor adherence. 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 at **5 µm thickness** and mounted on the label side of Leica BOND Plus slides or VWR Superfrost Plus Micro Slides (Figure 53) (figure not to scale; see the template on the Flow Cell Assembly Tool for a to-scale template).

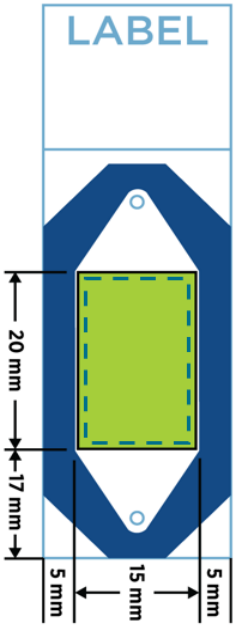


Figure 53: Tissue scan area (not to scale)

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. For best performance, ensure that some tissue-free glass is present in all four corners and within the scan area (the dashed teal line) (Figure 54).

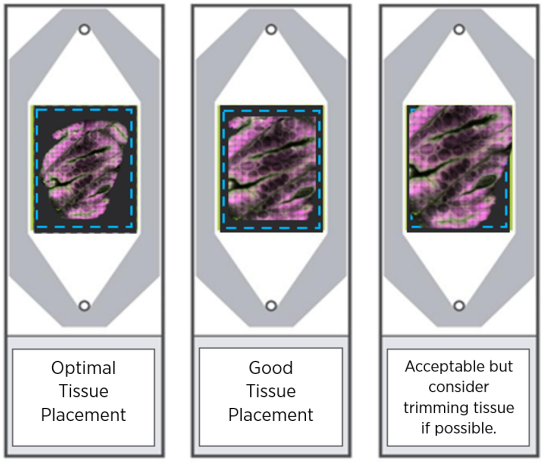


Figure 54: While all 3 examples are acceptable, Slide 1 shows optimal tissue placement; Slide 2 shows good tissue placement, and Slide 3 gives an example of a tissue that should be trimmed if possible as not all corners of the scan area have visible glass and there is minimal glass visible within the center of the scan area.

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 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 or poor sealing of the incubation frame.

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 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 µm thickness on a calibrated cryostat and mounted immediately on a BOND Plus slide (required for BOND/BOND RX^m semi-automated slide preparation) or VWR Superfrost Plus Micro slide (for manual slide preparation). During sectioning, it is important to cut across the tissue with a smooth, consistent turn of the hand wheel. Blocks may be sectioned up to 10 µm thickness; however, the instrument will only image the 5 µm closest to the slide.

> ⓘ **IMPORTANT**: Cryostat temperature should be set to -20°C. Place fresh frozen block inside of cryostat for a minimum of 30 minutes to equilibrate 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 (Figure 53).
- 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 minutes then store at -80°C with a desiccant.
- Slides can be stored at -80°C for several weeks before use.

Appendix II: Tissue Specific Slide Preparation Considerations

For the RNA Assay, optimal digestion buffer concentration and incubation time for target retrieval and digestion may differ for some tissue types and need to be empirically determined. With all tissue types, **begin with default experimental conditions** and modify as needed on an experiment basis. For additional information see Top 3 Tips for Successful CosMx™ SMI Single-cell Spatial Runs at 1000 plex.

Table 33: Suggested slide prep modifications based on tissue type and biology. With all tissue types, begin with default experimental conditions and modify as needed on an experiment basis.

	Tissue Types	Suggested Modification
1	**All types of tumors**, *especially those derived from epithelial tissues* such as colon, lung, breast, ovarian, kidney, bladder cancer, and cholangiocarcinoma.	**Default experimental conditions**: Target Retrieval Time: 15 minutes Digestion Buffer Concentration: 3 µg/mL Digestion Time: 30 minutes Fiducial Concentration: 0.001%
2	**All normal solid organs**: lymph node, liver, kidney etc.	Use **default experimental conditions.**
3	Tissues with higher levels of adipose, airways, loose connective tissue, delicate structures, normal breast, normal lung, organoids, retina.	These tissue types are prone to poor tissue adherence. **Begin with default experimental conditions**. If tissue begins to peel following Target Retrieval, reduce target retrieval time to 8 minutes. If peeling is observed following digestion, decrease digestion buffer concentration to 1 µg/mL.
4	Tissues with high density, such as cartilage and bone.	Due to the biology of these tissue types, they are prone to poor tissue adherence. **Begin with default experimental conditions**. If tissue begins to peel following Target Retrieval, reduce target retrieval time to 8 minutes. If peeling is observed following digestion, decrease digestion buffer concentration to 1 µg/mL.
5	Tissues with low pH, typically associated with stomach- related diseases and cancers.	Use **default experimental conditions**. However, due to the effect of low pH on these tissue types, the RNA counts will typically be lower.
6	Tissues with feces: normal intestine, colon, and inflammatory	Use **default experimental conditions**. However, due to the pre-fixation steps required for these tissue types, the counts

	Tissue Types	Suggested Modification
	bowel disease (IBD).	will typically be lower.
7	Tissues that exhibit high autofluorescence, such as the placenta and non-human primate (NHP) tissues.	Use **default experimental condition**s during slide preparation. A pre-bleaching configuration with a longer pre-bleaching time may be required. In addition, the use of a Hydrogen Peroxide pre- treatment may be needed (see High Autofluorescence in Tissue on page 121).
8	Bone marrow should be treated similarly to cancer tissue (1) due to the stability of marrow cells.	Use **default experimental conditions** during slide preparation. Due to the decalcification of the tissue, lower counts may be observed.
9	Human tissue with long ischemic time and samples where poor tissue quality is observed prior to sample preparation.	Consider using mouse tissue with the appropriate panel, which may allow shorter ischemic time, to improve data quality.
10	Cell pellet arrays	**Default experimental conditions**: Target Retrieval Time: 8 minutes Digestion Buffer Concentration: 1 µg/mL Digestion Time: 15 minutes Fiducial Concentration: 0.001%

Troubleshooting

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

Poor Tissue Adherence

Possible Causes: Inadequate baking time, inherent biology of sample type.

Suggested Actions:

- For FFPE tissue optimal performance, after sectioning and prior to use or storage, bake slides at 37°C overnight at an angle no greater than 45 degrees. Alternatively, slides can be baked at 37°C for 2 hours and then dried overnight at room temperature. A polyethylene slide holder (VWR, 82024524) can be used for overnight drying.
- For the RNA FFPE assay, the day before beginning sample prep, a second baking step is optimal for tissue adherence. Bake sections on slides overnight in a 60°C drying oven, vertically in a slide rack overnight or in a slide holder at a 45 degree angle.
- Additionally, although both Leica BOND Plus slides and VWR Superfrost Plus Micro Slide, Premium have been validated, better tissue adherence has been observed with the Leica BOND Plus slides which should be used for tissues with known poor adherence. Alternatively, although not yet validated, TOMO® Adhesion Microscope Slides, Matsunami Glass (VWR, 10748-166) have also shown improved tissue adherence in preliminary testing.
- A tissue section adhesive such as Epredia™ Tissue Section Adhesive (Fisher Scientific, 86014) can also be used to improve tissue adherence. 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.

Tissue Peeling following Digestion

Possible Causes: Digestion conditions are too harsh.

Suggested Action: If default digestion conditions result in tissue peeling, a milder digestion may improve tissue stability. Protease A digestion buffer can be used in lieu of the default digestion buffer. The digestion time remains the same.

Required Material: CosMx RNA Protease A Kit (NanoString, CMX-PRTA-R)

To prepare the Protease A digestion buffer:

1. **Resuspend Protease A** with 200 µL of Protease A Buffer.
2. **Create Protease A working stock**: dilute rehydrated Protease A stock 1:50 by adding 5 µL of Protease A to 245 µL of Protease A Buffer.
3. **Dilute working stock** to a concentration of 5 µg/mL by adding 82.5 µL of Protease A working stock into 1567.5 µL of Protease A Buffer.
4. Place slides into slide insert of hybridization tray and, using a P200 pipette, slowly **add 400 µL of Protease A digestion buffer** to the tissue within incubation frame. Gently move tray side to side as needed to **ensure that digestion buffer covers the entire tissue**.
5. Insert hybridization tray containing slides into hybridization oven and **incubate 40°C following the default digestion time**.
6. Continue with sample preparation as written.

High Autofluorescence in Tissue

Possible Causes: Inherent biology of sample type (e.g. liver, brain, lung), high RBC content.

Suggested Action: Highly autofluorescent tissues can be treated with hydrogen peroxide (H_2O_2) during the tissue deparaffinization step: Day 1: Deparaffinize FFPE Tissue Sections (20 minutes) on page 32. The H_2O_2 treatment has been shown to reduce false codes by about 50% in high autofluorescence tissues; however, a reduction to RNA counts was also observed.

Required Material:

- 50% H_2O_2 (Sigma, 516813) (protect from light, keep at 4°C when not in use)
- 10% Tween-20 (Teknova© ,T0710, or similar)
- 1 M Tris-HCl, pH 7.5 (ThermoFisher, 15567027)

Solution preparation:

Prepare a Tris-HCl-Tween solution (wash solution):

Table 34: Tris-HCl-Tween Wash Solution

Reagent	Initial Concentration	Final Concentration	Volume to Add (mL)
1 M Tris-HCl, pH 7.5	1M (1000 mM)	10 mM	2.5 mL
10% Tween-20	10%	0.05%	1.25 mL
DEPC-Treated Water	n/a	n/a	246.25 mL
Total Volume			250 mL

Procedure:

1. During Prepare Equipment and Washes step, fill staining jar with 50 mL of Tris-HCl wash solution and preheat to 60°C for at least 10 minutes in a water bath.
2. Proceed with xylene and ethanol washing of slides.
3. Dry slides in slide rack in 60°C oven for 5 minutes.
4. At 3 minutes after drying starts, add 250 µL of 50% H_2O_2 to preheated wash solution. Mix thoroughly. Keep solution heated at 60°C.
5. After drying finishes, move slides to H_2O_2-containing wash solution, **incubate at 60°C for 5 minutes**.
6. Transfer slides to a staining jar with fresh Tris-HCl wash solution (room temperature) and wash by gently moving slides up and down for 10 seconds.
7. Continue to Perform Target Retrieval (50 minutes) on page 36.

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