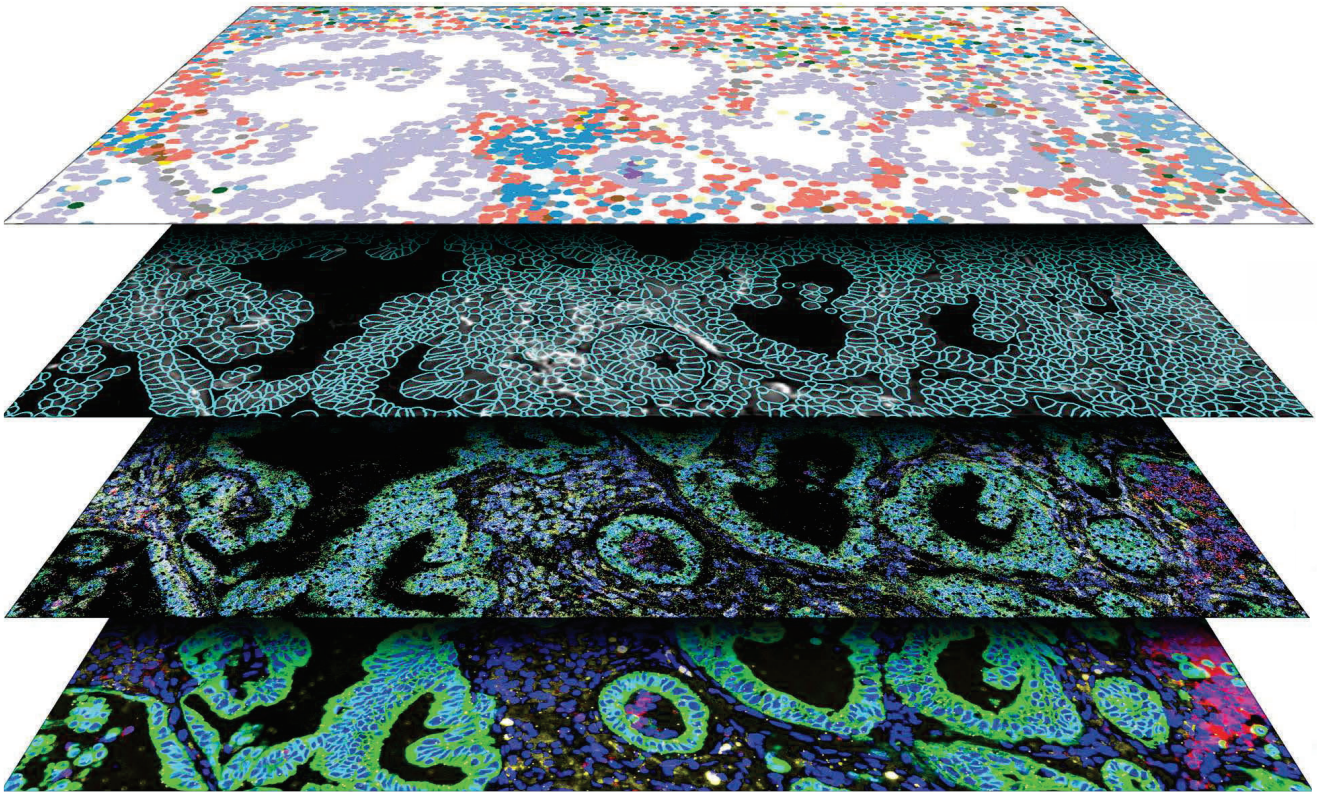
Manual Slide Preparation



MAN-10159-02 | July 2023

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NanoString Technologies, Inc.
530 Fairview Ave N
Seattle, Washington 98109

www.nanostring.com

T:888.358.NANO (6266)
F: 206.378.6288
E: support@nanostring.com

Sales Contacts

United States: us.sales@nanostring.com
EMEA: europe.sales@nanostring.com
Asia Pacific & Japan: apac.sales@nanostring.com
Other Regions: info@nanostring.com

EU Authorized Representative

NanoString Technologies
Germany Gmbh
Birketweg 31
80639 Munich
Germany

UK Authorized Representative

NanoString Technologies
Europe Limited
11th Floor Whitefriars
Lewins Mead
Bristol BS1 2NT
United Kingdom

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Table of Contents

Changes in this Revision

This CosMx SMI Manual Slide Preparation User Manual (MAN-10159) covers protein and RNA sample preparation using manual (non- automated) methods. The CosMx SMI Semi- Automated Slide Preparation User Manual (MAN-10160) covers sample preparation using semi-automated methods on the BOND RX/RX^{m} Fully Automated IHC/ISH Stainer from Leica Biosystems®.

Changes in this manual revision include:

- Addition of Protein Fresh Frozen Assay (validated for Mouse Neuro) on page 134.
- Launch of the Mouse Neuro Assay for Protein FFPE and fresh frozen tissue.
- Addition of Protein Custom Assay workflow. See Appendix IV: Adding Custom Barcoded Antibodies on page 177.
- Removal of second NHS- Acetate step to streamline workflow for RNA FFPE and RNA Fresh Frozen assays.
- Updated FFPE Slide Prep Kit (RNA) contents to reflect new commercial configuration.
- Extended acceptable protein storage time based on R&D testing.

Conventions

The following conventions are used throughout this manual and are described for your reference.

Bold text is typically used to highlight a specific button, keystroke, or menu option. It may also be used to highlight important text or terms.

Blue underlined text is typically used to highlight links and/or references to other sections of the manual. It may also be used to highlight references to other manuals and/or instructional material.

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

⚠ **WARNING**: This symbol indicates the potential for bodily injury or damage to the instrument if the instructions are not followed correctly. Always carefully read and follow the instructions accompanied by this symbol to avoid potential hazards.

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

Safety

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

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

Introduction to CosMx SMI Slide Preparation

The CosMx™ SMI platform is an integrated system with cyclic in situ hybridization chemistry, a high-resolution imaging readout instrument, and an interactive data analysis and visualization software. The CosMx SMI platform enables rapid quantification and visualization of up to 1,000 RNA and 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.

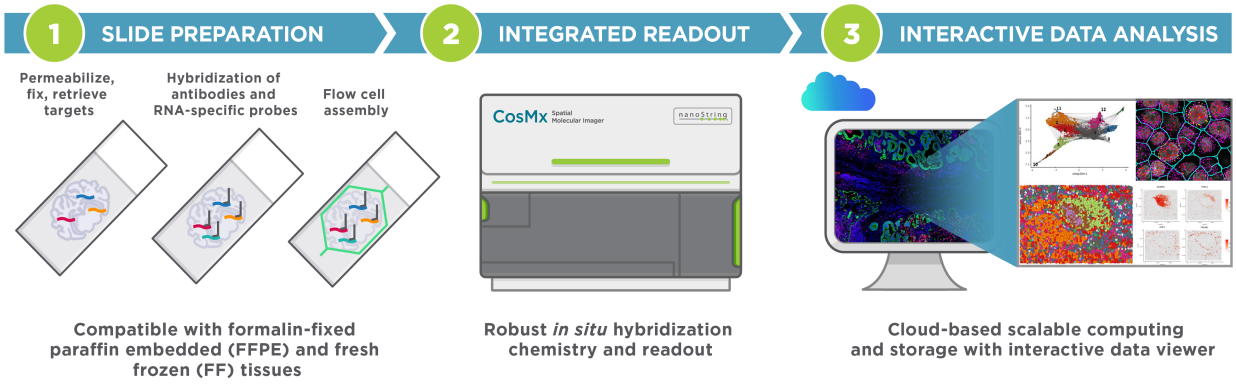


Figure 1: CosMx™ SMI is an integrated system that includes validated reagents and consumables, an instrument for chemistry and readout, and an interactive cloud-based software suite for data analysis.

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 for analysis and visualization. Within the AtoMx Platform, users can incorporate custom analysis workflows.

The CosMx™ Spatial Molecular Imager is the first platform to demonstrate simultaneous single-cell and sub-cellular detection of 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 User Manual MAN-10159 CosMx SMI Semi-Automated Slide Preparation User Manual MAN-10160
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 Spatial Informatics Platform User Manual (MAN-10170).

Panel and Cell Segmentation Marker Selection

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

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

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

- Swap in 7 to 10 user-defined genes to the 100-plex RNA panel or swap in 7 to 50 user-defined genes to the 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 turn-key solution that provides 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.

CosMx SMI Cell Segmentation Marker Selection

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

Table 1: CosMx SMI Cell Segmentation and Supplemental Marker Kits

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

Kit Description	Kit Components
CosMx™ Mouse Neuroscience Cell Segmentation Kit (Protein) Channels 1 and 2	CosMx DAPI Nuclear Stain, Ch1
	CosMx Mm S6 Neuro Marker, Ch2 (Protein)
CosMx™ Mouse Neuroscience Segmentation (Protein) Kit Channels 3 and 4 (required)	CosMx Mm GFAP Marker, Ch3
	CosMx Mm IBA1 Marker, Ch4 (Protein)
CosMx™ Mouse NeuN A La Carte Marker (Protein) Channel 5 (required)	CosMx Mm NeuN Marker, Ch5 (Protein)

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

Table 2: A La Carte Markers

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

Additional markers may be available to meet specific project needs.

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

RNA FFPE Manual Slide Preparation

CosMx SMI Slide Preparation Workflow

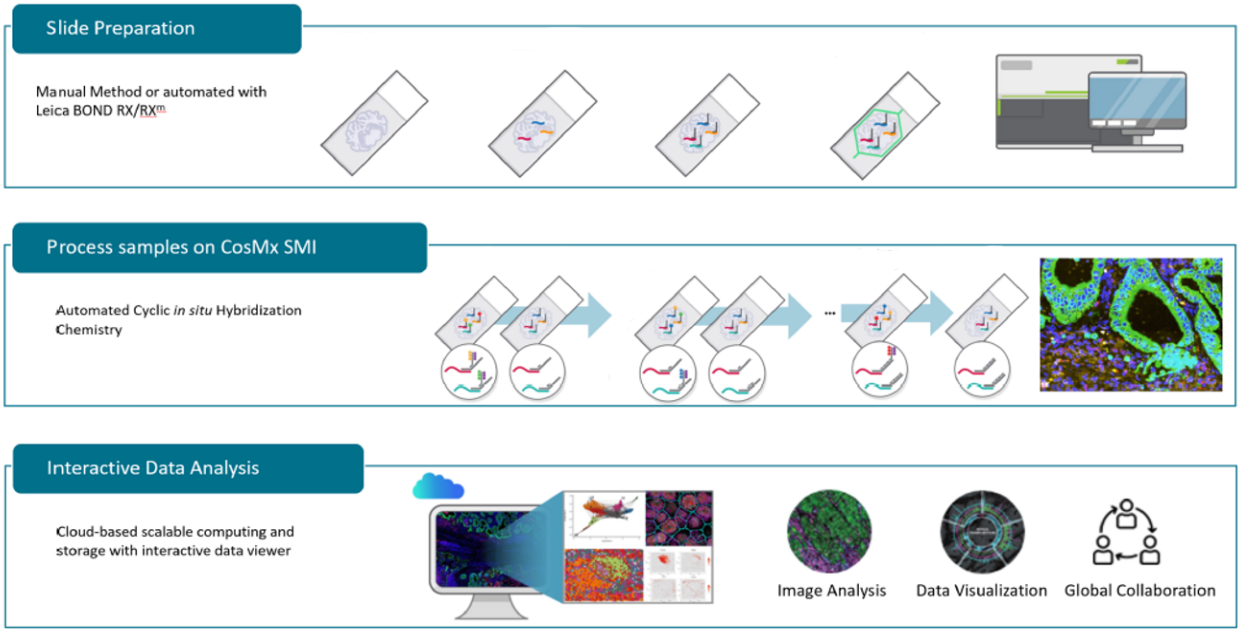


Figure 2: CosMx SMI Workflow Overview

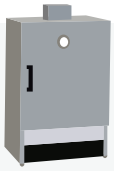
Day 1: Slide Preparation. Prepare slides and incubate biological targets with RNA specific probes. Prepare manually or using the BOND RX/RX ^{m} fully automated IHC/ISH stainer from Leica Biosystems (BOND RX/RX^{m}).

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.

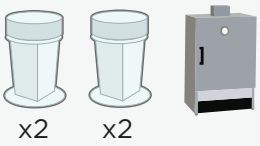
Day 0: Prepare Reagents and Overnight Tissue Bake

- Prepare shelf stable reagents

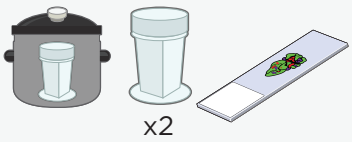


- Bake slides overnight at 60°C to improve tissue adherence

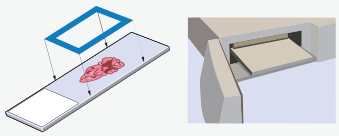
Day 1: Slide Preparation



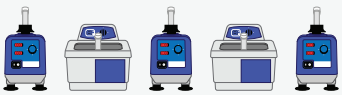
Remove Paraffin
- Xylene and EtOH washes
- Dry Slides 5 min at 60°C



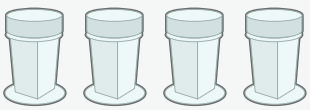
Target Retrieval
- 15 mins at 100°C
- H2O rinse and 3 min EtOH wash
- Dry for 30 min-1hour



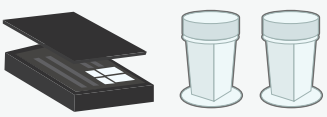
Protease Digestion
- Apply incubation frame
- Apply digestion buffer
- Incubate at 40°C for 30 mins
- Wash 2X in 2X SSC



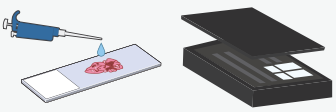
Apply Fiducials
- Prepare and apply fiducials
- Incubate for 5 minutes
- Wash with 1X PBS



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

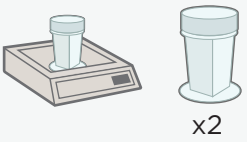


Blocking
- Prepare and apply NHS-Acetate
- Incubate for 15 mins.
- 2 washes, 5 mins each, in 2X SSC



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

Day 2: Wash and Stain Slide

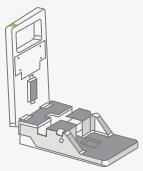


Stringent Washes
- 2 stringent washes, 25 mins each.
- 2 washes, 2 mins each, 2X SSC

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.

Prepare Instrument Reagents
- Add enzymes to Buffer 4.
- Add RNase Inhibitor to imaging tray.

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

Equipment, Materials, and Reagents

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

Equipment:

Table 3: Equipment not provided by NanoString

	Equipment	Source	Part Number(s)
☐	**Baking oven**	Quincy Lab,Inc.® (or comparable)	Example
☐	**Hybridization oven including hybridization chamber** • RapidFISH Slide Hybridizer **or** • HybEZ™ oven • Humidity control tray NOTE: These hybridization ovens are designed to keep the slides hydrated and maintain a precise temperature overnight. NanoString does not recommend the use of any other hybridization ovens for CosMx SMI slide preparation.	Boekel Scientific® or ACDBio™	240200 for 120V or 240200-2 for 230V 321710/321720 310012
☐	**6-quart pressure cooker** NOTE: Products from other vendors may require testing and optimization OPTIONAL: A steamer may be used in lieu of a pressure cooker and may be preferred with more fragile tissues. If a steamer is used, a thermometer will also be needed.	BioSB® TintoRetriever Nesco® Hamilton Beach®	BSB 7015 ST-25F 3753OZ
☐	**Ultrasonic bath** (500 mL capacity) NOTE: 40kHz frequency with timer	General Lab Supplier	Example (CPX-952-118R)
☐	**Vortex mixer**	General Lab Supplier	Various
☐	**Microcentrifuge** for 1.5 mL microcentrifuge tubes and 8-well PCR strip tubes	General Lab Supplier	Various

	Equipment	Source	Part Number(s)
☐	**Water bath** Temperature setting of 37°C	General Lab Supplier	Various
☐	**Thermal cycler** Must include a 96-well 200 µL tube block	General Lab Supplier	Various
☐	**Analytic scale with draft shield** NOTE: ensure scale can measure in milligrams (mg) so that reagents can be weighed accurately.	Various	Example

Materials:

Table 4: Materials not provided by NanoString

	Materials	Source	Part Number(s)
☐	**Pipettes for 2.0 – 1,000 µL**	General Lab Supplier	Various
☐	**Filter tips** (RNase/DNase Free)	General Lab Supplier	Various
☐	**2.0 mL Centrifuge tubes** (RNase/DNase Free)	General Lab Supplier	Various
☐	**0.2 mL PCR tubes** or PCR strip tubes	General Lab Supplier	Various
☐	**VWR® Superfrost® Plus Micro Slide, Premium** or **Leica BOND PLUS slides** NOTE: these slides have been validated by NanoString. Do not use other products.	VWR™ Leica® Biosystems	48311-703 S21.2113.A
☐	**Slide rack**	General Lab Supplier	Example
☐	**Polypropylene slide staining jars** (24 required) or	Ted Pella®	21029

	Materials	Source	Part Number(s)
	Slide staining station NOTE: due to the photo-sensitivity of this assay, the staining jars should be impermeable to light.	Amazon® Fisher Scientific	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 5: Reagents not provided by NanoString

	Reagent	Source / Part Number(s)	Storage Conditions
☐	**DEPC-treated water**	ThermoFisher Scientific, AM9922 (or comparable)	Room temperature
☐	**100% Ethanol** (EtOH): ACS grade or Better 	General Lab Supplier	Flammable Storage Room temperature
☐	**10X Phosphate Buffered Saline** pH 7.4 (PBS)	ThermoFisher Scientific, AM9625 (or comparable)	Room temperature
☐	**Xylene**  NOTE: Citrisolv can be used, however, follow the alternative workflow for Citrisolv for Day 1: Deparaffinize FFPE Tissue Sections (20 minutes) on page 33.	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: NHS-Acetate powder is shipped in a plastic bag with a desiccant and should be left in the bag and stored at -20°C until ready to use.	Fisher Scientific™, 26777	-20°C

	Reagent	Source / Part Number(s)	Storage Conditions
☐	**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) Box 1 of 2

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

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

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

CosMx FFPE Slide Prep Kit Box 2 of 2

Figure 4: CosMx Proteinase K (Box 2/2)

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

Kit Contents (Store at -20°C)
CosMx Proteinase K

CosMx RNase Inhibitor

Figure 5: CosMx RNase Inhibitor

Table 8: 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 Panel (see Panel and Cell Segmentation Marker Selection on page 12).

Figure 6: CosMx Human IO Panel (RNA)

Table 9: CosMx RNA Probe Mix

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

CosMx Segmentation and Supplemental Markers

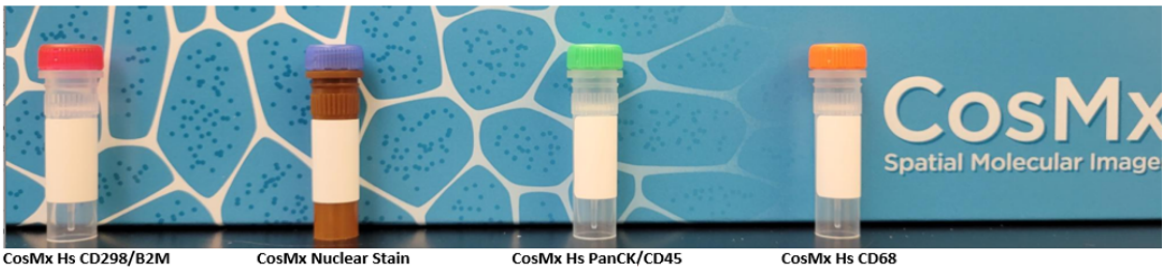


Figure 7: CosMx Segmentation and Supplemental Markers (RNA)

Table 10: CosMx Segmentation and Supplemental Markers (RNA)

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

Flow-Cell Assembly Tool and Kit

Figure 8: 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 Tissue Samples

Appendix I: CosMx SMI Sample Preparation Guidelines on page 170 covers FFPE block selection and sectioning in detail. Review these guidelines as needed prior to beginning the FFPE Slide Preparation procedure.

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

Tissue Sectioning and Slide Preparation:

FFPE blocks should be sectioned at **5 µm thickness** and mounted on the label side of VWR Superfrost Plus Micro Slides or Leica BOND PLUS 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) of the slide and be no larger than **20 mm Long by 15 mm Wide** (Figure 9) (image not to scale, see the template on 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 center of the scan area (the dashed teal line). For examples of tissue placement best practices, see Appendix I: CosMx SMI Sample Preparation Guidelines on page 170.

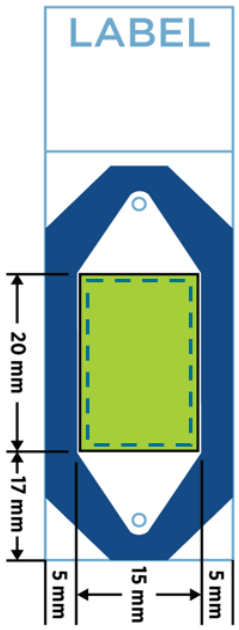


Figure 9: Tissue Scan Area (not to scale)

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 the slide preparation as outlined. Directly before applying the incubation frame, scrape off parts of the tissue exceeding the scan area. Scraping off tissue before target retrieval and ethanol drying could generate tissue folds that may result in staining/binding artifacts, while suboptimal scraping may result in poor incubation frame adherence. Applying the incubation frame over tissue could result in tissue damage when the frame is removed 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 guidelines.

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

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

Prepare shelf stable reagents. See Prepare RNA Assay Reagents on page 30.

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

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

> NOTE: the 60°C tissue baking step the day before slide preparation has shown to increase tissue adherence and stability during the slide preparation protocol. If overnight baking is not possible, bake slides for at least 2 hours on slide preparation day 1 before continuing to Day 1: Deparaffinize FFPE Tissue Sections (20 minutes) on page 33. The 2 hours bake has not been validated by NanoString but is preferable to skipping the second bake.

Prepare RNA Assay Reagents

> **IMPORTANT**: Take care to maintain nuclease-free conditions. The greatest risk of contamination comes from CosMx SMI RNA probes and other oligos. NanoString recommends the use of RNase AWAY (Thermo Fisher 7003PK), as it will limit contamination from oligos, detection probes, and nucleases. After using RNase AWAY, allow to air dry completely, or rinse with DEPC-treated water. See manufacturer's instructions for details.

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

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

Table 11: 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 kept at -20°C with a desiccant until use.	-20°C in dessicant

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

	Reagent	Dilution	Storage
		µg/mL by adding 2 µL of Proteinase K stock to 198 µL of 1X PBS. • **Step 2**: Dilute the 200 µg/mL solution made in step 1 to the target concentration of 3 µg/mL by adding 30.0 µL of the 200 µg/mL solution to 1970 µL of 1X PBS. • Mix thoroughly by inverting tube or pipetting up and down using a clean pipetter tip. **Do not vortex.** NOTE: Protease Mix should be prepared fresh daily and stored on ice until ready to use. *NOTE: This concentration may differ for some tissue types, including CPA samples (see Appendix II: Tissue Specific Digestion (RNA Assay) on page 175).	
☐	Fiducials and Hybridizaton Mix	These reagents have additional steps that will be covered in detail in their respective sections. Remove ISH Probe Mix from storage and keep on ice until ready to use. NOTE: Fiducials are light sensitive and should be kept stored, protected from light, until instructed to remove.	n/a
☐	Day 2 Reagents	These reagents have additional steps that will be covered in detail in their respective steps. Follow Day 2 procedure for preparation of these reagents.	n/a

Day 1: Deparaffinize FFPE Tissue Sections (20 minutes)

You will need the following materials and reagents for this step: **staining jars, Xylene and 100% EtOH. 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 RNA Assay Reagents on page 30).

Before beginning, slides should have already been baked overnight (see Slide Preparation Day 0: Prepare Shelf Stable Reagents and Overnight Tissue Bake on page 29). If slides were not baked overnight, slides can be baked for 2 hours at 60°C; however, this is not optimal and may have a negative impact on tissue adherence.

Prepare Equipment and Washes

1. **Prepare hybridization tray** by lining the bottom of the tray with Kimwipes and carefully wet the Kimwipes with 2X SSC or DEPC-treated water. Kimwipes should be thoroughly damp but standing buffer should not be present.
2. **Preheat hybridization chamber and tray to 40°C** following manufacturer's instructions.
3. Ensure baking oven is still set to 60°C.
4. **Prepare staining jars with enough Xylene and Ethanol to cover** tissue (Figure 10). Ensure you have sufficient buffer to cover all slides in container for the washes. Wash buffers need to completely cover the tissue on the slide but should be below the slide label. The washes may make the slide labels illegible if submerged.

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

5. 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 will be 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 will take 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 37.

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 10).

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

   - Place slides into **first Xylene jar and wash for 5 minutes**.
   - **Repeat** the 5 minute wash with a new staining jar containing Xylene.
   - After second Xylene wash, **transfer slides to first 100% Ethanol** wash and wash slides for **2 minutes***.
   - **Repeat** 2 minute* wash with a new staining jar containing Ethanol.

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

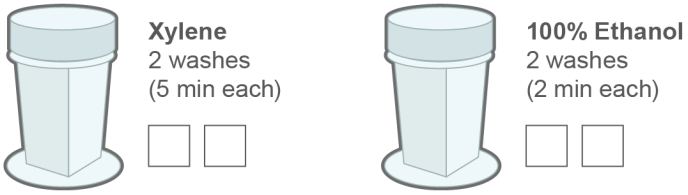


Figure 10: Deparaffinize FFPE Tissue Sections

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

2. **Dry slides** in slide rack in 60°C oven for **5 minutes.** After 5 minutes, remove slides from oven and leave at room temperature until target retrieval solution has been pre-heated to 100°C.
3. **If not already aliquoted, remove stock NHS-Acetate powder from -20°C freezer** and leave at room temperature for 1-2 hours before use in NHS-Acetate Preparation and Application (25 minutes) on page 47. Pre-aliquoted NHS-Acetate powder can remain at -20°C until instructed to remove on on page 47.

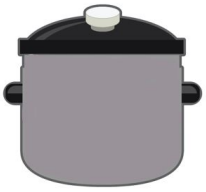
Perform Target Retrieval (50 minutes)

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% EtOH.**

> 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.

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

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

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.

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

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.**


Target Retrieval

3. Return the staining jar containing the slides **into the preheated pressure cooker.** Ensure that water level is well below lid of jar. About halfway up jar will be sufficient. If needed, a trivet may be used to raise the jar.

4. Reattach the pressure cooker lid, **open the pressure release valve to *Pressure Release* position** and return the pressure cooker to 100°C. For the BioSB, this can take up 20 minutes.

5. **Once the pressure cooker temperature returns to 100°C**, start timer and run for **15 minutes** for FFPE tissue or 8 minutes for cell pellet arrays (CPA).

6. When the timer reaches zero, press cancel on pressure cooker to stop heating, **carefully remove the pressure cooker lid and remove the staining jar**.

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

3. Remove the foil from the Target Retrieval jar and quickly transfer the slides to the Target Retrieval solution. Replace the foil, then replace steamer lid.

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

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 (see Figure 11).

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

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

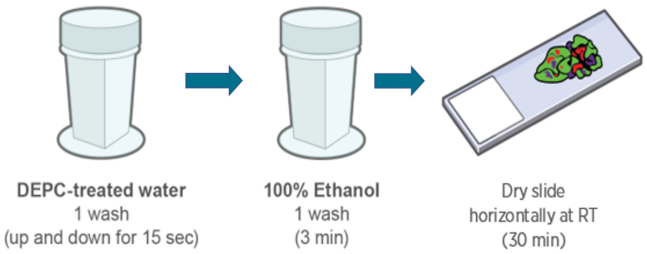


Figure 11: Ethanol Wash

11. While slides are drying, prepare digestion buffer (see Prepare RNA Assay Reagents on page 30).

Tissue Permeabilization (40 minutes)

You will need the following materials and reagents for this step: **staining jars, hybridization oven, hybridization tray, incubation frames** (CosMx FFPE Slide Prep Kit (RNA) Box 1/2, Stored at 4°C), **digestion buffer** (see Prepare RNA Assay Reagents on page 30), and **DEPC-treated water.**

1. If needed, trim the tissue following the template in **Prepare Tissue Samples on page 27**.
    - 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 12).
    - 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.

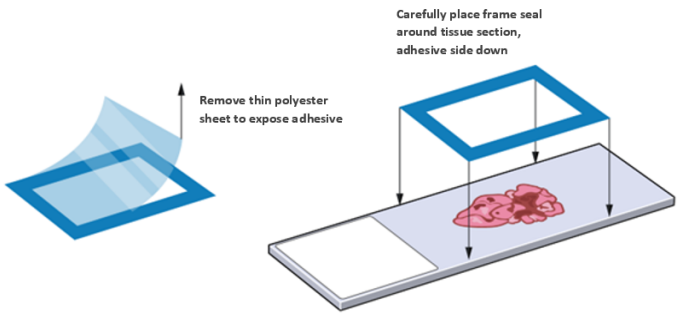


Figure 12: Apply Incubation Frame

5. With the slide still on a flat surface, use a clean razor blade to **carefully trim the long edges of the incubation frame to remove excess plastic** ensuring that there is no excess film extending over slide edges. Trim the short end of the incubation frame (opposite the slide label) as needed.
6. Remove digestion buffer from ice and **warm digestion buffer** by hand for about 3 minutes to bring the mixture to room temperature.
7. **Retrieve preheated hybridization tray** from hybridization oven.
8. Place slides into slide insert of hybridization tray and, using a P200 pipette, slowly **add 400 µL of digestion buffer to the tissue within incubation frame** (Figure 13). Gently move tray side to side as needed to **ensure that digestion buffer covers the entire tissue.**

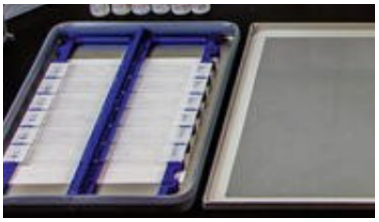


Figure 13: Hybridization Tray

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

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

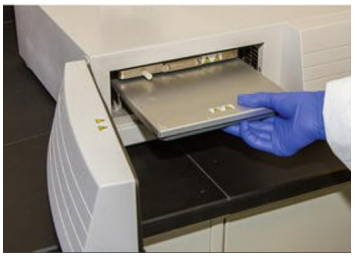


Figure 14: Hybridization Oven

Table 12: Digestion Times

Tissue Type	Digestion Time
Lymph node	15 minutes
Cell Pellet Array (CPA)	15 minutes
Tonsil	30 minutes

Tissue Type	Digestion Time
All other tissue types*	30 minutes

*NOTE: Incubation times and temperatures may differ by tissue and may need to be empirically determined. For this protocol, start with the default conditions: **30 minutes at 40°C** and adjust the time and concentration as needed. See Appendix II: Tissue Specific Digestion (RNA Assay) on page 175 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 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 43.
12. After incubation, tap off excess digestion buffer **one slide at a time** and transfer slides to staining jar containing fresh DEPC-treated water.
13. Move slides up and down 3-5 times to wash and **repeat with a new jar of DEPC-treated water (Figure 15).**

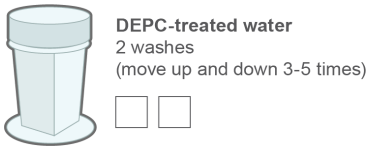


Figure 15: DEPC-treated water wash

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

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

Fiducial Preparation and Application (20 minutes)

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

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

1. **Use the graphic below to vortex and sonicate the fiducials prior to use** (Figure 16):

> ⓘ **IMPORTANT**: When sonicating the fiducial tube, be sure to not 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.

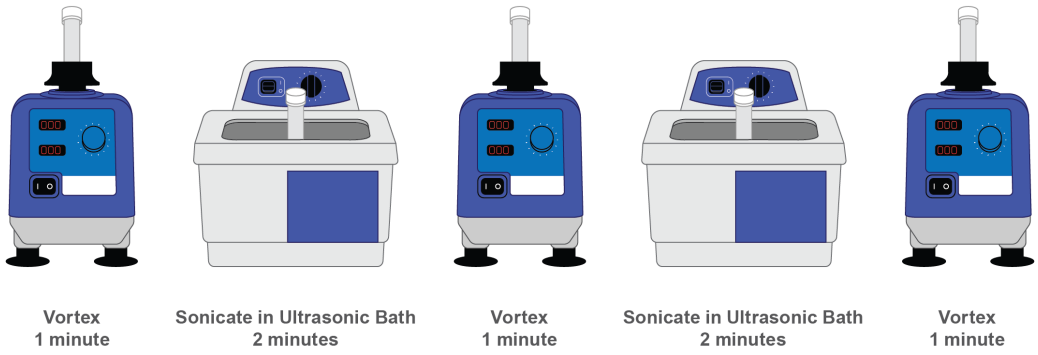


Figure 16: Fiducial preparation

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

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

NOTE:The concentration may differ for some tissue types and need to be empirically determined. For this protocol, start with the default concentration of 0.001% and adjust the concentration as needed. See Appendix III: Tissue Specific Fiducial Concentrations (RNA Assay) on page 176 for suggested tissue specific concentrations based on R&D preliminary testing.

3. Remove slide from DEPC and gently tap tissue on a clean Kimwipe to remove excess water. Lay slide horizontally in staining tray.
4. **Immediately before applying fiducials to slides, vortex tube for 1 additional minute**. Vortex fiducials for 30 seconds between slides to keep fiducials in suspension and ensure consistent concentration across all slides.
5. **Apply up to 250 µL of the final fiducial solution, ensuring the solution covers glass and tissue within the incubation frame**. Fiducials must be present on the glass within the scan area for consistent focusing during the instrument run.

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

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

6. **Incubate covered in staining tray for 5 minutes at room temperature.** During fiducial incubation, set up 1X PBS jar and post-fix reagents.

Figure 17: Incubate covered for 5 minutes

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

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

Proceed to next steps immediately.

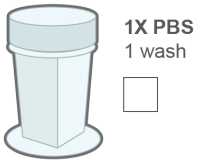


Figure 18: PBS Wash (1 minute)

Post-Fixation (20 minutes)

You will need the following materials and reagents for this step: **staining jars, 10% NBF, NBF Stop Buffer (Tris-Glycine Buffer)** and **1X PBS** (see Prepare RNA Assay Reagents on page 30 for more information).

> ⚠ **WARNING**: Use of appropriate personal protective equipment is advised. Used NBF Stop Buffer contains NBF and must be disposed of in the same manner as the NBF.

Post-fix the tissue by performing the following washes (Figure 19).

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

Figure 19: NBF Post Fix

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

NHS-Acetate Preparation and Application (25 minutes)

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

1. **Prepare 100 mM NHS-Acetate mixture** immediately before you are ready to apply the mixture onto the tissue (200 µL/sample). Ensure stock NHS-Acetate has reached room temperature before aliquoting to prevent condensation.
   a. NHS-Acetate powder is stored desiccated at -20°C. If not already done, prepare 25 mg aliquots (for a 4-slide preparation) of NHS-Acetate powder by weighing out the powder directly into 4 2.0 mL centrifuge tubes. Label tubes with the exact weight, seal tubes with parafilm, and store tubes at -20°C in desiccant for later use. See Prepare RNA Assay Reagents on page 30.

      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 NHS-Acetate powder by multiplying the weight of NHS-Acetate powder in mg by 38.5.
         - Example: for 25.0 mg of NHS-Acetate powder: 25.0 * 38.5 = 962.5 µL of buffer to add.
   c. Slowly pipette up and down to mix. Bubbles may occur. Do not fully dispense liquid from pipette while mixing.

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

2. Perform the following steps **one slide at a time** to prevent the tissue from drying out:
   - **Remove slide from 1X PBS,** gently tap slide on a clean kimwipe to remove excess buffer, and transfer to a clean staining tray.

- **Apply 200-250 µL of NHS-Acetate mixture** onto the tissue within the incubation frame. Gently rock the tray side to side as needed to ensure that the NHS-Acetate solution covers the entire tissue.
- Repeat with remaining slides and **incubate covered in staining tray for 15 minutes at room temperature** (Figure 20).

Figure 20: Incubate 15 minutes

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

Figure 21: 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)

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

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

ⓘ **IMPORTANT:** Take care to maintain nuclease-free conditions. Areas should be cleaned thoroughly with RNase AWAY after probe mix formulation (RNaseZap is only effective for enzymes, not oligos, and should not be used in place of RNase AWAY). Alternatively, mixes can be made in PCR workstations that have been decontaminated with UV light. Gloves should also be changed after handling any probe mixes to avoid cross-contamination.

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

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

Set the hybridization oven temperature to 37°C according to product instructions. The hybridization ovens listed in the Equipment section are not light-permeable. If your chamber is light-permeable, minimize light exposure (e.g., by wrapping the lid in aluminum foil).

1. Pre-heat thermal cycler and lid to 95°C.
2. Remove an **incubation frame cover** and clean with Ethanol. Dry with a clean Kimwipe and visually inspect the cover for dust. Use a new Kimwipe as needed to remove any dust. Lay **incubation frame cover** on a clean Kimwipe until use.
3. Flick to mix probes and centrifuge before using. **Do not vortex probes**.

4. **Denature CosMx RNA probe mixes** (RNA Probe Mix, RNA Add-On, rRNA Segmentation Markers (Mm Neuro Panel only), and any custom add-ons) by transferring totalvolumes needed for assay (Table 13), from stock tubes into clean 0.2 mL PCR tubes **(probes and add-ons should be kept separate during denaturing**).

> ⓘ **IMPORTANT**: Ensure accurate pipetting. When preparing 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. Make hybridization solution (Table 13).

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

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

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

If using the **Mouse Neuro Panel, an rRNA marker will be used in lieu of DEPC-treated water. Reference Table 27 on Page 93 in the Fresh Frozen Protocol for the hybridization solution used for the **Mouse Neuro assay**.

7. **Clean all equipment** and benchtop with RNase AWAY and allow to dry; or rinse with DEPC-treated water (see **IMPORTANT** note, above). **The hybridization chamber can be a key source of contamination by oligos**. Arrange fresh Kimwipes on bottom of the chamber. Change gloves and clean workspace with RNase AWAY.
8. **Wet the Kimwipes with 2X SSC or DEPC-treated water**. Take care that the Kimwipes and 2X SSC do not contact the slides. Kimwipes should be thoroughly wet, but standing buffer should not be present.
9. To prevent the tissue from drying, perform the following steps **one slide at a time.**
   - Remove slides from 2X SSC, **gently tap slide to remove excess liquid.**
   - Carefully remove the polyester frame backing (with the center square removed) from the incubation frame to expose the top adhesive layer of the incubation frame. **Ensure that the incubation frame does not lift from the slide when removing the polyester frame backing.**

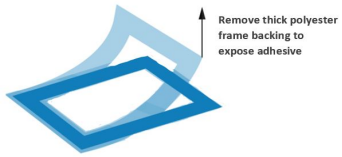


Figure 22: Remove Polyester Frame backing from Incubation Frame

   - 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 23).

     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 23). 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.**

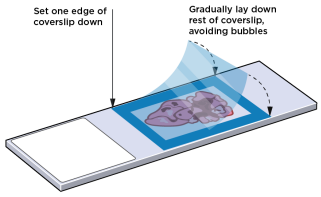


Figure 23: Apply Incubation Frame Cover

- Place the slide horizontally into the hybridization tray (Figure 24).

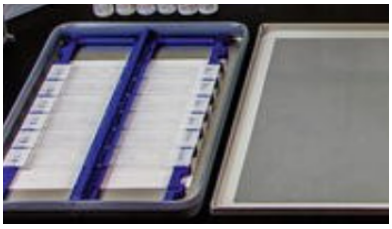


Figure 24: Hybridization Tray

- **Repeat step 9 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.

10. Close hybridization chamber, insert tray into oven, and clamp tray into place. **Incubate at 37°C overnight** (16 – 18 hours) (Figure 25).

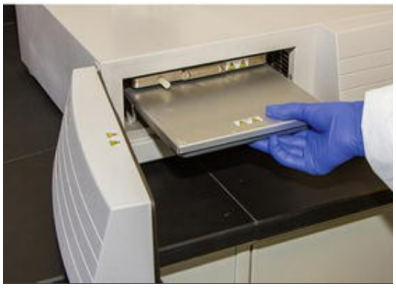


Figure 25: Incubate overnight at 37°C

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

If not already done, prepare 40 mL formamide aliquots following the instructions in Equipment, Materials, and Reagents on page 18 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 Assay Reagents on page 30).

> ⚠ **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**.

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

4. **Preheat staining jars containing freshly prepared stringent wash in 37°C water bath**. It will take about 30 minutes to preheat wash.
5. If nearing the 18 hour maximum overnight incubation time, while jars are preheating, **transfer slides to 2X SSC.**
6. Perform the following steps **one slide at a time** to prevent the tissue from drying.
   - With a clean pair of forceps, carefully remove the **incubation frame cover** from the incubation frame. Dip slide 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 26). After the last wash, the slides can be stored in 2X SSC for up to one hour.
   - Gently tap each slide one at a time on a clean Kimwipe to remove excess 2X SSC and place slides in the **first stringent wash for 25 minutes**. **Repeat** wash with the second staining jar.

- During second stringent wash, begin preparing reagents for Nuclear and Cell Segmentation Staining (2 hours) on page 56.
- Following stringent washes, immediately **transfer slides to 2X SSC and wash for 2 minutes**. **Repeat** with second jar of 2X SSC. Leave slides in 2X SSC as needed until reagents have been prepared for nuclear and cell segmentation staining.

Figure 26: Perform stringent wash

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

Nuclear and Cell Segmentation Staining (2 hours)

You will need the following materials and reagents for this step: **incubation frames, staining jars, 1X PBS, Blocking Buffer** (4°C), **Nuclear Stain Stock** (-80°C), and **Segmentation Marker Kit** (-80°C) (see Prepare RNA Assay Reagents on page 30).

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

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

> **IMPORTANT**: Avoid wiping the slide within the scan area as this could remove the fiducials needed for imaging. See scan area template on the flow cell assembly tool or follow the guidelines in Prepare Tissue Samples on page 27.

   - Carefully apply a new incubation frame following the instructions in Tissue Permeabilization (40 minutes) on page 40. 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 27).

Figure 27: Cover tray and incubate for 15 minutes

8. During nuclear stain incubation, **prepare segmentation and marker stain mix** using the following table where *n* = the number of slides (Table 15). **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 and marker stain mix.

Table 15: Prepare Staining Mix

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

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

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**.

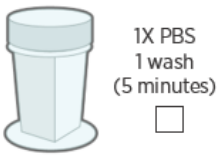


Figure 28: Wash for 5 minutes in 1X PBS

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 Staining Mix** directly to tissue. Gently move tray side to side as needed to ensure that the mix covers the entire tissue.
    - If needed, an incubation frame cover can be placed over the incubation frame to ensure that the mix completely covers the tissue.
    - Adjust volume to add as needed for tissues of varying sizes. The **segmentation and visualization stain mix needs to completely cover the tissue** but does not need to completely fill the incubation frame.
13. **Repeat** with remaining slides and cover tray (Figure 29).

Figure 29: Cover tray and incubate for 1 hour

14. **Incubate slides for 1 hour at room temperature** protected from light.
15. Following segmentation and visualization incubation, **transfer slides to 1X PBS and wash for 5 minutes** (Figure 30).

Figure 30: Wash 3x in 1X PBS

16. **Repeat wash 2 times** for a total of 3 PBS washes.
17. 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.

18. 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 Assemblyon page 165.

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).

RNA Fresh Frozen Manual Slide Preparation

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

CosMx SMI Slide Preparation Workflow

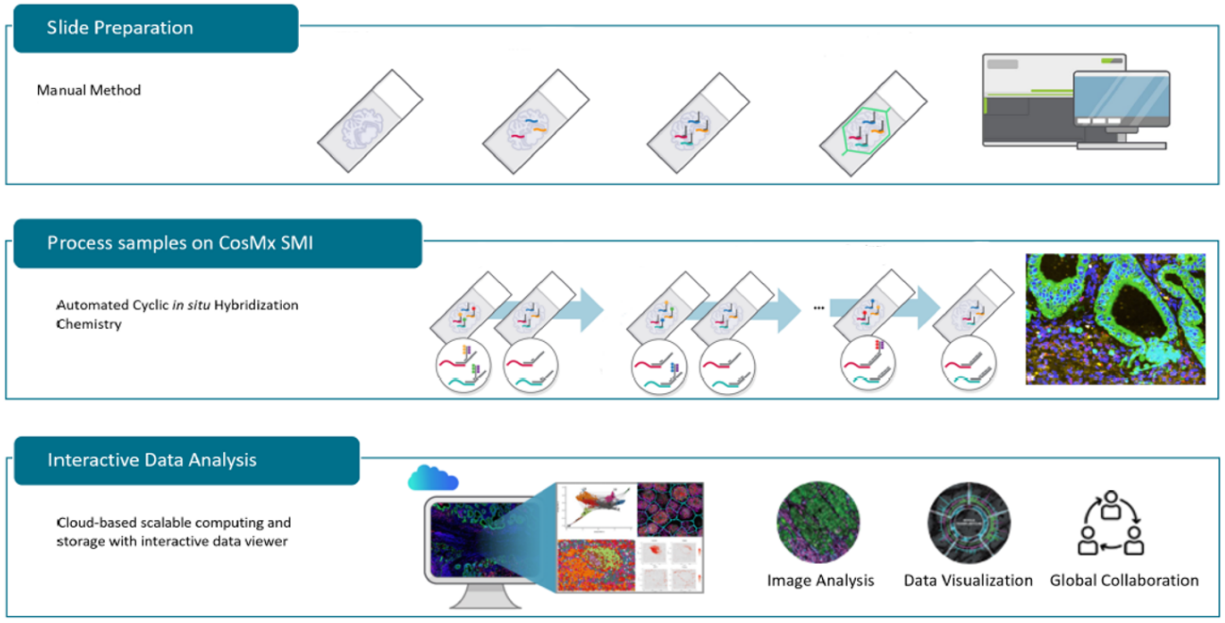


Figure 31: CosMx SMI Workflow Overview

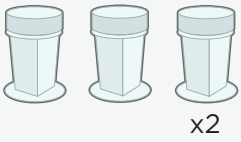
Day 1: Slide Preparation. Prepare slides and incubate biological targets with RNA specific probes.

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).

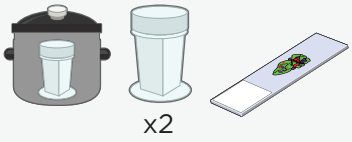
After run completion: Create a **Data Analysis** study in the AtoMx Spatial Informatics Platform (SIP) and perform quality-control checks, data analysis, and generate analysis plots.

Day 1: Slide Preparation

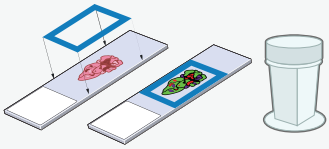
NBF Fixation and Bake
- 10% NBF Fixation
- 3 washes in 1X PBS
- 30-minute bake



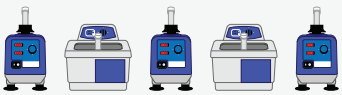
Wash and Rehydrate Tissue
- 3 washes in 1X PBS
- Wash in 4% SDS
- 3 washes in 1X PBS
- Wash in 50% EtOH
- Wash in 70% EtOH
- 3 washes in 100% EtOH



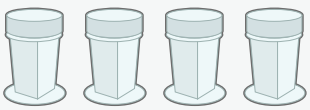
Target Retrieval
- 15 mins at 100°C
- H2O rinse and EtOH wash
- Dry for 30 min-1hour



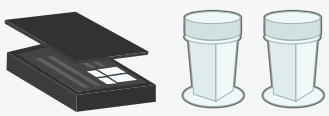
Protease Digestion
- Apply incubation frame
- Apply digestion buffer
- Incubate at RT for 30 mins
- 2 washes in 1X PBS



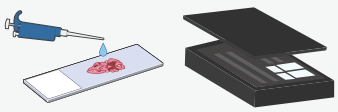
Apply Fiducials
- Prepare and apply fiducials
- Incubate for 5 minutes
- Wash with 1X PBS



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

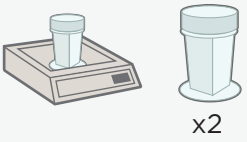


Blocking
- Prepare and apply NHS-Acetate
- Incubate for 15 mins.
- 2 washes, 5 mins each, in 2X SSC



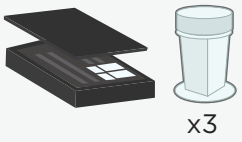
Overnight Hybridization
- Prepare and apply assay specific probes
- Incubate at 37°C overnight

Day 2: Wash and Stain Slides



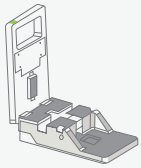
Stringent Washes
- 2 stringent washes, 25 mins each.
- 2 washes, 2 mins each, 2X SSC

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.

Prepare Instrument Reagents
- Add enzymes to Buffer 4.
- Add RNase Inhibitor to imaging tray.

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

Equipment, Materials, and Reagents

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

Equipment:

Table 16: Equipment not provided by Nanostring

	Equipment	Source	Part Number (s)
☐	**Baking Oven**	Quincy Lab, Inc (or comparable)	Various GC Models
☐	**Hybridization Oven including hybridization chamber** • RapidFISH Slide Hybridizer **or** • HybEZ oven • Humidity control tray NOTE: These hybridization ovens are designed to keep the slides hydrated and maintain a precise temperature overnight. NanoString does not recommend the use of any other hybridization ovens for CosMx SMI Sample Prep	Boekel Scientific or ACDBio	240200 for 120V or 240200-2 for 230V 321710/321720 310012
☐	**6-quart pressure cooker** NOTE: Products from other vendors may require testing and optimization OPTIONAL: A steamer may be used in lieu of a pressure cooker and may be preferred with more fragile tissues. If a steamer is used, a thermometer will also be needed.	BioSB® TintoRetriever Nesco® Hamilton Beach®	BSB 7015 ST-25F 3753OZ
☐	**Ultrasonic Bath** (500 mL capacity) NOTE: 40 kHz frequency with timer	General Lab Supplier	Example
☐	**Vortex mixer**	General Lab Supplier	Various

	Equipment	Source	Part Number (s)
☐	**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 17: Materials not provided by Nanostring

	Materials	Source	Part Number(s)
☐	**Pipettes for 2.0 – 1,000 µL**	General Lab Supplier	Various
☐	**Filter tips (RNase/DNase Free)**	General Lab Supplier	Various
☐	**2.0 mL Centrifuge Tubes (RNase/DNase Free)**	General Lab Supplier	Various
☐	**0.2 mL PCR tubes** or PCR strip tubes	General Lab Supplier	Various
☐	**VWR Superfrost Plus Micro Slide, Premium** or **Leica BOND PLUS slides** NOTE: these slides have been validated by NanoString. Do not use other products.	VWR Leica Biosystems	48311-703 S21.2113.A
☐	**Slide Rack**	General Lab Supplier	Example

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

Reagents:

Table 18: Reagents not provided by Nanostring Technologies Inc.

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

	Reagent	Source / Part Number(s)	Storage Conditions
	100% Ethanol (EtOH) (cont.)		Room temperature
☐	**10X Phosphate Buffered Saline pH 7.4 (PBS)**	ThermoFisher, AM9625 (or comparable)	Room temperature
☐	**SDS, 10% Solution, RNase-free**	ThermoFisher, AM9822	Room temperature
☐	**20X SSC (DNase, RNase free)**	Thermofisher, AM9763	Room temperature
☐	**Tris Base**	Sigma-Aldrich, 10708976001 (or comparable)	Room temperature
☐	**Glycine**	Sigma-Aldrich, G7126 (or comparable)	Room temperature
☐	**Sulfo NHS-Acetate powder** NOTE: NHS-Acetate powder is shipped in a plastic bag with a desiccant and should be left in the bag and stored at -20°C until ready to use.	Fisher Scientific, 26777	-20°C
☐	**10% Neutral Buffered Formalin (NBF)** 	EMS Diasum, Cat # 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)

Figure 32: Fresh Frozen Slide Preparation Kit (RNA) Box 1/2

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

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

CosMx Fresh Frozen RNA Slide Prep Kit Box 2 of 2

Figure 33: CosMx Proteinase K, Box 2/2

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

Kit Contents (Store at -20°C)
CosMx Proteinase K

CosMx RNase Inhibitor

Figure 34: 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 Probe Mix and/or Custom RNA Probe (see Panel and Cell Segmentation Marker Selection on page 12).

Figure 35: CosMx Mouse Neuro RNA Probe Mix

Table 22: CosMx Mouse Neuro RNA Probe Mix

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

CosMx Segmentation and Supplemental Markers

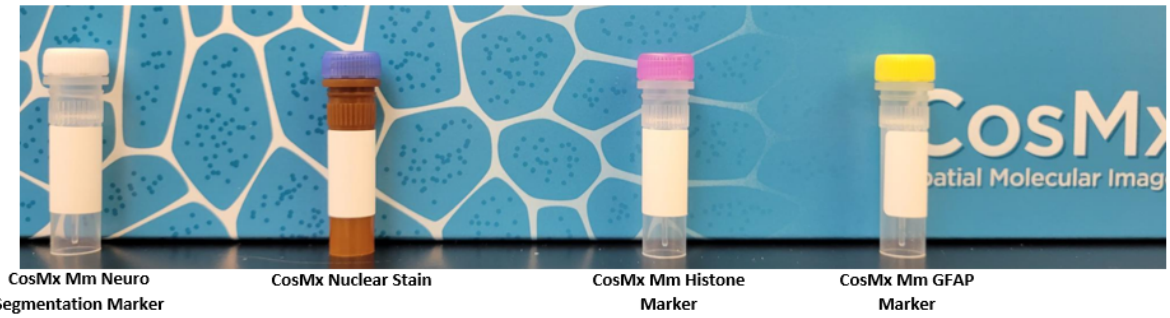


Figure 36: CosMx Segmentation and Supplemental Markers (Mouse Neuro FF RNA)

Table 23: CosMx Segmentation and Supplemental Markers (Mouse Neuro FF RNA)

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

* IMPORTANT: RNA Mm Neuro Segmentation Marker (rRNA) is added during the overnight hybridization step.

Flow-Cell Assembly Tool and Kit

Figure 37: Flow Cell Assembly Tool and Kit

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

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

Prepare Fresh Frozen Tissue Samples

Appendix I: CosMx SMI Sample Preparation Guidelines on page 170 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 VWR Superfrost Plus Micro Slides or Leica BOND PLUS 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 placed within the Scan Area (the green area) of the slide and be no larger than **20 mm Long by 15 mm Wide** (Figure 38) (image not to scale, see the template on 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 center of the scan area (the dashed teal line). For examples of tissue placement best practices, see Appendix I: CosMx SMI Sample Preparation Guidelines on page 170.

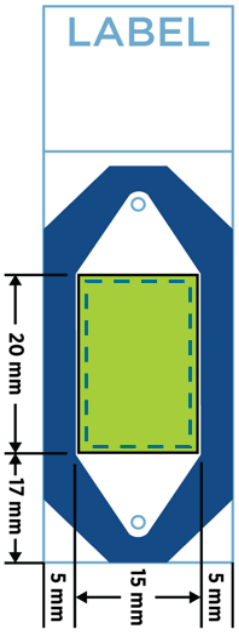


Figure 38: Tissue Scan Area (not to scale)

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 scannable area. If the tissue section is outside of the Scan Area, it will not be imaged.

Prepare RNA Fresh Frozen Assay Reagents

IMPORTANT: Take care to maintain nuclease-free conditions. The greatest risk of contamination comes from RNA probes and other oligos. NanoString recommends the use of RNase AWAY (Thermo Fisher 7003PK), as it will limit contamination from oligos, RNA detection probes, and nucleases. After using RNase AWAY, allow to air dry completely, or rinse with DEPC-treated water. See manufacturer's instructions for details.

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

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

Table 24: RNA Fresh Frozen Reagent Preparation

	Reagent	Dilution	Storage
☐	1X PBS (pH 7.4)	Prepare 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
☐	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

	Reagent	Dilution	Storage
☐	70% Ethanol (EtOH)	Prepare 50 mL of 70% ethanol by adding 15 mL of DEPC-treated water to 35 mL 100% Ethanol.	Make Fresh Daily
☐	50% Ethanol (EtOH)	Prepare 50 mL of 50% ethanol by adding 25 mL of DEPC-treated water to 25 mL 100% Ethanol.	Make Fresh Daily
☐	1X Target Retrieval Buffer	Prepare 50 mL fresh daily by adding 5 mL of CosMx Target Retrieval Buffer, 10X (provided by NanoString) to 45 mL DEPC-treated water. NOTE: NanoSting provides 20 mL of CosMx Target Retrieval Solution, 10X. A 50-100 mL capacity staining jar is recommended when preparing only 2 slides.	Make Fresh Daily
☐	Digestion Buffer	Prepare immediately before use. See Tissue Permeabilization (40 minutes) on page 83.	Make immediately before use.
☐	NHS-Acetate Mix	Individual aliquots can be prepared prior to slide preparation but should be stored at -20°C with a desiccant until use. **To prepare aliquots** (4-slide preparation, see note below): • Bring stock to room temperature for 1-2 hours prior to opening. • Prelabel 4 screw top 1.5 mL centrifuge tubes with the required information. • Using a weighing spatula, carefully weigh 25 mg of NHS-Acetate directly into the screw-top tube on an analytic scale. • Close the tube and label tube with final weight. Seal the tube with parafilm, and **return to -20°C in desiccant until use.**	-20°C in desiccant

	Reagent	Dilution	Storage
	NHS-Acetate Mix (cont.)	NOTE: If preparing only 2 slides, prepare 15 mg aliquots into 6 total tubes.	
☐	Fiducials and Hybridization Mix	These reagents have additional steps that will be covered in detail in their respective sections. Remove ISH Probe Mix from storage and keep on ice until ready to use. NOTE: Fiducials are light sensitive and should be kept stored, protected from light, until instructed to remove.	n/a
☐	Day 2 Reagents	These reagents have additional steps that will be covered in detail in their respective steps. Follow Day 2 procedure for preparation of these reagents.	n/a

Day 1: NBF Fixation and Bake

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

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

NBF Fixation:

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

Figure 39: 2 Hour NBF Fixation

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

Figure 40: 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 Rehydrate Fresh Frozen Tissue Sections (1 hour)

You will need the following materials and reagents for this step: **staining jars, 1X PBS, 4% SDS, 100% EtOH, 70% EtOH, 50% EtOH**, and **DEPC-treated water**. See Prepare RNA Fresh Frozen Assay Reagents on page 73 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 will be 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 will take 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 80.

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 rehydrate Fresh Frozen tissue sections:

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

1. After NBF fixation, **gently perform the following washes** using staining jars (Figure 41).

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

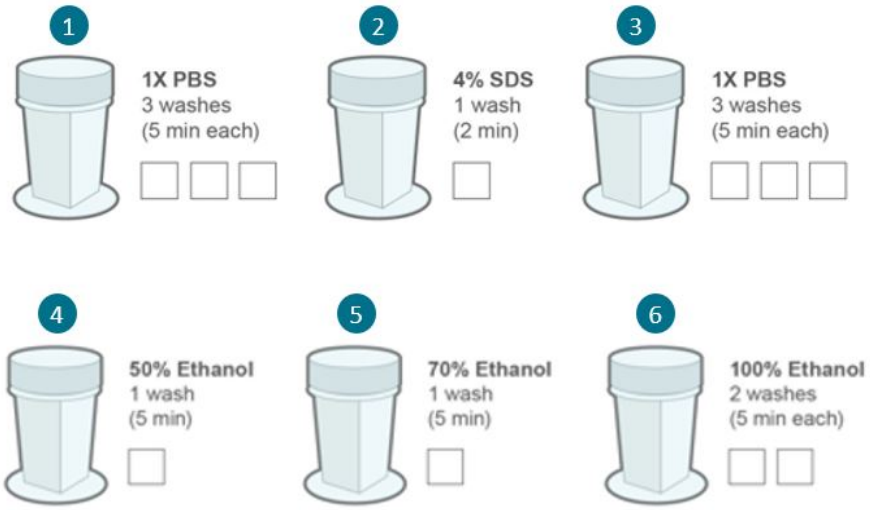


Figure 41: Rehydrate and Fix Tissue

2. **If not already aliquoted, remove NHS-Acetate powder from -20°C freezer** and leave at room temperature for 1-2 hours before use in NHS-Acetate Preparation and Application (25 minutes) on page 90.

Perform Target Retrieval (50 minutes)

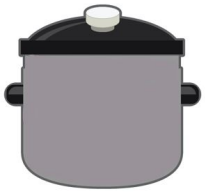
You will need the following materials and reagents for this step: **pressure cooker or steamer, staining jars, 1X Target Retrieval Solution** (pre-heated in the previous step), **DEPC-treated water** and **100% EtOH**.

> 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.

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



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

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.

⚠ WARNING Removing the steamer lid releases high-temperature steam. Wear protective heat resistant gloves to open lid and remove staining jar.

resistant gloves to open lid and remove staining jar.

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

3. Return the staining jar containing the slides **into the preheated pressure cooker.** Ensure that water level is well below lid of jar. About halfway up jar will be sufficient. If needed, a trivet may be used to raise the jar.

4. Reattach the pressure cooker lid, **open the pressure release valve to *Pressure Release* position** and return the pressure cooker to 100°C.

5. **Once the pressure cooker temperature returns to 100°C**, start timer and run for **15 minutes** for FFPE tissue or 8 minutes for cell pellet arrays (CPA).

6. When the timer reaches zero, press cancel on pressure cooker to stop heating, **carefully remove the pressure cooker lid and remove the staining jar**.

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

3. Remove the foil from the Target Retrieval jar and quickly transfer the slides to the Target Retrieval solution. Replace the foil, then replace steamer lid.

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

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 42).

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

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

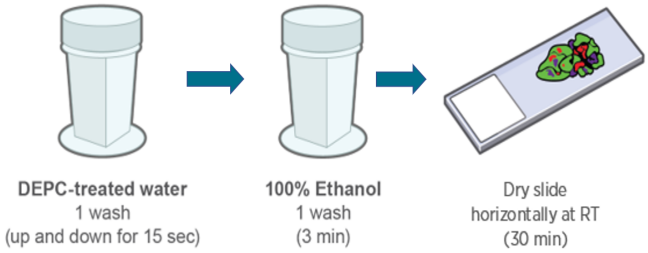


Figure 42: Ethanol Wash

11. While slides are drying, continue to next page to prepare incubation frame and digestion buffer (see Tissue Permeabilization (40 minutes) on page 83).

Tissue Permeabilization (40 minutes)

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

1. If needed, trim the tissue following the template in Prepare Fresh Frozen Tissue Samples on page 71.
    - 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 43).
    - 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.

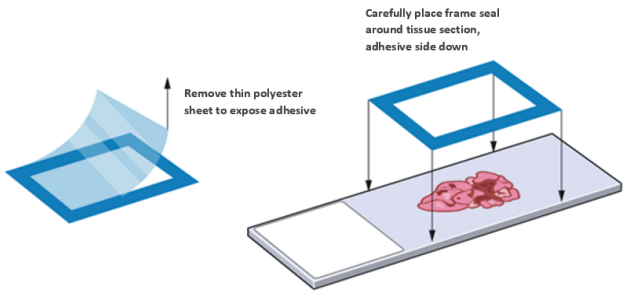


Figure 43: Apply Incubation Frame

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:

   - **Resuspend Protease A** with 200 µL of Protease A Buffer.

   NOTE: A 2-step serial dilution is recommended for both Protease A and Proteinase K. Accurate dilution of both proteases is critical for proper assay performance (Table 25). After each dilution, mix thoroughly by inverting tube or pipetting up and down using a clean pipetter tip. **Do not vortex.**

   - **Create Proteinase K working stock:** dilute 20 mg/mL stock to 100 µg/mL by adding 2 µL of Proteinase K to 398 µL of 1X PBS.
   - **Create Protease A working stock**: dilute rehydrated Protease 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 25: Digestion Buffer Preparation

Working Stocks		
Stock	2 µL Proteinase K (20 mg/mL)	5µL rehydrated Protease A
Dlluent	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	

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

7. With the slide on a clean, flat service, **apply 400 µL of digestion buffer to completely cover tissue within incubation frame**. Gently move the slide side to side as needed to ensure that digestion buffer covers the entire tissue.
8. **Incubate for 30 minutes at room temperature.**

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

9. During incubation, **remove fiducials and 2X SSC-T from 4°C and let come to room temperature, protected from light, for at least 10 minutes.**
10. Once fiducials have reached room temperature, prepare fiducial working solution following instructions for Fiducial Preparation and Application (20 minutes) on page 43.
11. After incubation, tap off excess digestion buffer *one slide at a time* and **transfer slides to staining jar containing fresh 1X PBS**. Wash for 5 minutes.

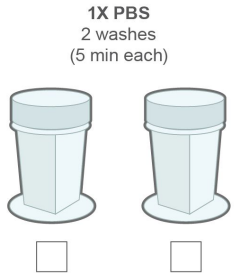


Figure 44: Wash 2X in 1X PBS

12. **Repeat with a new jar of 1X PBS.**
13. Slides can be stored in 1X PBS while fiducials are prepared.

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

Fiducial Preparation and Application (20 minutes)

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

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

1. **Prepare fiducials for use following the below steps** (Figure 45):

> **IMPORTANT**: When sonicating the fiducial tube, be sure to not 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.

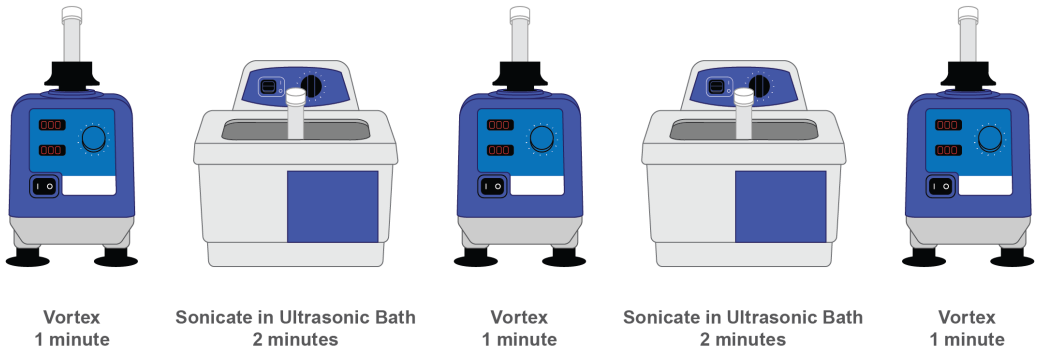


Figure 45: Fiducial preparation

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

2. Once fiducials are prepared, **dilute fiducial stock (0.1%) to the working concentration (0.00015%) in 2X SSC-T.** A 2-step serial dilution is recommended for fiducial preparation. Accurate dilution of 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 26).

Table 26: Fiducial Final Dilution

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

NOTE:The concentration may differ for some tissue types and need to be empirically determined. For this protocol, start with the default concentration of 0.00015% and adjust the concentration as needed. See Appendix III: Tissue Specific Fiducial Concentrations (RNA Assay) on page 176 for suggested tissue specific concentrations based on R&D preliminary testing.

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

Figure 46: Incubate covered for 5 minutes

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

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

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

8. Wash slides in staining jar with **1X PBS for 5 minutes**. During PBS wash, prepare staining jars for next step, Post-Fixation (20 minutes) on page 89.

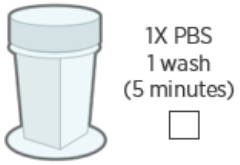


Figure 47: PBS Wash

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 73 for more information).

> ⚠ **WARNING**: Use of appropriate personal protective equipment is advised. Used NBF Stop Buffer contains NBF and must be disposed of in the same manner as the NBF.

Post-fix the tissue by performing the following washes (Figure 48).

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

Figure 48: NBF Post Fix

4. During PBS wash, remove **core probe mix** (or custom probe mix, if applicable), **RNase Inhibitor, and add-on probes** (if applicable) from -20°C and thaw on ice.
5. Remove segmentation markers from -80°C and thaw on ice.
6. **Remove Buffer R** from 4°C and bring to room temperature.

NHS-Acetate Preparation and Application (25 minutes)

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

1. **Prepare 100 mM NHS-Acetate mixture** immediately before you are ready to apply the mixture onto the tissue (200 µL/sample). Ensure stock NHS- Acetate has reached room temperature before aliquoting to prevent condensation.
   a. NHS-Acetate powder is stored desiccated at -20°C. If not already done, prepare 25 mg aliquots (for a 4-slide preparation) of NHS-Acetate powder by weighing out the powder directly into 4 2.0 mL centrifuge tubes. Label tubes with the exact weight, seal tubes with parafilm, and store tubes at -20°C in desiccant for later use. See Prepare RNA Fresh Frozen Assay Reagents on page 73.
      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 NHS- Acetate powder by multiplying the weight of NHS-Acetate powder in mg by 38.5.
         - Example: for 25.0 mg of NHS-Acetate powder: 25.0 * 38.5 = 962.5 µL of buffer to add.
   c. Slowly pipette up and down to mix. Bubbles may occur. Do not fully dispense liquid from pipette while mixing.

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

2. Perform the following steps **one slide at a time** to prevent the tissue from drying out:
   - **Remove slide from 1X PBS,** gently tap slide on a clean kimwipe to remove excess buffer, and transfer to a clean staining tray.

- **Apply 200-250 µL of NHS-Acetate mixture** onto the tissue within the incubation frame. Gently rock the tray side to side as needed to ensure that the NHS-Acetate solution covers the entire tissue.
- Repeat with remaining slides and **incubate covered in staining tray for 15 minutes at room temperature** (Figure 49).

Figure 49: Incubate 15 minutes

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

Figure 50: 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)

You will need the following materials and reagents for this step: **hybridization oven, hybridization tray, incubation frame covers** (CosMx FF Slide Prep Kit (RNA) Box 1/2, Stored at 4°C), **thermal cycler, ice bucket with ice, Buffer R, Core Probe Mix**(-20°C), **rRNA Segmentation Marker** (-80°C), **RNase Inhibitor** (-20°C), **custom stand-alone panel and probe add-on** (if applicable, -20°C), and **DEPC-treated water**.

Do not begin in situ hybridization step until within 16-18 hours of day 2 start time. If not within that time frame, slides can be stored, protected from light, in 2X SSC wash for up to 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.

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

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

Set the hybridization oven temperature to 37°C according to product instructions. The hybridization ovens listed in the Equipment section are not light-permeable. If your chamber is light-permeable, minimize light exposure (e.g., by wrapping the lid in aluminum foil).

1. Pre-heat thermal cycler and lid to 95°C.
2. Remove the **incubation frame cover** and clean with Ethanol. Dry with a clean Kimwipe and visually inspect the cover for dust. Use a new Kimwipe or air blower as needed to remove any dust. Lay incubation frame cover on a clean Kimwipe until use.
3. Flick to mix probes and centrifuge before using. **Do not vortex probes**.
4. **Denature CosMx core probe mix and rRNA segmentation marker*** by transferring total volumes needed for assay (Table 27), from stock tubes into clean 0.2 mL PCR tubes **(probes and segmentation markers must be kept separate during denaturing**).

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

*The rRNA Segmentation marker targets ISH probes and is used for upfront imaging. This probe is unique to the mouse neuro assay and is especially important for cell segmentation.

IMPORTANT: Ensure accurate pipetting. When preparing 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. Make hybridization solution (Table 27). Prepare hybridization mix no more than 20 minutes before tissue application.

Table 27: Hybridization Solution (4-slide configuration)

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

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

**Only the rRNA Segmentation marker is added during the overnight hybridization. All other segmentation markers will be added during Nuclear and Cell Segmentation Staining on page 98.

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

7. **Clean all equipment** with RNase AWAY and allow to dry; or, rinse with DEPC-treated water (see **IMPORTANT** note, above). **The hybridization chamber can be a key source of contamination by oligos**. Arrange fresh Kimwipes on bottom of the chamber. Change gloves and clean workspace with RNase AWAY.
8. **Wet the Kimwipes with 2X SSC or Deionized (DI) water**. Take care that the Kimwipes and 2X SSC do not contact the slides. Kimwipes should be thoroughly wet, but standing buffer should not be present.
9. To prevent the tissue from drying, perform the following steps **one slide at a time.**
   - Remove slides from 2X SSC, **gently tap slide to remove excess liquid.**
   - Carefully remove the thick polyester frame backing (with the center square removed) from the incubation frame to expose the top adhesive layer of the incubation frame (Figure 51). **Ensure that the incubation frame does not lift from the slide when removing the polyester frame backing.**

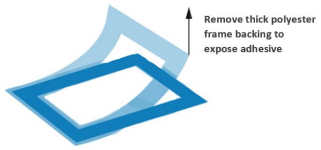


Figure 51: Remove polyester frame backing

   - **Place the slide into the hybridization tray in a horizontal position** (Figure 52).

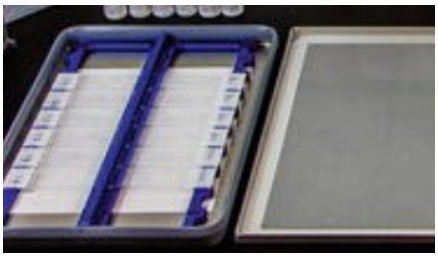


Figure 52: Hybridization Tray

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

     Start by adding the hybridization solution to bottom most part of the tissue within the frame. Applying the incubation frame cover will help move the hybridization solution across the tissue (Figure 53).

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

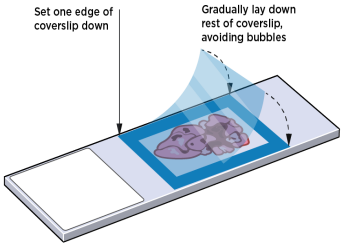


Figure 53: Apply Incubation Frame Cover

- **Repeat step 9 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.

10. Close hybridization chamber, insert tray into oven, and clamp tray into place. **Incubate at 37°C overnight** (16 – 18 hours) (Figure 54).

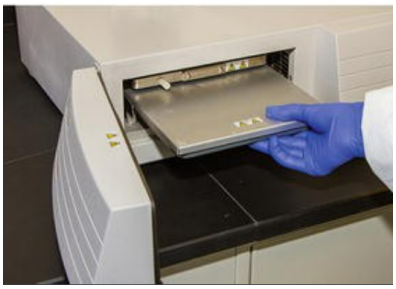


Figure 54: Incubate overnight at 37°C

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

Day 2: Perform Stringent Washes (90 minutes)

You will need the following materials and reagents for this step: **water bath, 4X SSC, 100% formamide,** and **2X SSC** (see Prepare RNA Fresh Frozen Assay Reagents on page 73).

> ⚠ **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 Supplemental Cell Segmentation Kit (if applicable) from the freezer and thaw on ice.
3. **Warm 100% formamide to room temperature for at least 30 minutes before opening**. Once formamide is at room temperature, **prepare stringent wash directly in staining jars by mixing equal parts 4X SSC and 100% formamide**.

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

4. **Preheat staining jars containing freshly prepared stringent wash in 37°C water bath**. It will take about 30 minutes to preheat wash.
5. While jars are preheating, **transfer slides to 2X SSC** and perform the following steps **one slide at a time** to prevent the tissue from drying. Dip slide back into 2X SSC as needed to avoid tissue drying.
   - With a clean pair of forceps, carefully remove the incubation frame cover from the incubation frame. Dip slide back into 2X SSC as needed to avoid tissue drying. If cover will not come off without removing incubation frame, remove the frame and cover, the frame can be reapplied in a later step.
   - 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.
6. Once both jars have pre-heated to 37°C and all incubation frame covers have been removed, perform the washes detailed below (Figure 55). After the last wash, the slides can be stored in 2X SSC for up to one hour.
   - Gently tap each slide, on at a time, on a clean Kimwipe to remove excess 2X SSC and place slides in the **first stringent wash and wash for 25 minutes**. **Repeat** with the second stringent wash.

- During the second stringent wash, begin preparing reagents for Nuclear and Cell Segmentation Staining on page 98.
- Following stringent washes, immediately **transfer slides to 2X SSC and wash for 2 minutes**. **Repeat** with second jar of 2X SSC. Leave slides in 2X SSC as needed until reagents have been prepared for nuclear and cell segmentation staining.

Figure 55: Perform stringent wash

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

Nuclear and Cell Segmentation Staining

You will need the following materials and reagents for this step: **incubation frames, staining jars, 1X PBS, Blocking Buffer** (4°C)**, DAPI Nuclear Stain Stock** (-80°C), and **supplemental markers** (if applicable, -80°C) (see Prepare RNA Fresh Frozen Assay Reagents on page 73).

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** 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 28).

Table 28: Dilute nuclear stain

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

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

     ⓘ **IMPORTANT**: Avoid wiping the slide within the scan area as this could remove the fiducials needed for imaging. See scan area template on the flow cell assembly tool or follow the guidelines in Prepare Fresh Frozen Tissue Samples on page 71.

   - If needed, carefully reapply the incubation frame following the instructions in Tissue Permeabilization (40 minutes) on page 83. 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. 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 DAPI 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 56).**

Figure 56: Cover tray and incubate for 15 minutes

8. During nuclear stain incubation, **prepare supplemental marker mix** (if applicable) using the following table where *n* = the number of slides (Table 29). **Flick each tube to mix and centrifuge before use**.

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

Table 29: Prepare Supplemental Marker Mix

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

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

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

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

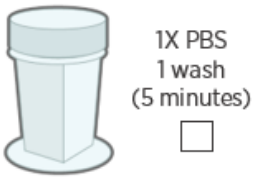


Figure 57: Wash for 5 minutes in 1X PBS

11. During PBS wash, add 2X SSC or deionzied (DI) water to the staining tray. Do not overfill. The water level should be well below the slides to avoid cross-contamination.
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 supplemental marker mix** directly to tissue. Gently move tray side to side as needed to ensure that the mix covers the entire tissue.
    - If needed, an incubation frame cover can be placed over the incubation frame to ensure that the mix completely covers the tissue.
    - Adjust volume to add as needed for tissues of varying sizes. The **supplemental marker mix needs to completely cover the tissue** but does not need to completely fill the incubation frame.
13. **Repeat** with remaining slides and cover tray (Figure 58).

Figure 58: Cover tray and incubate for 1 hour

14. **Incubate slides for 1 hour at room temperature** protected from light.

15. Following incubation, **transfer slides to 1X PBS and wash for 5 minutes** (Figure 59).

Figure 59: Wash 3X in 1X PBS

16. **Repeat 2 times** for a total of 3 PBS washes.
17. 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.
18. 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 Assemblyon page 165.

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).

Protein FFPE Manual Slide Preparation

CosMx SMI Slide Preparation Workflow

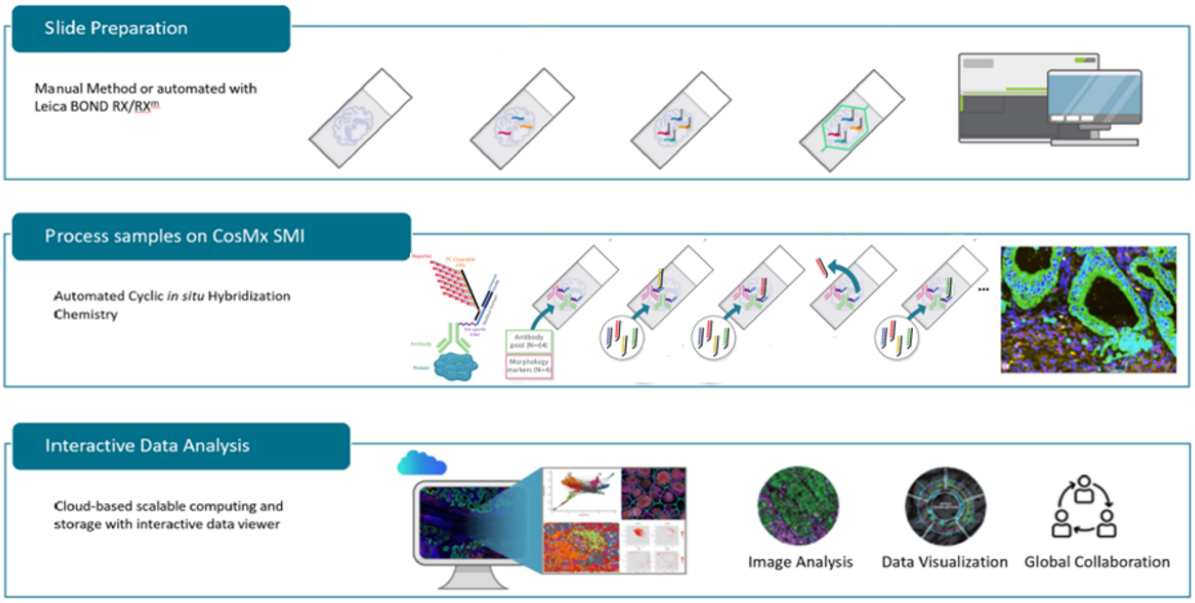


Figure 60: CosMx SMI Workflow Overview

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

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

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

Day 0: Prepare Reagents

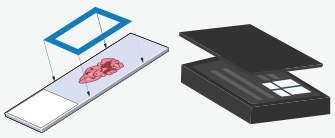
- Prepare shelf stable reagents

Day 1: Slide Preparation

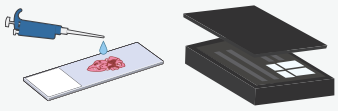
Bake Slides
- Bake Slides 1 hr at 65°C

Deparaffinize Tissue
- 2 washes in CitriSolv, 5 min each wash
- 2 washes in 100% EtOH, 10 min each wash
- 2 washes in 95% EtOH, 5 min each wash
- Wash 5 min in 70% EtOH
- 2 washes in 1X PBS, 5 min each wash

Target Retrieval
- 15 mins at 100°C
- Stand at RT and 3 washes in 1X PBS, 5 min each

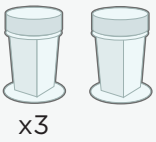


Blocking
- Apply incubation frame
- Apply Buffer W
- Incubate 1 hour at RT

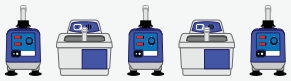


Overnight Incubation
- Prepare and apply assay specific antibodies and segmentation markers
- Incubate at 4°C overnight

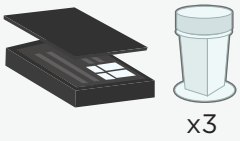
Day 2: Wash and Stain Slide



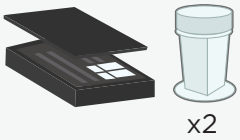
Remove Unbound Antibodies
- 3 washes in 1X TBS-T, 10 mins each wash.
- Wash in 1X PBS, 2 min



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



Post Fixation
- Apply 4% PFA to tissue
- Incubate for 15 minutes
- 3 washes in 1X PBS, 5 min

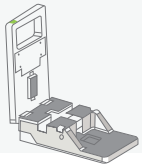


Nuclear Stain
- Prepare Nuclear Stain stock and apply to tissue
- Incubate 10 mins at RT
- 2 washes in 1X PBS for 5 mins each wash

Apply NHS-Acetate
- Prepare and apply NHS-Acetate
- Incubate for 15 mins.
- Wash in 1X PBS for 5 mins

Prepare Flow Cells and Load Instrument

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



Prepare Instrument Reagents
- Add enzymes to Buffer 4.
- Add custom reporters (if needed) to the imaging tray.

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

Equipment, Materials, and Reagents

The following equipment (Table 30), materials (Table 31), and reagents (Table 32) are required for this protocol but are not supplied by NanoString.

Equipment:

Table 30: Equipment not provided by Nanostring

	Equipment	Source	Part Number(s)
☐	**Baking Oven**	Quincy Lab, Inc (or comparable)	Various GC Models
☐	**6-quart Pressure Cooker** **NOTE**: Products from other vendors may require testing and optimization.	BioSB® TintoRetriever	BSB 7015
☐	**Ultrasonic Bath** (500 mL capacity) **NOTE**: 400kHz frequency with timer	General Lab Supplier	Example
☐	**Vortex Mixer**	General Lab Supplier	Various
☐	**Microcentrifuge** for 1.5 mL microcentrifuge tubes	General Lab Supplier	Various
☐	**Analytic Scale with draft shield** **NOTE**: ensure scale can weigh in milligrams (mg) for accuracy.	Various	Example

Materials:

Table 31: Materials not provided by Nanostring Technologies Inc.

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

Reagents:

Table 32: Reagents not provided by Nanostring Technologies Inc.

	Reagent	Source/Part Number(s)	Storage Conditions
☐	**DEPC-treated Water**	ThermoFisher, AM9922 (or comparable)	Room temperature
☐	**100% Ethanol (EtOH): ACS grade or Better** 	General Lab Supplier	Flammable Storage Room temperature
☐	**10X Phosphate Buffered Saline pH 7.4 (PBS)**	ThermoFisher, AM9625 (or comparable)	Room temperature
☐	**CitriSolv** 	VWR, 1601/89426-268	Flammable Storage Room temperature
☐	**TBS with Tween (TBS-T), 20X**	ThermoFisher, J77500.K2	Room temperature
☐	**Sulfo NHS-Acetate powder** NOTE: NHS-Acetate powder is shipped in a plastic bag with a desiccant and should be left in the bag and stored at -20°C until ready to use.	Fisher Scientific, 26777	-20°C
☐	**Paraformaldehyde (PFA) 16% Aqueous Solution** 	EMS, 15710	Room temperature

NanoString Supplied Reagents

CosMx SMI Protein Slide Preparation Kit

Figure 61: FFPE Protein Slide Preparation Kit

Table 33: CosMx FFPE Protein Slide Preparation Kit

Kit Contents (Store at 4°C)	
NHS-Acetate Buffer	Buffer W
10X Target Retrieval Solution	CosMx Fiducials
Incubation Frames	Incubation Frame Covers

CosMx Protein Panels (see Panel and Cell Segmentation Marker Selection on page 12).

- Human Immuno-Oncology Panel (Figure 62)
- CosMx Mouse Neuroscience Panel (not pictured)

Figure 62: CosMx Human Immuno-Oncology Panel (Protein)

Table 34: Available CosMx Protein Panels (store at -80°C)

CosMx Human Immuno-Oncology Panel	
Core Panel	**Custom Protein Add-On**
CosMx Human Immuno-Oncology Panel	Custom Protein Add-On Probes (if applicable) IMPORTANT: when custom Add-On antibodies are ordered, a custom probe kit configuration is generated and linked to the user's AtoMx SIP tenant. Any changes to the custom assay, including the addition or removal of custom Abs may require a new configuration file to be generated. This process requires a turn-around-time of one (1) business day.
CosMx Mouse Neuroscience Panel	
Core Panel	**Custom Protein Add-On**
CosMx Mouse Neuroscience Panel (not pictured)	Custom Protein Add-On Probes (if applicable)

CosMx Human Universal Segmentation and Supplemental Markers

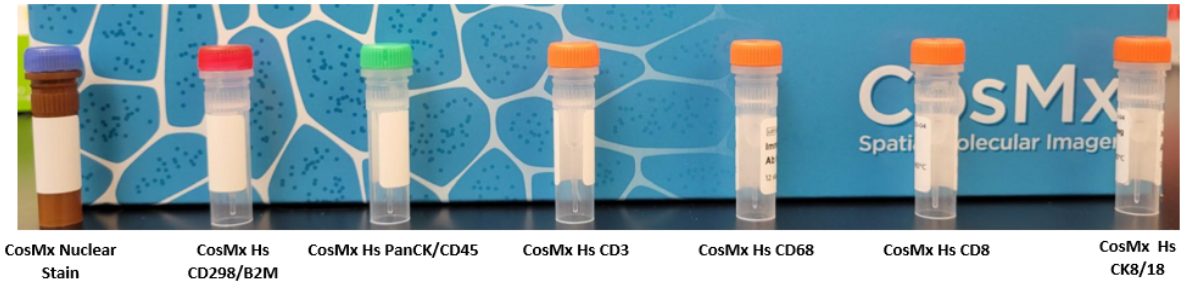


Figure 63: CosMx Segmentation and Supplemental Markers (Human IO Protein)

Table 35:CosMx Segmentation and Supplemental Markers (Human IO Protein)

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

CosMx Mouse Neuro Segmentation and Supplemental Markers

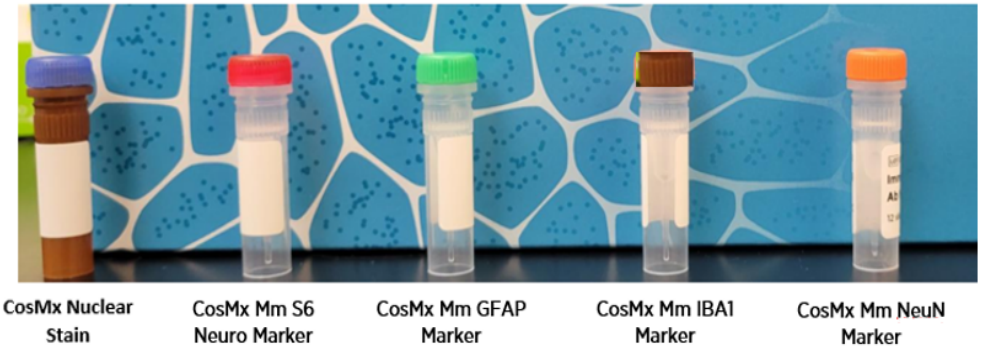


Figure 64: CosMx Segmentation and Supplemental Markers (Protein Mouse Neuro)

Table 36:CosMx Segmentation and Supplemental Markers (Protein Mouse Neuro)

Kit Contents (Store at -80°C) Each kit is sufficient for four (4) slides	
Kit Name	**Kit Component**
CosMx Mouse Neuroscience Cell Segmentation Ch1/2 (Protein) Kit	CosMx DAPI Nuclear Stain CosMx Mm S6 Neuro Marker Ch2 (Protein)
CosMx Mouse Neuroscience Segmentation Ch3/4 (Protein) Kit	CosMx Mm GFAP Marker Ch3 (Protein) CosMx Mm IBA1 Marker Ch4 (Protein)
CosMx Mouse NeuN A La Carte Marker Ch5 (Protein) Kit, Ch5	CosMx Mm NeuN Marker Ch5 (Protein)

Flow-Cell Assembly Tool and Kit

Figure 65: 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 Tissue Samples

Appendix I: CosMx SMI Sample Preparation Guidelines on page 170 covers FFPE block selection and sectioning in detail. Review these guidelines as needed prior to beginning the FFPE Slide Preparation procedure.

Tissue Sectioning and Slide Preparation:

FFPE blocks should be sectioned at **5 µm thickness** and mounted on the label side of VWR Superfrost Plus Micro Slides or Leica BOND PLUS 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 placed within the Scan Area (the green area) of the slide and be no larger than **20 mm Long by 15 mm Wide** (Figure 66) (image not to scale, see the template on 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 center of the scan area (the dashed teal line). For examples of tissue placement best practices, see Appendix I: CosMx SMI Sample Preparation Guidelines on page 170.

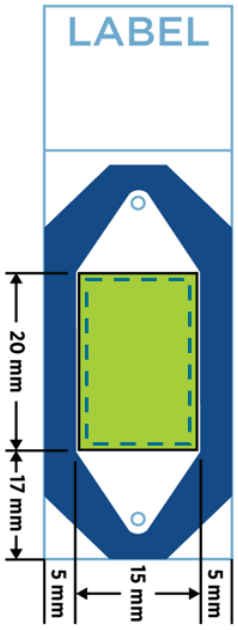


Figure 66: Tissue Scan Area (not to scale)

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 freshly sectioned tissue slides vertically at room temperature overnight. Tissues can be baked vertically overnight at 37°C to improved tissue adherence.

If sections are larger than the indicated size and/or placed off-center, continue with the slide preparation as outlined. Immediately before applying the incubation frame, scrape off parts of the tissue exceeding the scan area. Scraping off tissue before target retrieval 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.

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

Prepare Assay Reagents

Prepare the reagents using the instructions in the following table (Table 37).

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

Table 37: Prepare protein assay reagents

	Reagent	Dilution	Storage
☐	1X Tris-Buffered Saline with Tween 20 (TBS-T)	Dilute 100 mL of 10X TBS-T in 900 mL of DEPC-treated water.	Room Temperature
☐	1X PBS	Prepare 1 L of 1X PBS by diluting 100 mL of 10X PBS into 900 mL of DEPC-treated water.	Room Temperature
☐	NHS-Acetate Mix	Individual aliquots can be prepared prior to slide preparation but should be kept at -20°C with a desiccant until use. **To prepare aliquots:** • Bring stock to room temperature for 1-2 hours prior to opening. • Prelabel 4 screw top 1.5 mL centrifuge tubes with the required information. • Using a weighing spatula, carefully weigh 20-25 mg of NHS-Acetate directly into the screw-top tube on an analytic scale. • Label tube with final weight, close tube, seal the tube with parafilm, and return to -20°C until use.	-20°C in desiccant
☐	1X Target Retrieval Solution	Dilute 25 mL of 10X Target Retrieval Solution into 225 mL of DEPC-treated water. Must be prepared on the day of slide preparation. Do not prepare ahead of time.	Make fresh daily

	Reagent	Dilution	Storage
☐	95% Ethanol (EtOH)	Prepare 500 mL of 95% ethanol by adding 25 mL of DEPC-treated water to 475 mL 100% Ethanol. Prepare fresh on the day of slide preparation.	Make fresh daily
☐	70% Ethanol (EtOH)	Prepare 500 mL of 70% ethanol by adding 150 mL of DEPC-treated water to 350 mL 100% Ethanol. Prepare fresh on the day of slide preparation.	Make fresh daily
☐	Segmentation Markers and Target Antibodies	These reagents have additional steps that will be covered in detail in their respective sections. Remove these reagents from storage and keep on ice until ready to use.	n/a
☐	Day 2 Reagents	These reagents have additional steps that will be covered in detail in their respective steps. See Day 2: Wash Off Unbound Antibodies (50 minutes) on page 126 for preparation of these reagents.	n/a
☐	4% Paraformaldehyde (PFA)	Prepare 1 mL of 4% PFA by adding 250 µL of 16% PFA to 750 µL of DEPC-treated water.	Prepare Fresh

Day 1: Deparaffinize and Rehydrate FFPE Tissue Sections (1 hour)

You will need the following materials and reagents for this step: **staining jars, humidity (staining) tray, pressure cooker, 1X Target Retrieval Solution, Citrisolv, 100% EtOH, 95% EtOH, 70% EtOH**, and **1X PBS**. See Prepare Assay Reagents on page 114 for more details.

Prepare Equipment and Bake Slides

1. Bake slides vertically in a slide rack at 65°C for **1 hour**. Slides can be baked for up to 3 hours to improve tissue adherence.
2. **Prepare the humidity chamber (staining tray) according to product instructions**. If your chamber is light-permeable, minimize light exposure (e.g., by wrapping the lid in aluminum foil).
3. While slides are baking, prepare the pressure cooker and preheat the Target Retrieval Solution to 100°C:

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

- **Fill the pressure cooker** with water to the correct level per the manufacturer's instructions (4-8 cups depending on model used).
- **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 will be sufficient. If needed, a trivet may be used to raise the jar.
- 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**.
- **Preheat the pressure cooker to 100°C,** with the pressure valve closed, following the model specific instructions below: Pressure cooker preheating will take 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 (1 hour) on page 119.

Deparaffinize and rehydrate FFPE tissue sections.

After slides have baked for at least 1 hour, gently perform the following washes (Figure 67) using staining jars. Slides should be dipped up and down gently several times when placing in and before removing from staining jars.

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

Figure 67: Deparaffinize and Rehydrate FFPE tissue sections

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

Perform Target Retrieval (1 hour)

You will need the following materials and reagents for this step: **staining jars, pressure cooker,** and **1X PBS**. See Prepare Assay Reagents on page 114 for more details.

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

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

3. Carefully place the staining jar containing the slides back into the preheated pressure cooker (Figure 68). Ensure that water level is well below lid of jar. About halfway up jar will be sufficient. If needed, a trivet may be used to raise the jar.

Figure 68: Target Retrieval

4. Attach the pressure cooker lid and **close the pressure valve.** Return the pressure cooker to 100°C.
5. **Once the pressure cooker temperature returns to 100°C**, start timer and run for **15 minutes**.

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.

6. When the timer reaches zero, press cancel on the pressure cooker to stop heating, release the pressure valve to release pressure. **Carefully and slowly remove the pressure cooker lid and remove the staining jar.**

7. Leave the staining jar containing sample slides at **room temperature for 25 minutes** (max one hour) (Figure 69).

Figure 69: Stand at Room Temperature.

8. Once the slides have equilibrated to room temperature, transfer the slides to 1X PBS and **wash the slides in 1X PBS** for **5 minutes** (Figure 70).

Figure 70: Wash 3X in 1X PBS

9. **Repeat wash 2 times** for a total of 3 washes.
10. Immediately proceed to next step (Blocking (1 hour) on page 121).

Blocking (1 hour)

You will need the following materials and reagents for this step: **incubation frames, humidity chamber**, and **Buffer W**. See Prepare Assay Reagents on page 114 for more details.

1. **Fill the humidity chamber** with enough water to cover the bottom of the trough. Do not overfill as splashing while moving the tray chamber should be avoided.
2. Prepare the Incubation Frame:
    - Separate an individual frame from the strip by tearing along the perforations. Each frame is sandwiched between a thin polyester sheet and a thick polyester frame backing (with the center square removed).
3. If needed, trim the tissue following the template in **Prepare Tissue Samples on page 112**.
4. Using an absorbent wipe, ensure that the surface of the slide that will come in contact with the incubation frame is **dry and clean. Take care not to damage the tissue and work quickly to minimize dehydration of the sample.**
5. Apply the incubation frame (Figure 71).
    - 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, **carefully place the incubation frame** around each tissue section careful not to touch the adhesive surface. Lightly press along the border of the incubation frame to ensure that it is well adhered to the slide. Be careful to avoid applying any pressure in the center of the frame so as not to damage the tissue.

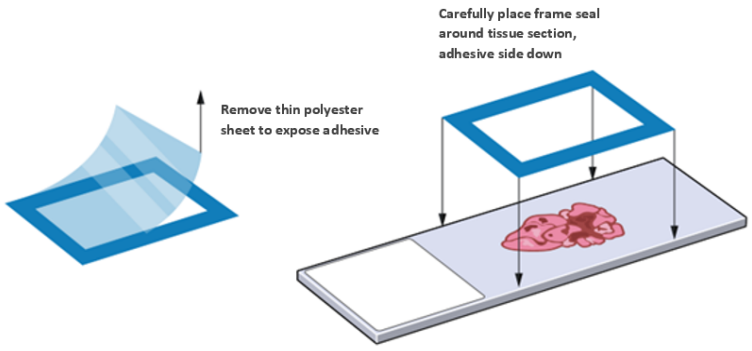


Figure 71: Apply Incubation Frame

6. Place the slides in the humidity chamber in a horizontal position. Add **200 µL Buffer W** directly to the tissue within the incubation frame and cover the humidity chamber (Figure 72).

Figure 72: Incubate for 1 hour

7. **Incubate in Buffer W for 1 hour** at room temperature (RT) in a closed humidity chamber.
8. Remove Protein Panel, cell segmentation kits, and any **supplemental markers** (if applicable) from freezer and **thaw mix on ice.** Thaw custom **barcoded antibodies** (if applicable). Refer to Appendix IV: Adding Custom Barcoded Antibodies on page 177 for custom antibody preparation instructions.

Primary Antibody Incubation (overnight)

You will need the following materials and reagents for this step: **humidity chamber, protein antibody mix, Cell Segmentation Markers, Supplemental Markers** (if applicable), and **Buffer W, incubation frame covers**. See Prepare Assay Reagents on page 114 for more information. If using custom protein, see Appendix IV: Adding Custom Barcoded Antibodies on page 177 for preparation guidelines.

1. **Mix** the protein panel (antibody mix) and any add-ons by flicking the tube and spin down using the microcentrifuge. **Do not vortex**.

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

2. Make a working antibody solution by diluting **protein antibody mix, cell segmentation markers, any supplemental or à la carte markers** (if applicable), and **diluted custom antibodies** (if applicable, see appendix) into **Buffer W** (n = number of slides). Adjust volumes to cover the number of slides to be prepared (125 µL per slide). **Table 38 is specific to the Protein Hs IO Panel. See Table 39 or the antibody solution calculation for the Mouse Neuro panel.**

Human IO Panel - Antibody Solution:

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

Table 38: Antibody Solution Calculations (n = number of slides)

Hs IO Protein Antibody Mix	CD298 / B2M Segmentation Marker	PanCK/ CD45 Marker *(optional)*	A la carte marker *(optional)*	Diluted Custom Antibodies* (if applicable)	Buffer W (µL)	Total Volume (µL)
62.5 µL x *n*	5 µL x *n*	5 µL x *n*	5 µL x *n*	1.25 µL x *n*	up to final volume of 125 µL x *n*	125 µL x *n*

*Volume is per antibody. **NOTE**: If using an antibody with a target concentration of **4 µg/mL, 2.5 µL x *n* must be used.**

Mouse Neuroscience Panel - Antibody Solution:

- Antibody mix: 1:5 dilution for each. Note, the 1:5 dilution is specific to the Mouse Neuro Antibody Mix.
- Cell Segmentation and supplemental markers: 1:25 dilution for each marker.

Table 39: Mouse Neuro Antibody Calculations (n = number of slides)

Mouse Neural Cell Typing Core Panel	Mouse Alzheimer's Pathology Module	Mm S6 Neuro Segmentation Marker	GFAP Marker	IBA1 Marker	A la carte marker (NeuN)	Custom Antibodies* (if applicable)	Buffer W	Total Volume (µL)
25 µL x *n*	25 µL x *n*	5 µL x *n*	5 µL x *n*	5 µL x *n*	5 µL x *n*	1.25 µL x *n*	up to final volume of 125 µL x *n*	125 µL x *n*

* Volume is per antibody. **NOTE**: If using an antibody with a target concentration of **4 µg/mL, 2.5 µL x *n* must be used.**

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

3. Carefully remove the thick polyester frame backing (with the center square removed) from the incubation frame to expose the top adhesive layer of the incubation frame (Figure 73). **Ensure that the incubation frame does not lift from the slide when removing the polyester frame backing.**

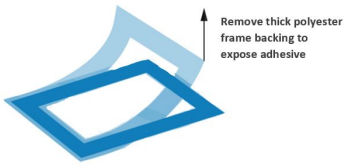


Figure 73: Remove polyester frame backing

4. **Place the slide into the humidity chamber in a horizontal position.**
5. Remove slides from the humidity chamber, **gently tap slide on a clean Kimwipe to remove excess Buffer W.**
6. **Add 125 µL of the diluted antibody solution** directly to the tissue within the incubation frame.

Start by adding the antibody solution to the edge of the tissue within the frame. Applying the incubation frame cover will help move the antibody solution across the tissue (Figure 74).

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

7. **Carefully apply incubation frame cover** (Figure 74). Start by setting one edge of the cover down on the incubation frame edge, then gradually lay down the rest of the cover. Press around the edges of the cover (along the border of the incubation frame) to ensure good adherence. **Do not press the center of the cover as it could damage the tissue.**

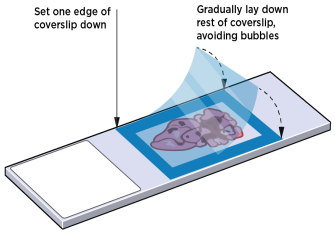


Figure 74: Apply Incubation Frame Cover

8. **Repeat step 3-7 for each slide.**
9. 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.
10. Transfer the humidity chamber to the 4°C refrigerator and incubate overnight (16-18 hours). Minimize exposure to light and ensure the humidity chamber stays level to avoid losing antibody solution (Figure 75).

Figure 75: Incubate overnight at 4°C

Day 2: Wash Off Unbound Antibodies (50 minutes)

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

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

You will need the following materials and reagents for this step: **staining jars, 1X TBS-T, ultrasonic bath, vortex, fiducials** (at RT), **1X PBS** and **4% PFA**. See Prepare Assay Reagents on page 114 for more details.

1. **Gently tap off each slide** on a fresh, clean, disposable surface (e.g., Kimwipes) to remove excess solution.
2. Using clean forceps, **carefully remove the incubation frame cover from the incubation frame**. Dip slide into 1X TBS-T as needed to avoid tissue drying. If cover will not come off without removing incubation frame, remove the frame and cover, the frame can be reapplied in a later step.
3. Place the slides into the staining jar containing 1X TBS-T and wash for **10 minutes**.

Figure 76: Wash 3X in TBS-T

4. During first wash, remove fiducials from 4°C storage and bring to room temperature for 10 minutes.
5. If not already done, remove the stock PFA from the -20°C and dilute to the 4% working concentration. See Prepare Assay Reagents on page 114.
6. **Repeat TBS-T wash 2 times** for a total of 3 TBS-T washes (Figure 76).
7. During the final washing step, **prepare fiducials** (Fiducial Prep and Application (20 minutes) on page 127).

Fiducial Prep and Application (20 minutes)

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

1. **Prepare fiducials for use following the below steps** (Figure 77):

> **IMPORTANT**: When sonicating the fiducial tube, be sure to not 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.

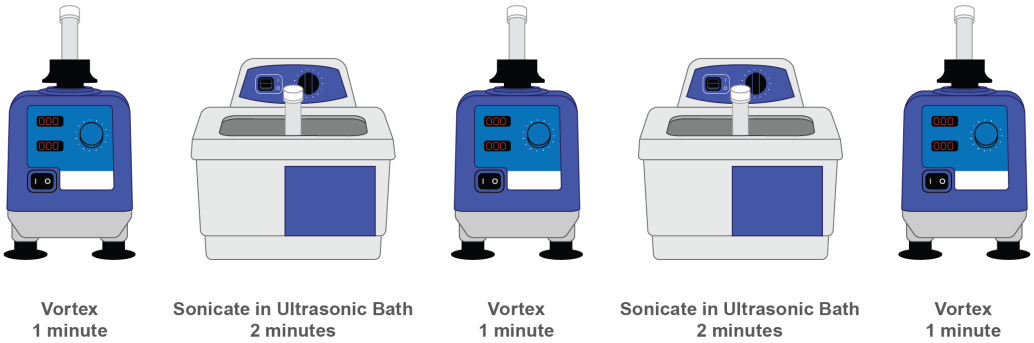


Figure 77: Prepare Fiducials

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

2. Once fiducials are prepared, **dilute fiducial stock (0.1%) to the working concentration (0.00005%) in 1X TBS-T.** A 2-step serial dilution is recommended for fiducial preparation. Accurate dilution of fiducialsis critical for proper assay performance.
   - Dilute stock to 0.01% by adding **10 µL of the fiducial stock to 90 µL of 1X TBS-T**. Label tube as *Dilution 1 (D1)*.
   - Cover *Dilution 1* and **leave at room temperature for 10 minutes** protected from light.
   - After 10 minutes, vortex and quick spin *Dilution 1* and dilute to the final working concentration (0.00005%) using the following table (Table 40).

Table 40: Fiducial Final Dilution

Number of Slides	Dilution 1 (D1) (0.01% Fiducials)	1X TBS-T	Final Volume
2-slides	2.5 µL	497.5 µL	500 µL
4-slides	5 µL	995 µL	1000 µL

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

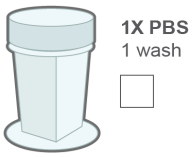


Figure 78: Wash for 2 minutes in 1X PBS

4. **Remove the slides one at a time** from 1X PBS and, if needed, carefully reapply the incubation frame following the instructions in Blocking (1 hour) on page 121. Before application, carefully **dry the slide around the tissue using a clean Kimwipe**. Ensure that the surface of the slide that will come into contact with the incubation frame is clean and dry while being careful to not touch the tissue.
5. Ensure that the frame is well adhered to the slide by gently pressing around the frame with clean forceps.
6. Lay the slides horizontally in the staining tray.
7. **Immediately before applying fiducials to tissue, vortex for 1 minute.** Vortex fiducials for 30 seconds between slides to keep fiducials in suspension and to ensure consistent concentration across all slides.
8. Apply up to 200 µL of **the final fiducial solution, ensuring the solution covers glass and tissue within the incubation frame**. Fiducials must be present on the glass within the scan area for consistent focusing during the instrument run.
9. **Incubate slides in humidity tray for 5 minutes at room temperature. Ensure that fiducial solution completely covers the entire area within the incubation frame.**

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

10. Following fiducial incubation, **transfer slides to fresh 1X PBS.** Wash slides for 5 minutes in the 1X PBS.

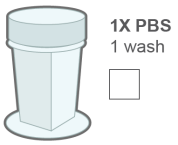


Figure 79: Wash 5 minutes in 1X PBS

11. After PBS wash, transfer slides into the humidity chamber and add **up to 200 µL 4% PFA directly to tissue. Incubate covered for 15 minutes** in the humidity chamber at room temperature (Figure 80).

Figure 80: Incubate 15 minutes

12. After PFA incubation, wash with **3 changes of 1X PBS for 5 minutes each** (Figure 81).

Figure 81: Wash 3X with 1X PBS

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

Nuclear Staining (20 minutes)

You will need the following materials and reagents for this step: **humidity chamber, staining jars, Nuclear Stain,** and **1X PBS**. See Prepare Assay Reagents on page 114 for more details.

1. If you have not already, **remove Nuclear Stain** from the freezer (stored at -80°C) and **warm to room temperature on bench** for at least 10 minutes before use.
2. **Prepare 200 µL x *n* of Nuclear Stain Buffer by diluting the nuclear stain stock 1:40 (where *n* equals the number of slides).**

- **Vortex, then centrifuge thawed nuclear stain** for at least **1 minute** to bring the solution to the bottom of the vial and precipitate insoluble particles. *Do not pipette from the bottom of the vial.*
- Add 5 µL x *n* of Nuclear Stain Stock into 195 µL x *n* of 1X PBS. Adjust volume as needed for more than 1 slide.
- Mix by pipetting up and down 3-5 times. Do not vortex.

Table 41: Prepare Nuclear Stain

Nuclear Stain Stock	1X PBS	Total Volume
5 µL x *n*	195 µL x *n*	200 µL x *n*

3. **Place slides in humidity chamber** in a horizontal position and **slowly apply 200 µL of Nuclear Stain Buffer** directly to tissue within the incubation frame. Gently move the tray side to side as needed to ensure that the buffer covers the entire tissue.

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

4. **Incubate slide for 10 minutes** at room temperature in the covered humidity chamber (Figure 82).

Figure 82: Incubate 10 minutes at RT

5. Following nuclear stain incubation, **wash slides for 5 minutes in 1X PBS** (Figure 83).

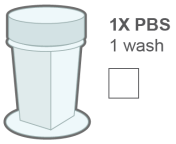


Figure 83: 1X PBS Wash

6. **Transfer** to a staining jar with **fresh 1X PBS and repeat wash** (Figure 84).

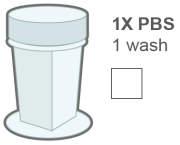


Figure 84: 1X PBS Wash

NHS-Acetate Preparation and Application (25 minutes)

You will need the following materials and reagents for this step: **scale, 2.0 mL centrifuge tube, staining jar, NHS-Acetate powder, NHS-Acetate buffer** (provided by NanoString), and **1X PBS**.

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

   a. NHS-Acetate powder is stored desiccated at -20°C. If not already done, prepare 25 mg aliquots of NHS-Acetate powder by weighing out the powder directly into 2.0 mL centrifuge tubes. Label tubes with the exact weight, seal tubes with parafilm, and store tubes at -20°C in desiccant for later use. See Prepare Assay Reagents on page 114.

   NOTE: If preparing only 2 slides, pre-weigh 11 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 NHS-Acetate powder by multiplying the weight of NHS-Acetate power in mg by 38.5.

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

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

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

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

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

Figure 85: Incubate 15 minutes

- Repeat with remaining slides and **incubate covered in staining tray for 15 minutes at room temperature** (Figure 85).

3. Carefully remove the incubation frame using clean forceps. Tap off excess liquid and submerge slides in **1X PBS** for a minimum of 5 minutes.
   - If samples need to be stored overnight and loaded onto the instrument the next day, remove the incubation frame following the guidelines from Day 2: Wash Off Unbound Antibodies (50 minutes) on page 126. Ensure the entire incubation frame is removed then store according to Safe Storage Guidelines for Protein Slides below.
   - If samples will be loaded onto the instrument the same day, continue to Flow Cell Assembly on page 165.

Safe Storage Guidelines for Protein Slides

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

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

Protein Fresh Frozen Manual Slide Preparation

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

CosMx SMI Slide Preparation Workflow

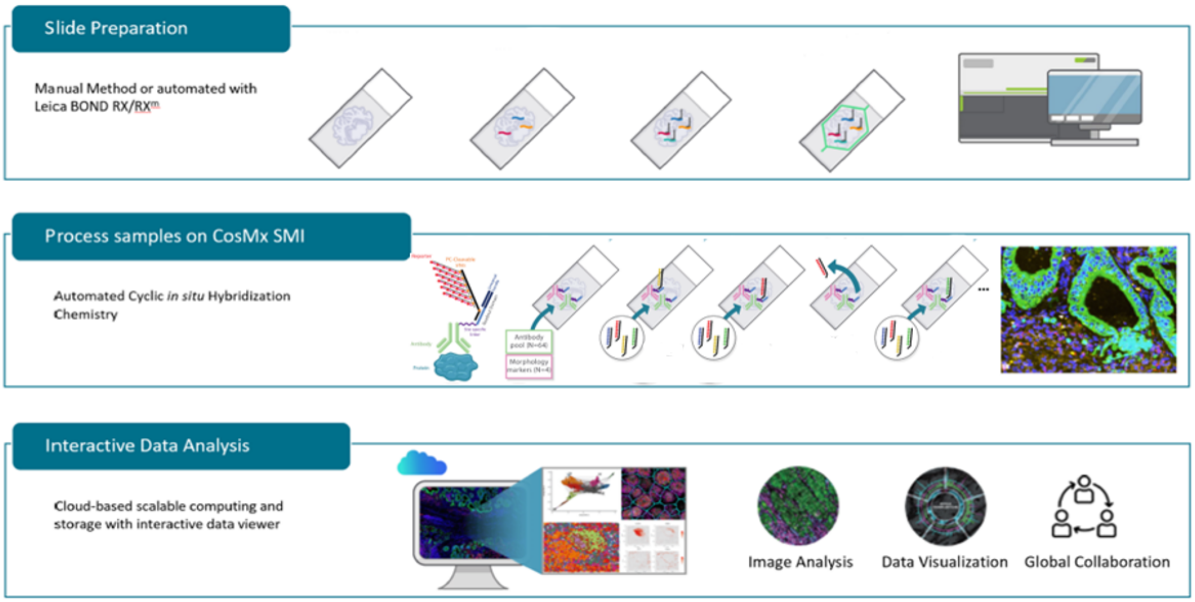


Figure 86: CosMx SMI Workflow Overview

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

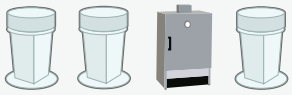
Day 2: Process Slides on CosMx SMI. Apply fiducials and nuclear stain and assemble the flow cells. Load prepared flow cells into the CosMx SMI instrument and enter flow cell/study information. Tissue is scanned to capture protein expression and morphology imaging within user-designated fields of view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

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

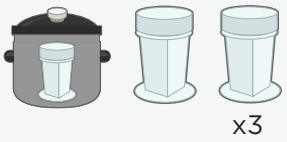
Day 0: Prepare Reagents

- Prepare shelf stable reagents

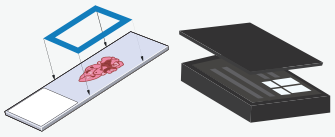
Day 1: Slide Preparation



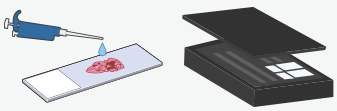
NBF Fix and Bake
- Wash 2 hours in pre-cooled NBF at 4°C
- 3 washes in 1X PBS, 5 min each wash
- Bake at 60°C for 30 min
- 3 washes in 1X TBS-T, 5 min each wash



Target Retrieval
- 7 mins at 80°C
- Stand at RT and 3 washes in 1X TBS-T, 5 min each

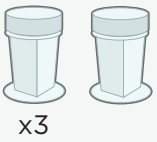


Blocking
- Apply incubation frame
- Apply Buffer W
- Incubate 1 hour at RT

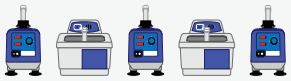


Overnight Incubation
- Prepare and apply assay specific antibodies and segmentation markers
- Incubate at 4°C overnight

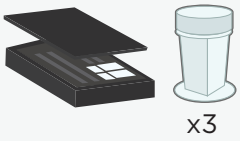
Day 2: Wash and Stain Slide



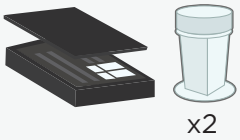
Remove Unbound Antibodies
- 3 washes in 1X TBS-T, 10 mins each wash.
- Wash in 1X PBS, 2 min



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



Post Fixation
- Apply 4% PFA to tissue
- Incubate for 15 minutes
- 3 washes in 1X PBS, 5 min

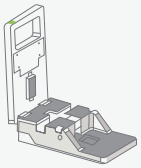


Nuclear Stain
- Prepare Nuclear Stain stock and apply to tissue
- Incubate 10 mins at RT
- 2 washes in 1X PBS for 5 mins each wash

Apply NHS-Acetate
- Prepare and apply NHS-Acetate
- Incubate for 15 mins.
- Wash in 1X PBS for 5 mins

Prepare Flow Cells and Load Instrument

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



Prepare Instrument Reagents
- Add enzymes to Buffer 4.
- Add custom reporters (if needed) to the imaging tray.

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

Equipment, Materials, and Reagents

The following equipment (Table 42), materials (Table 43), and reagents (Table 44) are required for this protocol but are not supplied by NanoString.

Equipment:

Table 42: Equipment not provided by Nanostring

	Equipment	Source	Part Number(s)
☐	**Baking Oven**	Quincy Lab, Inc (or comparable)	Various GC Models
☐	**6-quart Pressure Cooker** **NOTE**: Products from other vendors may require testing and optimization	BioSB® TintoRetriever	BSB 7015
☐	**Ultrasonic Bath** (500 mL capacity) **NOTE**: 400kHz frequency with timer	General Lab Supplier	Example
☐	**Vortex Mixer**	General Lab Supplier	Various
☐	**Microcentrifuge** for 1.5 mL microcentrifuge tubes	General Lab Supplier	Various
☐	**Analytic Scale with draft shield** **NOTE**: ensure scale can weigh in milligrams (mg) for accuracy.	Various	Example

Materials:

Table 43: Materials not provided by Nanostring Technologies Inc.

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

Reagents:

Table 44: Reagents not provided by Nanostring Technologies Inc.

	Reagent	Source/Part Number(s)	Storage Conditions
☐	**10% Neutral Buffered Formalin** (NBF) 	EMS Diasum®, 15740 (or comparable)	Room temperature
☐	**DEPC-treated Water**	ThermoFisher, AM9922 (or comparable)	Room temperature
☐	**10X Phosphate Buffered Saline pH 7.4 (PBS)**	ThermoFisher, AM9625 (or comparable)	Room temperature
☐	**TBS with Tween (TBS-T), 20X**	ThermoFisher, J77500.K2	Room temperature
☐	**Tris Base**	Sigma-Aldrich 10708976001 (or comparable)	Room temperature
☐	**EDTA**		Room temperature
☐	**Tween 20**		Room temperature
☐	**Sulfo NHS-Acetate powder** NOTE: NHS-Acetate powder is shipped in a plastic bag with a desiccant and should be left in the bag and stored at -20°C until ready to use.	Fisher Scientific, 26777	-20°C
☐	**Paraformaldehyde (PFA) 16% Aqueous Solution** 	EMS,15710	-20°C

NanoString Supplied Reagents

CosMx SMI Protein Slide Preparation Kit

Figure 87: CosMx Protein Slide Preparation Kit

Table 45: CosMx Protein Slide Preparation Kit

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

CosMx Protein Mouse Neuro Protein Kit (see Panel and Cell Segmentation Marker Selection on page 12).

Figure 88: CosMx Mouse Neuro Protein Kit

Table 46: CosMx Mouse Neuro Protein Kit

Kit Contents (Store at -80°C)	
CosMx Mouse Neuro Cell Typing Core Ab Mix	CosMx Mouse Alzheimer's Pathology Module Ab Mix

CosMx Segmentation and Supplemental Markers

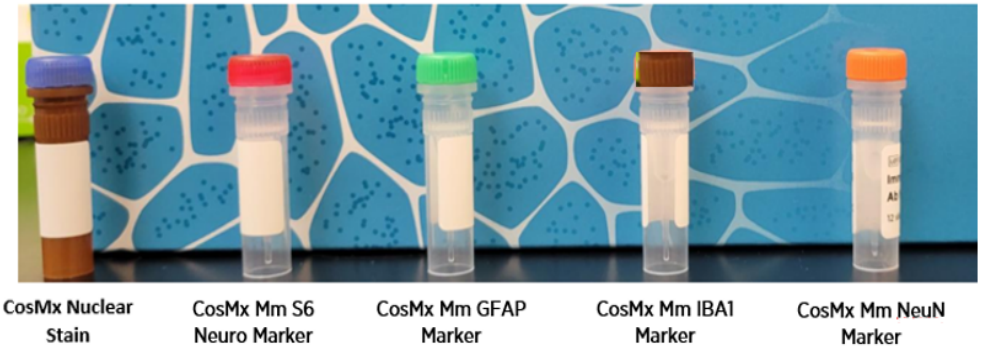


Figure 89: CosMx Segmentation and Supplemental Markers (Protein FF Mm Neuro)

Table 47:CosMx Segmentation and Supplemental Markers (Protein FF Mm Neuro)

Kit Contents (Store at -80°C) Each kit is sufficient for four (4) slides	
Kit Name	**Kit Component**
CosMx Mouse Neuroscience Cell Segmentation Ch1/2 (Protein) Kit	CosMx DAPI Nuclear Stain CosMx Mm S6 Neuro Marker Ch2 (Protein)
CosMx Mouse Neuroscience Segmentation Ch3/4 (Protein) Kit	CosMx Mm GFAP Marker Ch3 (Protein) CosMx Mm IBA1 Marker Ch4 (Protein)
CosMx Mouse NeuN A La Carte Marker Ch5 (Protein) Kit, Ch5	CosMx Mm NeuN Marker Ch5 (Protein)

Flow-Cell Assembly Tool and Kit

Figure 90: Flow Cell Assembly Tool and Kit

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

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

Prepare Fresh Frozen Tissue Samples

Appendix I: CosMx SMI Sample Preparation Guidelines on page 170 covers Fresh Frozen block selection and sectioning in detail. Please review sample preparation guidelines prior to beginning the Protein 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 VWR Superfrost Plus Micro Slides or Leica BOND PLUS 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 placed within the Scan Area (the green area) of the slide and be no larger than **20 mm Long by 15 mm Wide** (Figure 91) (image not to scale). For best performance, ensure that some tissue-free glass is present in all four corners and within the center of the scan area (the dashed teal line). For examples of tissue placement best practices, see Appendix I: CosMx SMI Sample Preparation Guidelines on page 170.

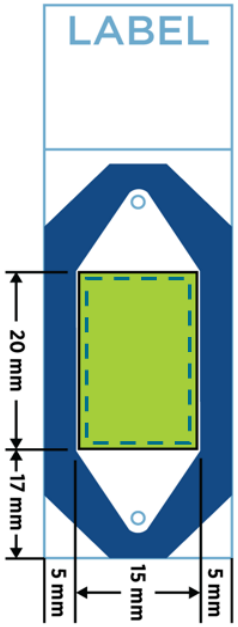


Figure 91: Tissue Scan Area (not to scale)

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 slide at -80°C.

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 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 scannable area. If the tissue section is outside of the Scan Area, it will not be imaged.

Prepare Assay Reagents

Prepare the reagents using the instructions in the following table (Table 48).

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

Table 48: Prepare protein assay reagents

	Reagent	Dilution	Storage
☐	10% NBF **NOTE**: 10% NBF must be pre-chilled before use.	n/a Pre-chill NBF at 4°C for a minimum of 30 minutes prior to use (up to overnight).	4°C (overnight)
☐	1X Tris-Buffered Saline with Tween 20 (TBS-T)	Dilute 100 mL of 10X TBS-T in 900 mL of DEPC-treated water.	Room Temperature
☐	Tris-EDTA buffer (pH 9.0)	• Combine 1.21 g of Tris and 0.37 g of EDTA in 1 L of DEPC-treated water. • Mix to dissolve and adjust pH to 9.0. • Add 0.5 mL of Tween 20 and mix well.	Room Temperature
☐	1X PBS	Prepare 1 L of 1X PBS by diluting 100 mL of 10X PBS into 900 mL of DEPC-treated water.	Room Temperature
☐	NHS-Acetate Mix	Individual aliquots can be prepared prior to slide preparation but should be kept at -20°C with a desiccant until use. **To prepare aliquots:** • Bring stock to room temperature for 1-2 hours prior to opening. • Prelabel 4 screw top 1.5 mL centrifuge tubes with the required information. • Using a weighing spatula, carefully weigh 20-25 mg of NHS-Acetate directly into the screw-top tube on an analytic scale. • Label tube with final weight, close	-20°C in desiccant

	Reagent	Dilution	Storage
		tube, seal the tube with parafilm, and return to -20°C until use.	
☐	Segmentation Markers and Target Antibodies	These reagents have additional steps that will be covered in detail in their respective sections. Remove these reagents from storage and keep on ice until ready to use.	n/a
☐	Day 2 Reagents	These reagents have additional steps that will be covered in detail in their prospective steps. See Day 2: Wash Off Unbound Antibodies (50 minutes) on page 126 for preparation of these reagents.	n/a
☐	4% Paraformaldehyde (PFA)	Prepare 1 mL of 4% PFA by adding 250 µL of 16% PFA to 750 µL of DEPC-treated water.	Prepare Fresh

Day 1: NBF Fixation and Bake

You will need the following materials and reagents for this step: **Staining jars, prechilled 10% NBF, 1X PBS, pressure cooker, Tris-EDTA buffer (pH 9.0), 1X TBS-T.**

- **Pre-cool a staining jar** filled with 10% NBF to 4°C for a minimum of 30 minutes, up to overnight. Ensure that you have sufficient buffer to cover all slides in container for the washes. Wash buffers need to completely cover the tissue on the slide but should be below the slide label. The washes may make the slide labels illegible if submerged.
- **Preheat oven** to 60°C.
- Remove sample slides from -80°C freezer and place onto dry ice to carry sample slides to slide preparation area.

NBF Fixation

1. Remove slides from dry ice and immediately **transfer to pre-cooled 10% NBF**.
2. Incubate slides in **10% NBF for 2 hours at 4°C.**

Figure 92: 2 Hour NBF Fixation

3. Following NBF fixation, **transfer slides to 1X PBS** and wash for 5 minutes.
4. **Repeat PBS wash twice**, using a new staining jar for each wash, for a total of 3 washes.

Figure 93: Wash 3x with 1X PBS

5. After final PBS wash, place slides into slide holder and bake vertically at 60°C for 30 minutes. During 30 minute bake, prepare the pressure cooker and staining tray.

Prepare Equipment

6. **Prepare the humidity chamber (staining tray) according to product instructions**. If your chamber is light-permeable, minimize light exposure (e.g., by wrapping the lid in aluminum foil).
7. Fill a new staining jar with **Tris-EDTA buffer (pH 9.0)**. When closing the jar, **do not fully seal the lid.**
8. Prepare the pressure cooker and preheat the **Tris-EDTA buffer (pH 9.0)** to 80°C:

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

- **Fill the pressure cooker** with water to the correct level per the manufacturer's instructions (4-8 cups depending on model used).
- **Place the staining jar containing freshly prepared Tris- EDTA buffer (pH 9.0) into the pressure cooker to preheat**. Ensure that water level is well below lid of jar, about halfway up jar will be sufficient. If needed, a trivet may be used to raise the jar.
- **Preheat the pressure cooker to 80°C** following the model specific instructions below: Pressure cooker preheating will take about 1 hour.

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 80°C.**
- After the second cycle is complete, continue to Perform Target Retrieval (1 hour) on page 150.

9. **Following the 30 minute bake**, carefully remove the slides from the over and place into a staining jar containing **1X TBS-T**. Wash slides for **5 minutes**.

Figure 94: Wash 3x in 1X TBS-T for 5 minutes

10. **Repeat wash twice**, using a new staining jar for each wash, for a total of 3 washes.
11. Continue immedediately to Perform Target Retrieval (1 hour) on page 150.

Perform Target Retrieval (1 hour)

You will need the following materials and reagents for this step: **staining jars, steamer, preheated Tris-EDTA buffer (pH 9.0)**, and **1X TBS-T**. See Prepare Assay Reagents on page 145 for more details.

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

> ⓘ **IMPORTANT**: Once the lid is removed, the temperature of the buffers will start to fall rapidly. Try to limit the time the pressure cooker is uncovered to 30 seconds (maximum uncovered time is 2 minutes). Reproducible results rely upon minimizing this variation in temperature.

1. **Following the final TBS-T wash, carefully remove the pre-heated Tris-EDTA buffer (pH 9.0),** and add the slides to the pre-heated solution. When closing the jar, **do not fully seal the lid**. This will prevent pressure from building within the jar and potentially damaging the slides.

2. Carefully place the staining jar containing the slides back into the preheated pressure cooker (Figure 95). Ensure that water level is well below lid of jar. About halfway up jar will be sufficient. If needed, a trivet may be used to raise the jar.

Figure 95: Target Retrieval

3. Attach the pressure cooker lid and **close the pressure valve.** Return the pressure cooker to 80°C .
4. **Once the pressure cooker temperature returns to 80°C**, start timer and run for **7 minutes**.

5. When the timer reaches zero, press cancel on the pressure cooker to stop heating, release the pressure valve to release pressure. **Carefully and slowly remove the pressure cooker lid and remove the staining jar.**

6. Leave the staining jar containing sample slides at **room temperature for 5 minutes** (Figure 96).

Figure 96: Stand at Room Temperature.

7. Once the slides have equilibriated to room temperature, transfer the slides to 1X TBS-T and **wash the slides in 1X TBS-T** for **5 minutes** (Figure 97).

Figure 97: Wash 3X in 1X TBS-T

8. **Repeat wash 2 times** for a total of 3 washes.
9. Immediately proceed to next step (Blocking (1 hour) on page 121).

Blocking (1 hour)

You will need the following materials and reagents for this step: **incubation frames, humidity chamber**, and **Buffer W**. See Prepare Assay Reagents on page 145 for more details.

1. **Fill the humidity chamber** with enough water to cover the bottom of the trough. Do not overfill as splashing while moving the tray chamber should be avoided.
2. Prepare the Incubation Frame:
    - Separate an individual frame from the strip by tearing along the perforations. Each frame is sandwiched between a thin polyester sheet and a thick polyester frame backing (with the center square removed).
3. If needed, trim the tissue following the template in Prepare Fresh Frozen Tissue Samples on page 143.
4. Using an absorbent wipe, ensure that the surface of the slide that will come in contact with the incubation frame is **dry and clean. Take care not to damage the tissue and work quickly to minimize dehydration of the sample.**
5. Apply the incubation frame (Figure 98).
    - 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, **carefully place the incubation frame** around each tissue section careful not to touch the adhesive surface. Lightly press along the border of the incubation frame to ensure that it is well adhered to the slide. Be careful to avoid applying any pressure in the center of the frame so as not to damage the tissue.

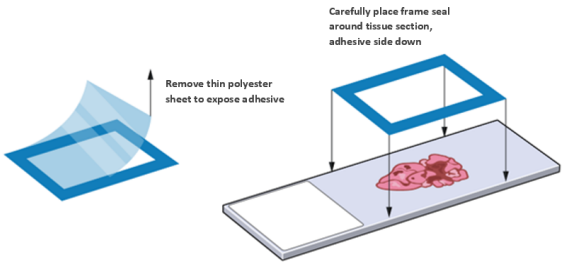


Figure 98: Apply Incubation Frame

6. Place the slides in the humidity chamber in a horizontal position. Add **200 µL Buffer W** directly to the tissue within the incubation frame and cover the humidity chamber (Figure 99).

Figure 99: Incubate for 1 hour

7. **Incubate in Buffer W for 1 hour** at room temperature (RT) in a closed humidity chamber.
8. Remove Protein Panel, and cell segmentation kits, and any supplemental markers (if applicable) from freezer and **thaw mix on ice.** Once thawed, prepare antibody mixture following the instructions found in Primary Antibody Incubation (overnight) on page 154.

Primary Antibody Incubation (overnight)

You will need the following materials and reagents for this step: **humidity chamber, protein antibody mix, Cell Segmentation Markers, Supplemental Markers** (if applicable), **Buffer W**, and **incubation frame covers**. See Prepare Assay Reagents on page 145 for more information.

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

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

2. Make a working antibody solution by diluting **protein probe mix, cell segmentation markers, any supplemental or à la carte markers** (if applicable), and **diluted custom antibodies** (if applicable) into Buffer W (n = number of slides) (Table 49). Adjust volumes to cover the number of slides to be prepared (125 µL per slide).

   - Antibody mix: 1:5 dilution for each. Note, the 1:5 dilution is specific to the Mouse Neuro Antibody Mix.
   - Cell Segmentation and supplemental markers: 1:25 dilution for each marker.

Table 49: Mouse Neuro Antibody Calculations (n = number of slides)

Mouse Neural Cell Typing Core Panel	Mouse Alzheimer's Pathology Module	Mm S6 Neuro Segmentation Marker	GFAP Marker	IBA1 Marker	A la carte marker (NeuN)	Custom Antibodies* (if applicable)	Buffer W	Total Volume (µL)
25 µL x *n*	25 µL x *n*	5 µL x *n*	5 µL x *n*	5 µL x *n*	5 µL x *n*	1.25 µL x *n*	up to final volume of 125 µL x *n*	125 µL x *n*

* Volume is per antibody. **NOTE**: If using an antibody with a target concentration of **4 µg/mL, 2.5 µL x *n* must be used.**

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

3. Remove slides from the humidity chamber, **gently tap slide on a clean Kimwipe to remove excess Buffer W.**

4. Carefully remove the thick polyester frame backing (with the center square removed) from the incubation frame to expose the top adhesive layer of the incubation frame (Figure 100). **Ensure that the incubation frame does not lift from the slide when removing the polyester frame backing.**

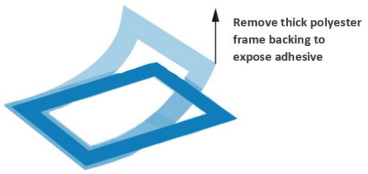


Figure 100: Remove polyester frame backing

5. **Place the slide into the humidity chamber in a horizontal position.**

6. **Add 125 µL of the diluted antibody solution** directly to the tissue within the incubation frame.

   Start by adding the antibody solution to the edge of the tissue within the frame. Applying the incubation frame cover will help move the antibody solution across the tissue (Figure 101).

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

7. **Carefully apply incubation frame cover** (Figure 101). Start by setting one edge of the cover down on the incubation frame edge, then gradually lay down the rest of the cover. Press around the edges of the cover (along the border of the incubation frame) to ensure good adherence. **Do not press the center of the cover as it could damage the tissue.**

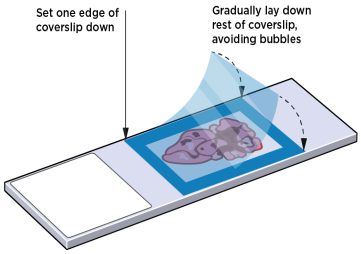


Figure 101: Apply Incubation Frame Cover

8. **Repeat step 3-7 for each slide.**
9. 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.
10. Transfer the humidity chamber to the 4°C refrigerator and incubate overnight (16-18 hours). Minimize exposure to light and ensure the humidity chamber stays level to avoid losing antibody solution (Figure 102).

Figure 102: Incubate overnight at 4°C

Day 2: Wash Off Unbound Antibodies (50 minutes)

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

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

You will need the following materials and reagents for this step: **staining jars, 1X TBS-T, ultrasonic bath, vortex, fiducials** (at RT), **1X PBS** and **4% PFA**. See Prepare Assay Reagents on page 145 for more details.

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

Figure 103: Wash 3X in TBS-T

4. During first wash, remove fiducials from 4°C storage and bring to room temperature for 10 minutes.
5. If not already done, remove the stock PFA from the -20°C and dilute to the 4% working concentration. See Prepare Assay Reagents on page 145.
6. **Repeat TBS-T wash 2 times** for a total of 3 TBS-T washes (Figure 103).
7. During the final washing step, **prepare fiducials** (Fiducial Prep and Application (20 minutes) on page 158).

Fiducial Prep and Application (20 minutes)

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

1. **Prepare fiducials for use following the below steps** (Figure 104):

> **IMPORTANT**: When sonicating the fiducial tube, be sure to not 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.

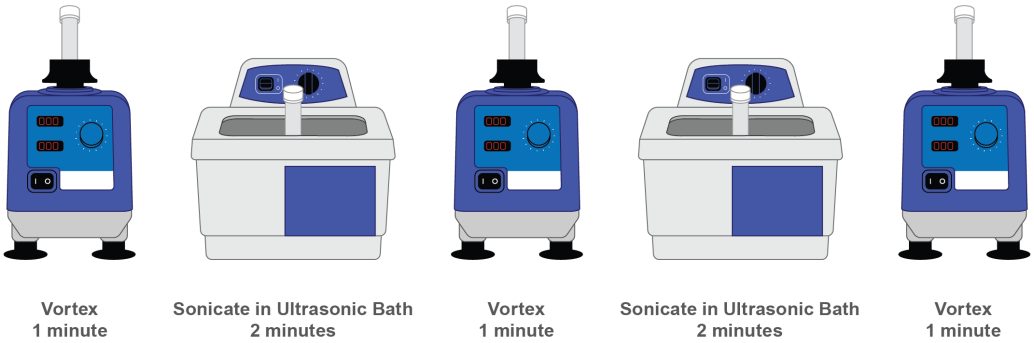


Figure 104: Prepare Fiducials

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

2. Once fiducials are prepared, **dilute fiducial stock (0.1%) to the working concentration (0.00005%) in 1X TBS-T.** A 2-step serial dilution is recommended for fiducial preparation. Accurate dilution of fiducialsis critical for proper assay performance.
   - Dilute stock to 0.01% by adding **10 µL of the fiducial stock to 90 µL of 1X TBS-T**. Label tube as *Dilution 1 (D1)*.
   - Cover *Dilution 1* and **leave at room temperature for 10 minutes** protected from light.
   - After 10 minutes, vortex and quick spin *Dilution 1* and dilute to the final working concentration (0.00005%) using the following table (Table 50).

Table 50: Fiducial Final Dilution

Number of Slides	Dilution 1 (D1) (0.01% Fiducials)	1X TBS-T	Final Volume
2-slides	2.5 µL	497.5 µL	500 µL
4-slides	5 µL	995 µL	1000 µL

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

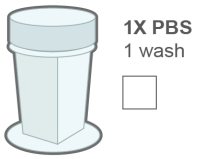


Figure 105: Wash for 2 minutes in 1X PBS

4. **Remove the slides one at a time** from 1X PBS and carefully **dry the slide around the tissue using a clean Kimwipe**. Ensure that the surface of the slide that will come into contact with the incubation frame is clean and dry while being careful to not touch the tissue.
5. If needed, carefully reapply the incubation frame following the instructions in Blocking (1 hour) on page 152. Ensure that the frame is well adhered to the slide by gently pressing around the frame with clean forceps.
6. Lay the slides horizontally in the staining tray.
7. **Immediately before applying fiducials to tissue, vortex for 1 minute.** Vortex fiducials for 30 seconds between slides to keep fiducials in suspension and to ensure consistent concentration across all slides.
8. Apply up to 200 µL of **the final fiducial solution, ensuring the solution covers glass and tissue within the incubation frame**. Fiducials must be present on the glass within the scan area for consistent focusing during the instrument run.
9. **Incubate slides in humidity tray for 5 minutes at room temperature. Ensure that fiducial solution completely covers the entire area within the incubation frame.**

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

10. Following fiducial incubation, **transfer slides to fresh 1X PBS.** Wash slides for 5 minutes in the 1X PBS.

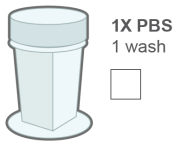


Figure 106: Wash 5 minutes in 1X PBS

11. After PBS wash, transfer slides into the humidity chamber and add **up to 200 µL 4% PFA directly to tissue. Incubate covered for 30 minutes** in the humidity chamber at room temperature (Figure 107).

Figure 107: Incubate 30 minutes

12. After PFA incubation, wash with **3 changes of 1X PBS for 5 minutes each** (Figure 108).

Figure 108: Wash 3X with 1X PBS

13. During PBS washes, **remove Nuclear stain and NHS-Acetate from the freezer and bring to room temperature**.

Nuclear Staining (20 minutes)

You will need the following materials and reagents for this step: **humidity chamber, staining jars, Nuclear Stain,** and **1X PBS**. See Prepare Assay Reagents on page 145 for more details.

1. If you have not already, **remove Nuclear Stain** from the freezer (stored at -80°C) and **warm to room temperature on bench** for at least 10 minutes before use.
2. **Prepare 200 µL x *n* of Nuclear Stain Buffer by diluting the nuclear stain stock 1:40 (where *n* equals the number of slides).**
   - **Vortex, then centrifuge thawed nuclear stain** for at least **1 minute** to bring the solution to the bottom of the vial and precipitate insoluble particles. *Do not pipette from the bottom of the vial*.
   - Add 5.0 µL x *n* of Nuclear Stain Stock into 195 µL x *n* of 1X PBS. Adjust volume as needed for more than one slide.
   - Mix by pipetting up and down 3-5 times. Do not vortex.

Table 51: Prepare Nuclear Stain

Nuclear Stock	1X PBS	Total Volume
5 µL x *n*	195 µL x *n*	200 µL x *n*

3. **Place slides in humidity chamber** in a horizontal position and **slowly apply 200 µL of Nuclear Stain** directly to tissue within the incubation frame. Gently move the tray side to side as needed to ensure that the stain covers the entire tissue.

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

4. **Incubate slide for 15 minutes** at room temperature in the covered humidity chamber (Figure 109).

Figure 109: Incubate 15 minutes at RT

5. Following nuclear stain incubation, **wash slides for 5 minutes in 1X PBS** (Figure 110).

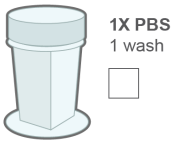


Figure 110: 1X PBS Wash

6. **Transfer** to a staining jar with **fresh 1X PBS and repeat wash** (Figure 111).

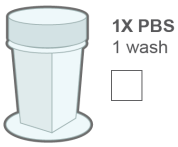


Figure 111: 1X PBS Wash

NHS-Acetate Preparation and Application (25 minutes)

You will need the following materials and reagents for this step: **scale, 2.0 mL centrifuge tube, staining jar, NHS-Acetate powder, NHS-Acetate buffer** (provided by NanoString, 4°C), and **1X PBS**.

1. **Prepare 100 mM NHS-Acetate mixture** immediately before you are ready to apply the mixture onto the tissue (200 µL/sample). Ensure stock NHS- Acetate has reached room temperature before opening stock bottle to prevent condensation.
   a. NHS- Acetate powder is stored desiccated at -20°C. If not already done, prepare 25 mg aliquots of NHS-Acetate powder by weighing out the powder directly into 2.0 mL centrifuge tubes. Label tubes with the exact weight, seal tubes with parafilm, and store tubes at -20°C in desiccant for later use. See Prepare Assay Reagents on page 145.
   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 NHS-Acetate powder by multiplying the weight of NHS-Acetate power in mg by 38.5.
         - Example: for 25.0 mg of NHS-Acetate powder: 25.0 * 38.5 = 962.5 µL of buffer to add.

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

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

Figure 112: Incubate 15 minutes

- Repeat with remaining slides and **incubate covered in staining tray for 15 minutes at room temperature** (Figure 112).

3. Carefully remove the incubation frame using clean forceps. Tap off excess liquid and submerge slides in **1X PBS** for a minimum of five (5) minutzes.
   - 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: Wash Off Unbound Antibodies (50 minutes) on page 157. Ensure the entire incubation frame is removed then store according to Safe Storage Guidelines for Protein Slides below.
   - If samples will be **loaded onto the instrument the same day**, remove the incubation frame following the guidelines from Day 2: Wash Off Unbound Antibodies (50 minutes) on page 157, then continue to Flow Cell Assemblyon page 165.

Safe Storage Guidelines for Protein Slides

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

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

Flow Cell Assembly

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

The provided flow cell assembly tool is a clamshell design that applies uniform force to adhere the flow cell coverslip onto the prepared slide (Figure 113).

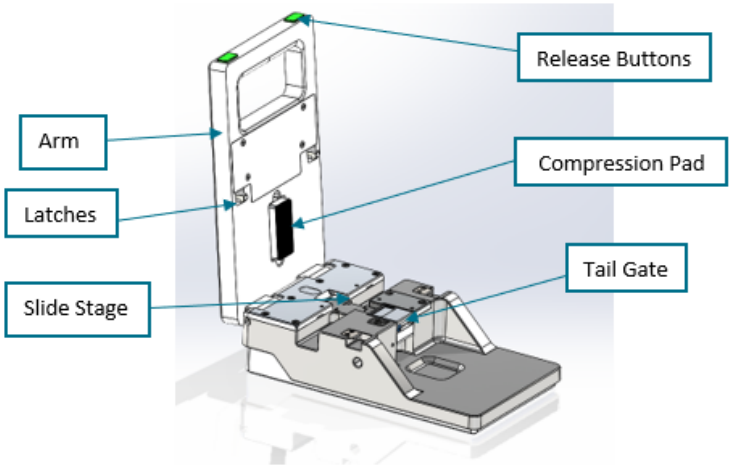


Figure 113: Flow Cell Assembly Tool

Before beginning flow cell assembly, verify tissue placement using the template provided on the flow cell assembly tool (Figure 114, not to scale). The maximum tissue allowable area is 17mm x 20.8mm as represented by the solid bright green rectangle.

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

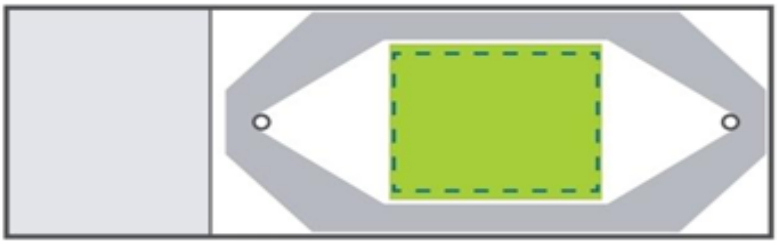


Figure 114: Tissue Allowable Area

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 to not wipe the slide within the imaging area** (shown in green) as this could remove fiducials required for on instrument imaging.
   - If using an adhesive slide label, ensure the label is less than 295 μm thick and is not folded over on itself. If label extends over frosted label area of the slide, carefully trim using a clean razor blade. Labels over the maximum thickness or labels that are not properly adhered may result in slide or flow cell damage during flow cell assembly and/or instrument loading.

4. **Lower the tailgate** (tool marker 1) on the flow cell assembly tool (Figure 115). 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).

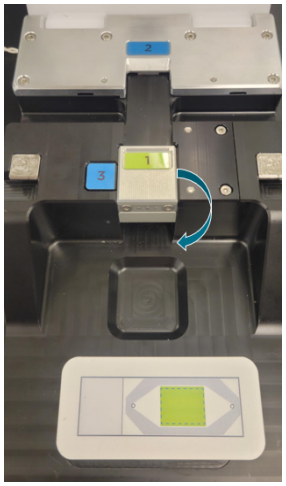


Figure 115: Lower Tailgate

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 (Figure 116). The air gaps should be reduced and signs of adhesion (dark patches) should be present along the edges.

Figure 116: 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 117). Once engaged, both green release buttons will pop out

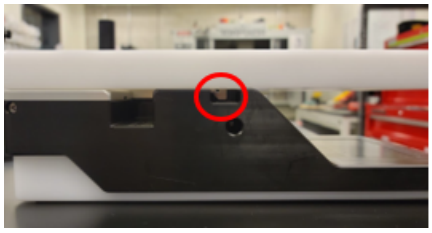


Figure 117: Fully Engaged Latch

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 or 1X PBS) 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 instrument Protein and RNA assays, care should be taken to preserve sample integrity in all steps. The integrity of FFPE samples can be impacted by many factors, including time from excision to fixation, storage conditions, tissue type, and sample age. It is important to take such factors into consideration when selecting samples for the CosMx SMI assay. Samples with poor integrity are likely to give low signal, particularly in the 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.

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 all-inclusive guide on sectioning. Please refer to your local pathologist, histologist, or core facility for training on sectioning.

- For both the Protein and RNA assays, it is important to avoid any scratches and folds in the tissue section. These scratches and folds can be magnified by the subsequent slide washes on the CosMx SMI instrument resulting in tissue loss.
- Sections should be cut at 5 μm thickness on a calibrated microtome. Blocks may be sectioned up to 10 μm thickness; however, the instrument will only image the 5 μm closest to the slide.
- Always discard the first few sections from the block face.
- Sections should be mounted in the center of the slide scannable area (Figure 118) while allowing adequate room for flow cell coverslip adhesives at the edges of the Scan Area.
- NanoString recommends the use of VWR® SuperFrost™ Plus Micro slides (for manual slide preparation) or BOND PLUS slides (for BOND RX/RX^{m} semi-automated slide preparation). If mounting multiple sections per slide, ensure that all tissues are at least 2–3 mm apart and still contained within the scan area.
- Any water trapped under the wax or tissue section should be removed by gently touching a folded Kimwipe onto the corner of the wax section. The Kimwipe should not contact the tissue.
- It is recommended to use mounted sections within two weeks for best results. Older sections (1–2 months) may produce reasonable results, but this may be tissue or block dependent and should be tested empirically. Slides should be stored at room temperature in a desiccator or at 4°C prior to processing.

1. Unstained tissue sections should be sectioned at **5 µm thickness** and mounted on the label side of VWR Superfrost Plus Micro Slides or Leica BOND PLUS slides (Figure 118) (figure not to scale, see the template on the Flow Cell Assembly Tool for a to-scale template). Blocks may be sectioned up to 10 µm thickness; however, the instrument will only image the 5 µm closest to the slide.

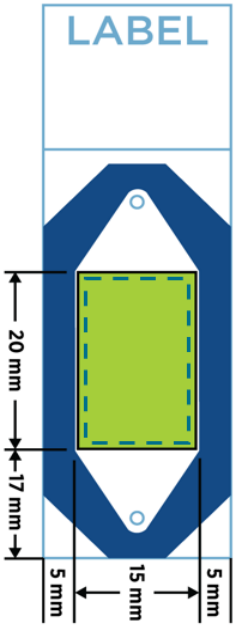


Figure 118: Tissue Scan Area (not to scale)

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 center of the scan area (the dashed teal line) (Figure 119).

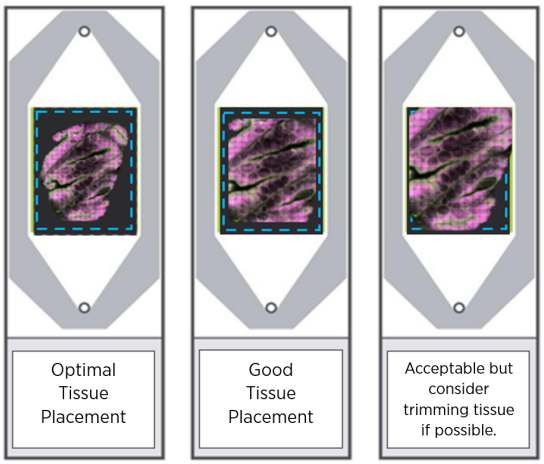


Figure 119: While all 3 examples are acceptable, Slide 1 shows optimal tissue placement; Slide 2 shows good tissue placement, and Slide 3 gives an example of a tissue that should be trimmed if possible as not all corners of the scan area have visible glass and there is minimal glass visible within the 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 5-10 μm thickness on a calibrated cryostat and mounted immediately on a VWR Superfrost Plus Micro slide (for manual slide preparation) or BOND PLUS slide (for BOND/BOND RX^{m} semi-automated slide preparation). During sectioning, it is important to cut across the tissue with a smooth, consistent turn of the hand wheel.

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

- Sections should be centered within the scan area as shown above (Figure 118).
- 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 Digestion (RNA Assay)

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

Table 52: Proteinase Digestion

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

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

Appendix III: Tissue Specific Fiducial Concentrations (RNA Assay)

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

Table 53: Tissue Specific Fiducial Concentration

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

Appendix IV: Adding Custom Barcoded Antibodies

This protocol is for the addition of custom antibodies barcoded through NanoString's Protein Barcoding Service. For additional guidance in selecting and optimizing the use of custom antibodies, please see the white paper: *Selection and Validation of CosMx™ Custom-Labeled Antibodies* available on the Protein Barcoding Service page.

> **IMPORTANT**: when custom Add-On antibodies are ordered, a custom probe kit configuration is generated and linked to the user's AtoMx SIP tenant. Any changes to the custom assay, including the addition or removal of custom antibodies may require a new configuration file to be generated. If a new files is needed, please request a new kit from AtoMxKitAdmin@nanostring.com. This process requires a turn-around-time of 1-3 business days.

Preliminary Screening via Chromogenic Detection

Barcoded antibodies should be screened via 3,3′-Diaminobenzidine (DAB) staining prior to use in a CosMx assay to determine the optimal antibody concentration and to confirm staining specificity (Figure 120). NanoString recommends the Abcam® Mouse and Rabbit Specific HRP/DAB IHC Detection Kit (Abcam, Cat. ab236466) for manual staining or Leica Biosystems' BOND Polymer Refine Detection (Leica Biosystems®, Cat. DS9800) for staining with the Leica BOND System. Start with a range of dilutions around the antibody vendor IHC recommendations for concentration of the unconjugated antibody. Since the modification with a barcode will naturally result in a decreased binding efficiency of the antibody to its antigen, a higher concentration of conjugated antibody than unconjugated may be required.

Conjugated (barcoded) antibodies are provided at a concentration of 200 µg/mL in a buffer of PBS and sodium azide. A test aliquot of 40 µL is provided for the purpose of post-conjugation testing. If there is no recommendation from the supplier, start with a test concentration for the initial range between 0.2 µg/mL and 4 µg/mL. If high background or non-specific staining is observed, it's recommended to test lower antibody concentrations; higher concentrations can be tested if there is no specific and strong signal observed on the tissue of interest; however, a high concentration of antibody will also limit the total number of slides that can be tested. If after adjusting the concentration to 8 µg/mL, no specific or strong signal is present, a different antibody clone should be considered.

In general, the concentration of antibody to use in the CosMx assay is half of the optimal concentration used in DAB staining (Table 54). However, signal intensity and sources of background will differ between the DAB assay and CosMx assay. DAB is an amplification-based method and relies on chromogenic detection, which differs from the fluorescent, amplification-free detection method on CosMx.

Table 54: Suggested conversion between chromogenic (DAB) staining results and concentrations used in the CosMx protein assay for custom conjugates. For illustration purposes only.

Assay	High expressor	Medium expressor		Low expressor
Post-conjugation chromogenic detection by DAB staining	1 µg/mL	2 µg/mL	4 µg/mL	8 µg/mL
CosMx Protein	0.5 µg/mL	1 µg/mL	2 µg/mL	4 µg/mL

In the example on the next page (Figure 120), pre-conjugated and post-conjugated antibodies are screened in control tissues and IHC staining patterns are reviewed by a pathologist to ensure that conjugation has not affected the pattern of binding (top). Post-conjugation titration is performed to determine the optimal antibody concentration and to confirm staining specificity (bottom). Select the lowest concentration that gives specific measurable signal. In the following example (Figure 120), 4 µg/mL was determined to be the optimal concentration via DAB staining, so 2 µg/mL was used in the CosMx assay. In this example, E-cad was found to have faint staining in the pre-conjugation IHC assay and no staining post-conjugation, suggesting that a different clone should be selected, or concentrations should be adjusted.

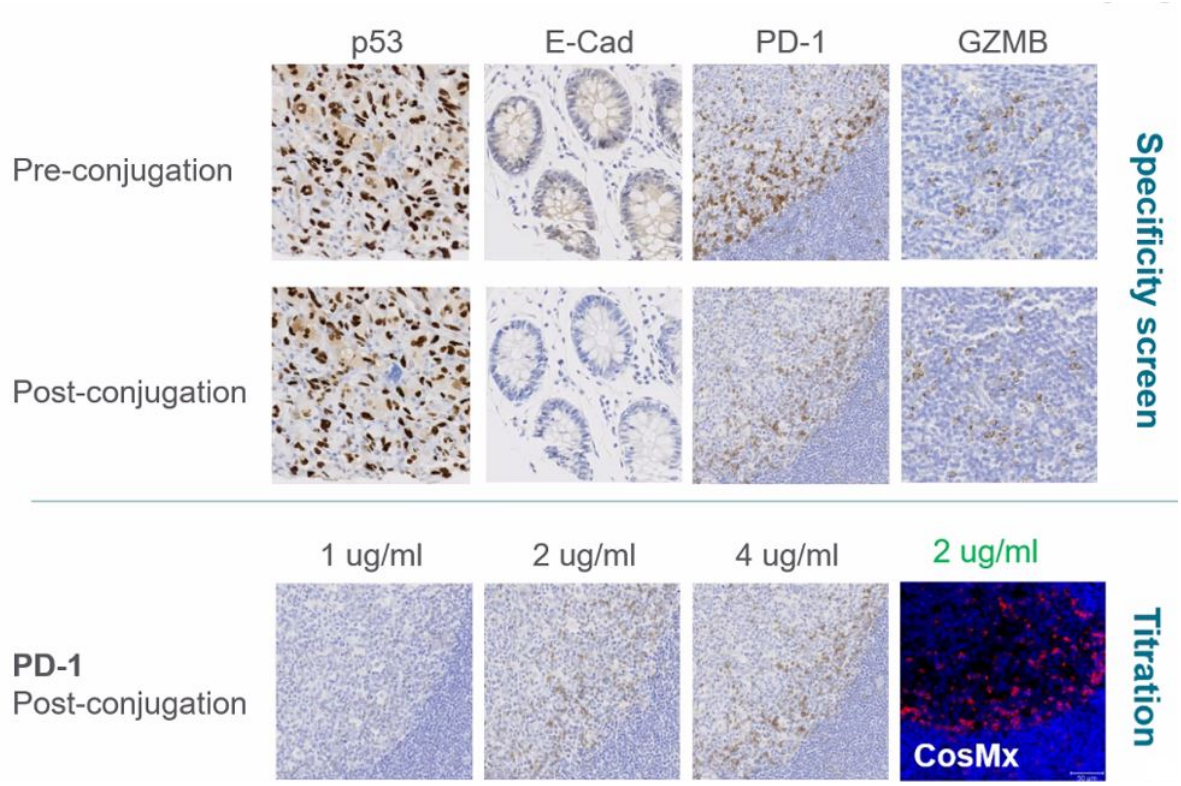


Figure 120: Pre- and post- conjugation antibody screen. Antibody specificity should be evaluated by examining serial sections for comparable staining patterns. Barcoded (post-conjugation) antibodies typically have weaker staining than unconjugated antibodies. If specificity is acceptable, then a titration should be performed to find an optimal antibody concentration. For instance, E-cad shows no specific staining following conjugation, which indicates that conjugation impacts antibody functionality.

Adding Custom Antibodies

1. Thaw custom antibodies. Note which reporter each antibody is associated with (A10 or B10). This is indicated on the stock vial for each antibody and will determine which reporter set(s) to add at instrument run setup.
2. Dilute each custom antibody.
    - Dilute stock antibody in Buffer W as indicated in the table below (Table 55). If DAB staining has not been performed, it is recommended to use 2 µg/mL for initial testing.

Table 55: Custom antibody dilution. Volume prepared is sufficient for up to 4 slides.

Target on-slide Concentration	Antibody	Buffer W	Diluted stock concentration
0.5 µg/mL	2 µL	6 µL	50 µg/mL
1 µg/mL	4 µL	4 µL	100 µg/mL
2 µg/mL or higher	no interim dilution required		200 µg/mL

3. Perform a 1:100 dilution by adding (1.25 µL x n) of each diluted antibody from the table above (Table 55) to the working antibody solution (Primary Antibody Incubation (overnight) on page 123 for FFPE or Primary Antibody Incubation (overnight) on page 154 for Fresh Frozen tissue).
4. Reduce the Buffer W volume added to the working solution accordingly.
5. Discard any unused diluted stock of custom antibody.

> ⓘ **IMPORTANT**: Prior to freezing the master stock for each antibody, it is recommended to further aliquot to minimize the number of freeze-thaw cycles. Store stock antibody -80°C.

NanoString Technologies, Inc.
530 Fairview Ave North
Seattle, Washington 98109 USA
www.nanostring.com

CONTACT US
info@nanostring.com
Tel: +1 888 358 6266
Fax: +1 206 378 6288

SALES CONTACTS
United States: us.sales@nanostring.com
EMEA: europe.sales@nanostring.com
Asia Pacific & Japan: apac.sales@nanostring.com
Other regions: info@nanostring.com