GeoMx® DSP

Automated Slide Preparation

User Manual

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Changes in This Revision

The GeoMx DSP Slide Preparation User Manuals were restructured for clarity and simplicity. This **GeoMx DSP Automated Slide Preparation User Manual** (MAN-10151) covers protein and RNA sample preparation using semi- and fully-automated methods on the BOND RX/RX^m Fully Automated IHC/ISH Stainer from Leica Biosystems®, for assays with NGS or nCounter readout. The GeoMx DSP Manual Slide Preparation User Manual (MAN-10150) covers manual (non-automated) methods. These manuals replace GeoMx-NGS Slide Preparation User Manual (SEV-00153-05) and GeoMx-nCounter Slide Preparation User Manual (SEV-00172-08).

NGS-specific information is separated from nCounter-specific information with colored text boxes, as described in **Conventions on page 8**.

Other changes in this manual revision include:

- Updated items and links in Equipment, Materials, and Reagents lists **on page 13**, **38** and **64**
- Updated slide dimensions diagrams (example **on page 19**) to clarify GeoMx DSP scan area
- Added guidance for epitope retrieval and Proteinase K conditions for mouse tissue **on page 53**, **54**, and **82**, and updated cell pellet Proteinase K digestion conditions **on page 54** and **83**
- Extended safe storage guidelines to 3 months for protein slides **on page 36** and 7 days for RNA slides **on page 62** and **86**
- Added a fully automated slide preparation protocol for RNA assays with NGS or nCounter readout **on page 64**
- Revised storage and handling guidance for RNA probe mixes in step 1 **on page 57** and **77**
- Updated fresh frozen sample preparation guidance **on page 89**
- Updated fixed frozen sample preparation guidance **on page 93**
- Improved guidance for including custom barcoded antibodies in GeoMx protein assays **on page 105**
- Added guidance to use secondary antibodies in morphology marker labeling **on page 108**
- Improved guidance for tyramide signal amplification (TSA) in morphology marker labeling **on page 110**
- Added a section on Troubleshooting **on page 123**

Conventions

The following conventions are used in the GeoMx DSP user manuals 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 or instructional material.

A gray box indicates general information that may be useful for improving assay performance. These notes aim to clarify other instructions or provide guidance to improve the efficiency of the assay workflow.

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

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.

For NGS readout: Content in blue boxes denotes steps or information specific to NGS readout of GeoMx DSP. Follow these instructions if using Illumina® NGS to read out GeoMx DSP counts.

For nCounter readout: Content in green boxes denotes steps or information specific to nCounter readout of GeoMx DSP. Follow these instructions if using nCounter® MAX/FLEX, Pro, or SPRINT to read out GeoMx DSP counts.

GeoMx DSP Workflow

The GeoMx Digital Spatial Profiler (DSP) is a novel platform developed by NanoString. This product relies on antibody or nucleic acid probes coupled to photocleavable oligonucleotide tags. After probes hybridize to targets in slide-mounted tissue sections, the oligonucleotide tags are released from discrete regions of the tissue via UV exposure. Released tags are quantitated by nCounter technology or Illumina Next Generation Sequencing (NGS). Counts are mapped back to tissue location, yielding a spatially resolved digital profile of analyte abundance (see Figure 1).

- **Day 1: Slide Staining.** Prepare slides and incubate biological targets with UV-cleavable probes. Prepare manually or using the BOND RX/RX^{m} Fully Automated IHC/ISH Stainer from Leica Biosystems®.
- **Day 2: Process Slides on GeoMx DSP.** Load prepared slides into the GeoMx DSP instrument and enter slide/study information. Slides are scanned to capture fluorescent images used to select regions of interest (ROIs). The instrument collects UV-cleaved oligos from the ROIs into the wells of a collection plate.

For NGS readout:

Day 3: Transfer the collected aspirates to a PCR plate and perform **Library Prep** with Seq Code primers. Pool and purify the products, then **Sequence** on an Illumina NGS instrument.

Day 4: Process FASTQ sequencing files into digital count conversion (DCC) files using **NanoString's GeoMx NGS Pipeline** on Illumina DRAGEN™ accessed via BaseSpace™ Sequence Hub, or using GeoMx NGS Pipeline standalone software. Upload DCC files on to the GeoMx DSP.

For nCounter readout:

Day 2, continued: Transfer the collected aspirates to a hybridization plate along with GeoMx Hyb Code reagents. Hybridization occurs overnight.

Day 3: Pool wells and **Process on an nCounter MAX/FLEX or Pro Analysis System or SPRINT Profiler.** Upload reporter count conversion (RCC) files to the GeoMx DSP.

- **Day 4 or 5**: Create a **Data Analysis** study in the Data Analysis suite and perform quality-control checks and data analysis, and generate analysis plots.

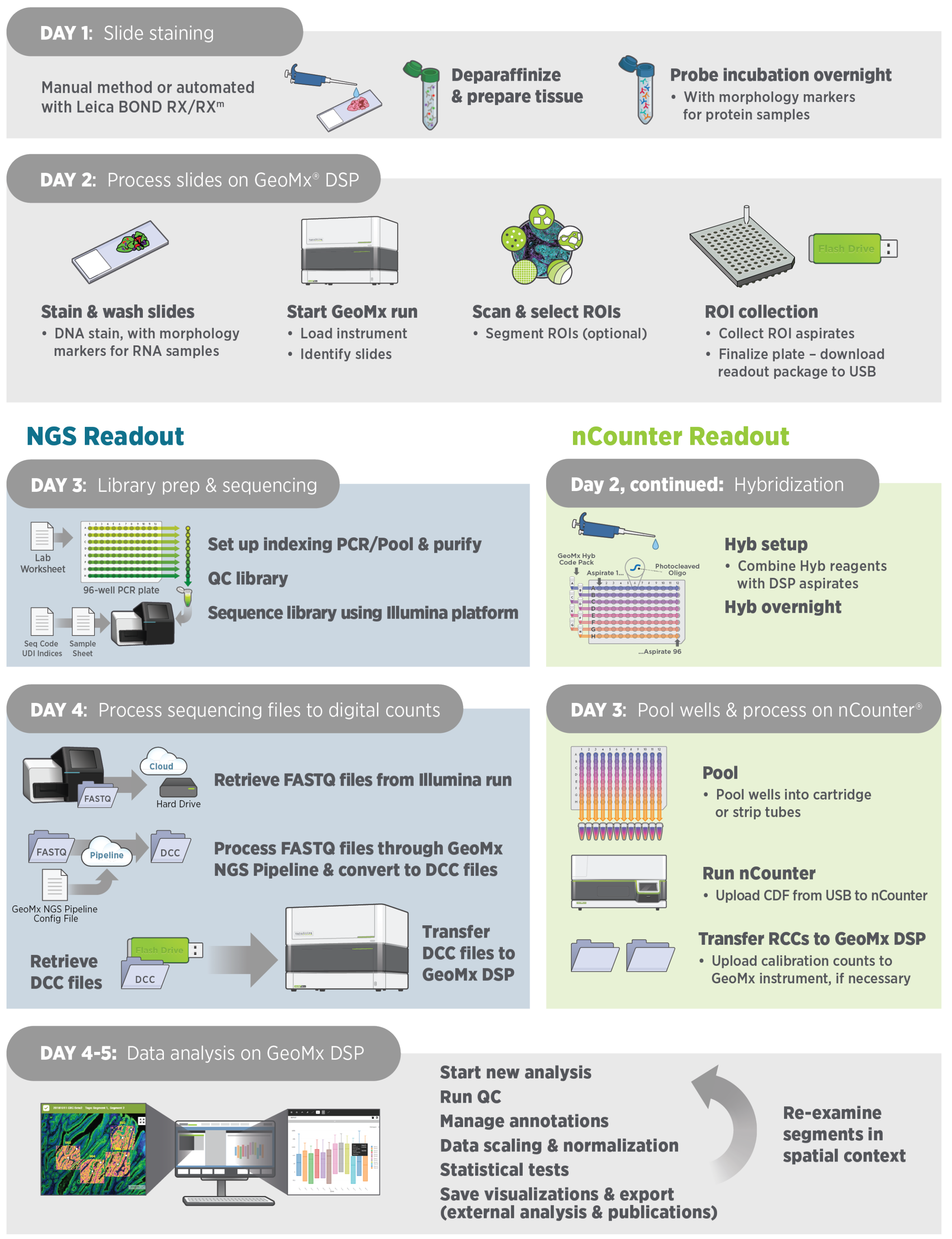

Figure 1: GeoMx DSP workflow summary

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User Manuals and Resources

The GeoMx DSP workflow is divided into the following user manuals:

Workflow Step 1	GeoMx DSP Manual Slide Preparation User Manual MAN-10150 GeoMx DSP Automated Slide Preparation User Manual MAN-10151	
Workflow Step 2	GeoMx DSP Instrument User Manual MAN-10152	
Workflow Step 3	**For NGS readout:** GeoMx DSP NGS Readout User Manual MAN-10153	**For nCounter readout:** GeoMx DSP nCounter Readout User Manual MAN-10089
Workflow Step 4	GeoMx DSP Data Analysis User Manual MAN-10154	

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 available in NanoString University.

For NGS readout: For documentation specific to the Illumina platform, see https://support.illumina.com.	**For nCounter readout:** For documentation specific to the nCounter Pro, MAX/FLEX, and SPRINT instruments, see https://www.nanostring.com/support/support-documentation/ or the NanoString University Document Library at https://university.nanostring.com.

Slide Prep Introduction

Slide preparation is the first step of the GeoMx DSP workflow. Tissue sections are processed for staining, followed by the addition of morphology reagents and biological probes (see Figure 2).

- **Morphology reagents** are antibody-fluorophore complexes that bind to specific targets on the tissue. Tissue structure and cell components most important to your analysis are illuminated using the fluorescence imaging on the DSP system. Alternative labeling techniques are described in the Appendices.
- **Biological probes** are either antibodies or *in situ* hybridization (ISH) probes that bind to protein or RNA targets, respectively. Each probe is coupled to a photocleavable oligonucleotide. These oligos, when exposed to the GeoMx DSP instrument's UV light, will be released into solution and aspirated into a collection plate well for downstream processing.

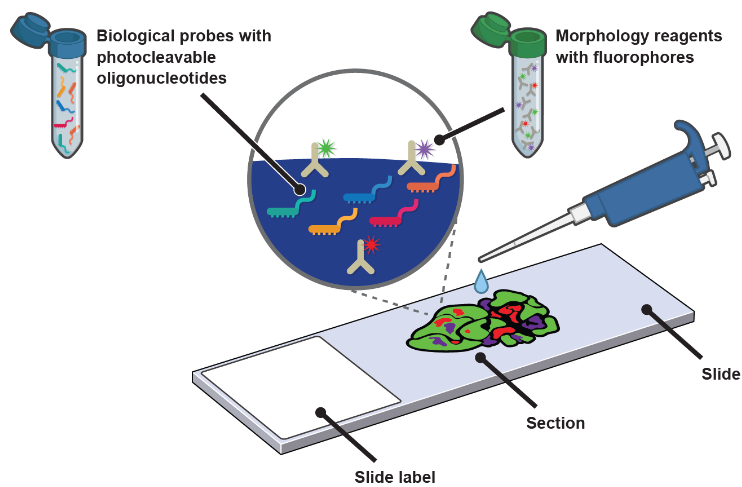

Figure 2: Slide preparation with ISH probes

Slide Prep Equipment, Materials, and Reagents

Required equipment, materials, and reagents are listed at the start of each slide preparation process in the manual. Individual **Equipment, Materials, and Reagents** lists for every application of the GeoMx DSP are available in the NanoString University Document Library (https://university.nanostring.com).

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Semi-Automated Protein Slide Preparation Protocol (FFPE)

This protocol requires the BOND RX or RX^{m} running BOND RX Version 6.0 software (or later).

This is not a fully automated protocol. After processing on the BOND RX/RX^{m}, protein probe application, stringent washes, and morphology marker application are performed manually.

> **IMPORTANT:** NanoString recommends testing your system by performing GeoMx DSP with your BOND RX/RX^{m} on well-characterized samples prior to use on experimental samples.

> **IMPORTANT:** For any given study, NanoString recommends using only one sample type preparation method (e.g., FFPE or fresh frozen samples, but not a combination of sample types).

1 Equipment, Materials, and Reagents

The following tables list equipment, materials, and reagents that are required for this protocol but are **not supplied by NanoString**.

Table 1: Equipment for protein slide prep not provided by NanoString.

Equipment	Source	Part No.
BOND RX Fully Automated IHC/ISH Stainer **or** BOND RX^{m} Compact Fully Automated IHC/ISH Stainer	Leica Biosystems	Contact Leica Biosystems
BOND RX Controller, running BOND RX software version 6.0 or above	Leica Biosystems	Contact Leica Biosystems
Baking oven	Quincy Lab, Inc.(or comparable)	Various GC models

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Table 2: Materials for protein slide prep not provided by NanoString.

Materials	Source	Part No.
BOND Research Detection System (includes 6 x 30 mL Open Containers)	Leica Biosystems	DS9455
BOND Titration Kit (includes 50 inserts)	Leica Biosystems	OPT9049
BOND Universal Covertiles	Leica Biosystems	S21.2001
BOND Open Containers 30 mL (if additional needed)	Leica Biosystems	OP309700
BOND Titration Container Inserts (if additional needed)	Leica Biosystems	OPT9719
Pipettes for 5–1,000 µL	Various	Various
12-channel P20 multi-channel pipetter	Various	Various
Filter tips (DNase/RNase free)	Various	Various
Microcentrifuge tubes (DNase/RNase free)	Various	Various
Apex BOND Adhesive slides	Leica Biosystems	3800040
Slide staining jars (Coplin jars) (recommended number: 12) and slide holder inserts	VWR (or comparable)	25608-904, 25608-868
Humidity chamber	Simport	M920-2 (select black lid)
Hydrophobic barrier pen	Vector Labs (or comparable)	H-4000
RNase AWAY® or 10% Bleach (RNaseZap® is not a substitute)	Thermo Fisher	7003PK
Razor blades	Various	Various
Cover slips (optional)	Various	Various
USB drive v3.0, 64 GB or higher (ability to be NTFS formatted)	SanDisk (or comparable)	SDCZ800-128G-G46

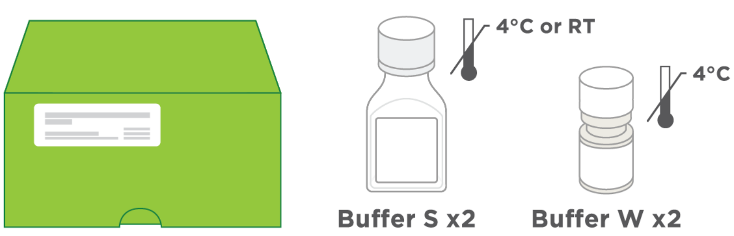
Table 3: Reagents for protein slide prep not provided by NanoString.

Reagents	Source, Part No.	Storage
BOND Dewax Solution, 1 L	Leica Biosystems, AR9222	4°C or RT
BOND Epitope Retrieval 1, 1 L	Leica Biosystems, AR9961	4°C
BOND Wash Solution 10X Concentrate, 1 L	Leica Biosystems, AR9590	4°C
DEPC-treated water	Thermo Fisher, AM9922 (or comparable) NOTE: As an alternative to commercial DEPC-treated water, prepare your own following standard protocols.	RT
10X tris-buffered saline (TBS)	Cell Signaling Technologies, 12498S	RT
10X TBS with Tween-20 (TBS-T)	Cell Signaling Technologies, 9997S	RT
4 or 16% paraformaldehyde (PFA)	Thermo Fisher, 4% concentration, FB002, R37814 16% stock (must be diluted to 4%), 28906, 28908 (or comparable)	4°C (or manufacturer instructions)
1X phosphate buffered saline pH 7.4 (PBS) NOTE: Used *only* to dilute 16% PFA	Sigma-Aldrich, P5368-10PAK, P5368-5X10PAK (or comparable)	RT
Fluoromount-G mounting media (optional)	SouthernBiotech, 0100-01	RT

NanoString Reagents

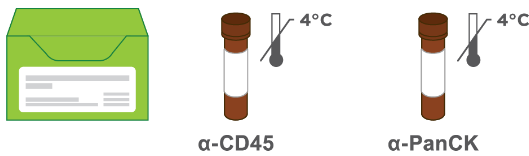
The following reagents are **supplied by NanoString.** Contact your NanoString Sales Representative to use our reagent planning tools to calculate required quantities.

GeoMx Protein Slide Prep Kit

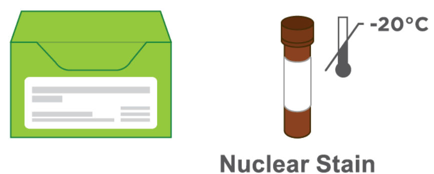


GeoMx Morphology Kit — Human or Mouse Protein compatible (various available)

Example morphology kit (Solid Tumor TME)



GeoMx Nuclear Stain Morphology Kit



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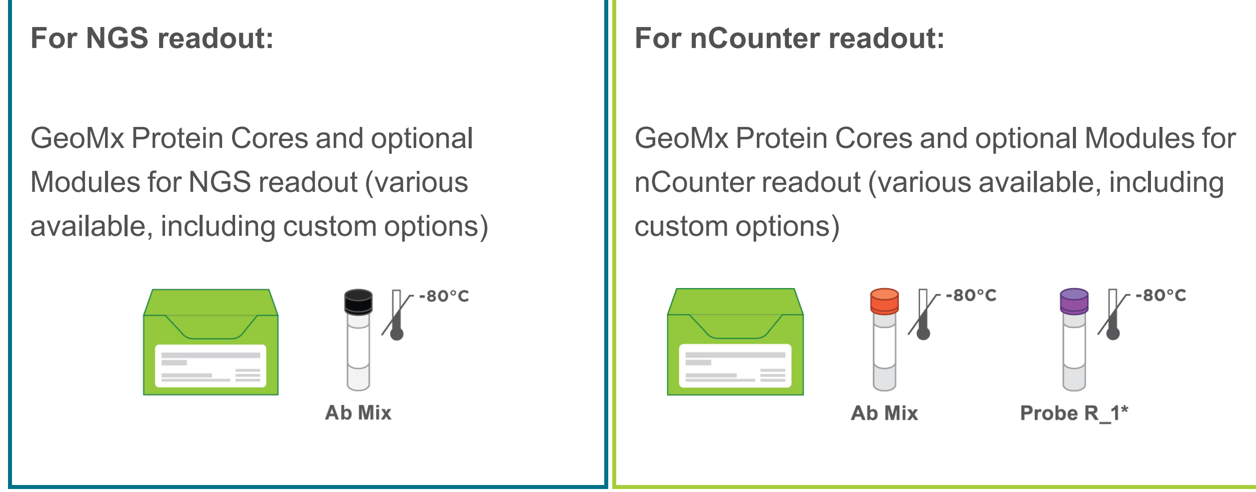

*Each Protein Core and Module for nCounter readout includes a Probe R reagent, from Probe R_1 to Probe R_9. **DO NOT combine two modules with a common Probe R_number in the same experiment run**. Substitute Probe R reagents are available for assays with overlapping Probe R_ numbers. Refer to Appendix IV: Substitute Probe R Guidance on page 104 for more information and to plan Probe R usage in your experiment.

> Each tube of detection antibody (Ab) mix contains sufficient reagent for 12 slides (112 µL). If you are using the entire Ab mix in one week, then store at 4°C. If not, **aliquot the Ab mix** into 4-slide aliquots (37 µL + 37 µL + 38 µL) and freeze unused aliquots at -80°C. Do not exceed more than 2 freeze/thaw cycles and do not freeze diluted antibody.
>
> The morphology marker antibodies are stored at 4°C. Aliquoting is not required.

Prepare reagents

Prepare the reagents using the dilution instructions in **Table 4**. Buffers can be stored in BOND Open containers at 4°C unless otherwise noted. Use DEPC-treated water for all dilutions. If the BOND RX/RX^{m} is also used to prepare slides for GeoMx RNA assays, use DEPC-treated water to dilute BOND Wash Solution.

Table 4: Reagent preparation for Protein Leica Biosystems slide preparation

Reagent	Dilution	Storage
1X BOND Wash Solution	Dilute 10X BOND Wash Solution with distilled water (or DEPC-treated water if ever in contact with RNA slides). Prepare 30 mL for the first run and replenish as needed.	RT
4% paraformaldehyde (PFA)	If using 16% stock, dilute from 16% to 4% in 1X PBS, aliquot, and store.	4°C (or manufacturer instructions)
1X tris-buffered saline with Tween-20 (TBS-T)	Prepare 1 L by diluting 100 mL of 10X TBS-T in 900 mL DEPC-treated water. Total volume needed for slide prep depends on volume on staining jars to be used.	RT
1X TBS	Dilute 500 µL of 10X TBS in 4.5 mL DEPC-treated water in order to prepare a total of 5 mL 1X TBS.	RT

2 Prepare tissue samples

Appendix I: Selecting and Sectioning FFPE Samples on page 87 covers FFPE block selection and sectioning in detail. Review it prior to beginning the Protein Slide Preparation protocol.

GeoMx has been validated for sample blocks up to 3 years old prepared from tissues with a cold ischemic time of less than 1 hour using 10% NBF or similar fixative. For best results, do not use FFPE blocks 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.

Slide Preparation

1. Tissue sections should be **5 µm** thick and mounted on **Apex BOND Adhesive slides**. Tissue sections must **fit within the white dots** marking the boundary on the Apex BOND slides, and be within the **Scan Area** (shown in green) (see Figure 3). Sections can be no larger than 35.3 mm long by 14.1 mm wide and should not overlap the slide gasket (shown in blue) or the Tip Calibration area (shown in red). If mounting multiple sections per slide, ensure that tissues are at least 2–3 mm apart and still fit within the Scan Area.

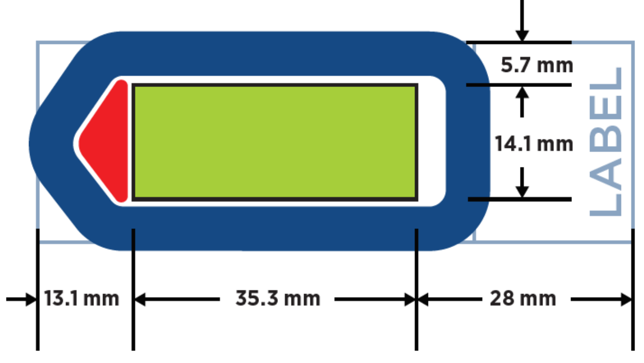

Figure 3: Slide dimensions

If sections are too large and/or placed off-center, continue with slide preparation as usual. Just before loading the slide in the instrument slide tray, scrape off parts of the tissue exceeding the scan area, making sure the slide gasket and tip calibration area are free of tissue. Scraping off tissue before slide preparation could generate tissue folds that may result in staining or binding artifacts.

IMPORTANT: The GeoMx DSP instrument will only image the area inside the Scan Area. **Tissue outside of the Scan Area will not be imaged and may cause problems in tissue detection by the optical system.**

2. **Bake sections on slides** in a 60°C drying oven for 30 minutes to 3 hours prior to deparaffinization. Stand slides vertically during baking to allow excess paraffin to flow off. Longer baking times may be necessary for some tissues to adhere to the slide; this should be empirically tested.

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3 Initial set up of the BOND RX

See Figure 4 for an overview of the BOND RX/RX^m user interface. Refer to the **BOND RX/RX^m User Manual**, accessible from the **Help** icon in the instrument software, for additional information.

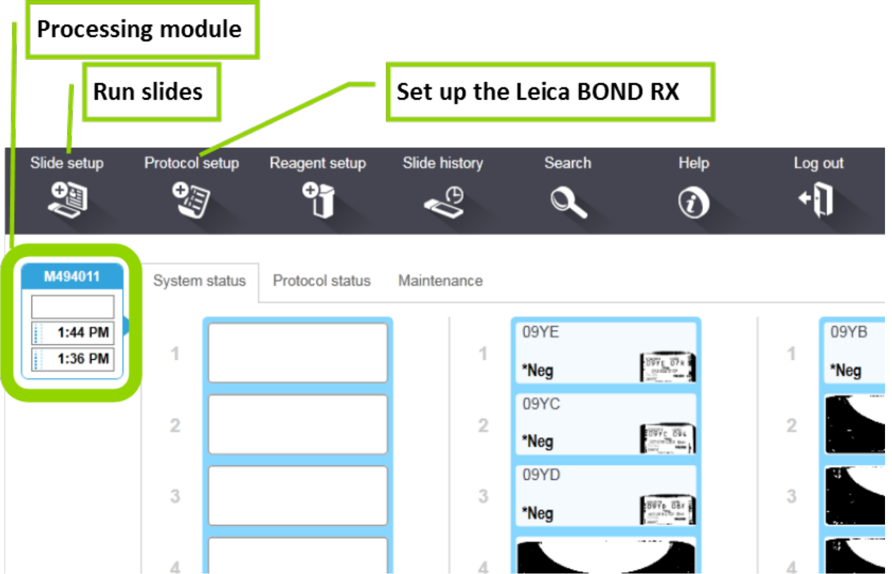

Figure 4: BOND RX/RX^m user interface

To set up the BOND RX/RX^m, you must setup the reagents, register the Detection System, set up the protocols to be used, and register the reagents and the containers in which they will be loaded.

Reagent Setup

If Home Buffer and Buffer W are not registered in the system, you will need to add them.

1. Select the **Reagent Setup** icon (see Figure 5).

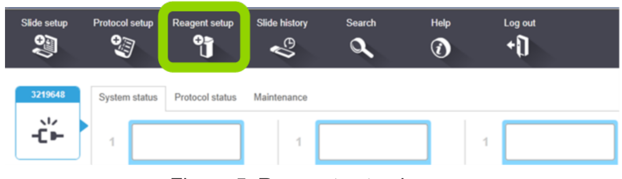

Figure 5: Reagent setup icon

2. From the **Setup** tab, click the **Add** button (see Figure 6).

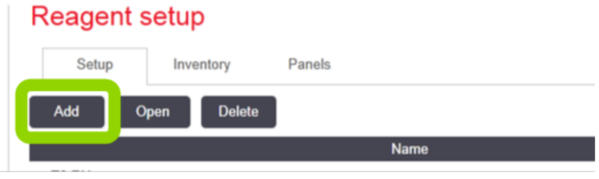

Figure 6: Add reagent button

3. In the **Add Reagent** window (see Figure 7), enter a unique name in the **Name** field. To register Home Buffer, enter *Home Buffer*.
4. Enter a unique abbreviated name in the **Abbreviated Name** field (up to eight characters). To register Home Buffer, enter *HomeBuff* or other abbreviated name.

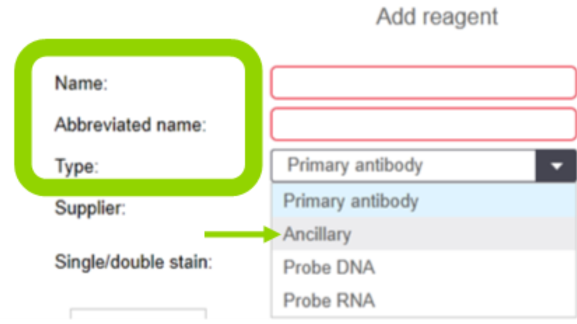

Figure 7: Add reagent window - Home Buffer

5. Select **Ancillary** for Home Buffer.
6. Click **Save**.
7. Register Buffer W as Primary Antibody, if applicable: BOND RX/RX^m systems that have been updated to BOND-RX-Ext-v29 (or later) already include Buffer W as an ancillary reagent, so this step can be skipped.
    a. From the **Setup** tab, click the **Add** button.
    b. In the **Add Reagent** window, enter Buffer W in the **Name** field (see Figure 8).
    c. Enter a unique abbreviated name in the **Abbreviated Name** field (up to eight characters).
    d. From the **Type** drop-down list, select **Primary antibody**.
    e. Select the **Default staining protocol**: **IHC DSP Protocol**.
    f. Select **Default HIER Protocol**: ***HIER 20 min with ER1**.
    g. Leave **Default enzyme protocol** blank.
    h. Check the **Preferred** box.
    i. Click **Save**.

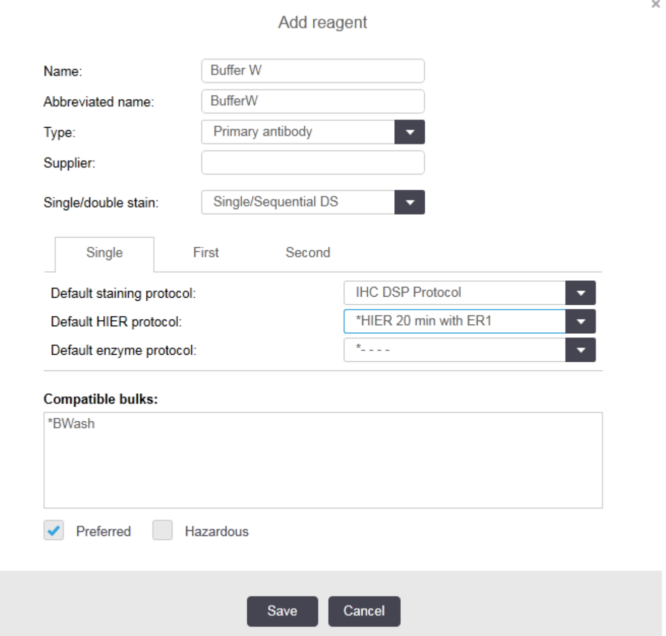

Figure 8: Add reagent window - Buffer W as Primary Antibody

Register the BOND Research Detection System

The BOND Research Detection System is a barcoded tray that holds containers of reagents.

1. **Remove any containers** from the BOND Research Detection System.
2. **Scan the barcode** on the side of the system (see Figure 9).

Figure 9: Research detection system barcode

3. In the **Add research reagent system** window (see Figure 10), enter the information for your reagent system.
   - Enter a **Name** for your BOND Research Detection System (if one has been previously registered, select the name from the drop down menu).
   - Enter the **Lot No.** (optional). Enter an **Expiration Date** (one well in the future).
   - Highlight **Row 1** and **scan the front barcode of a new 30 mL BOND Open Container** (the barcode will appear in the field for Row 1). Place the container in position 1 of the BOND Research Detection System tray.
   - Select **Home Buffer** from the drop down menu in the Reagent column.
   - Click **Add**.

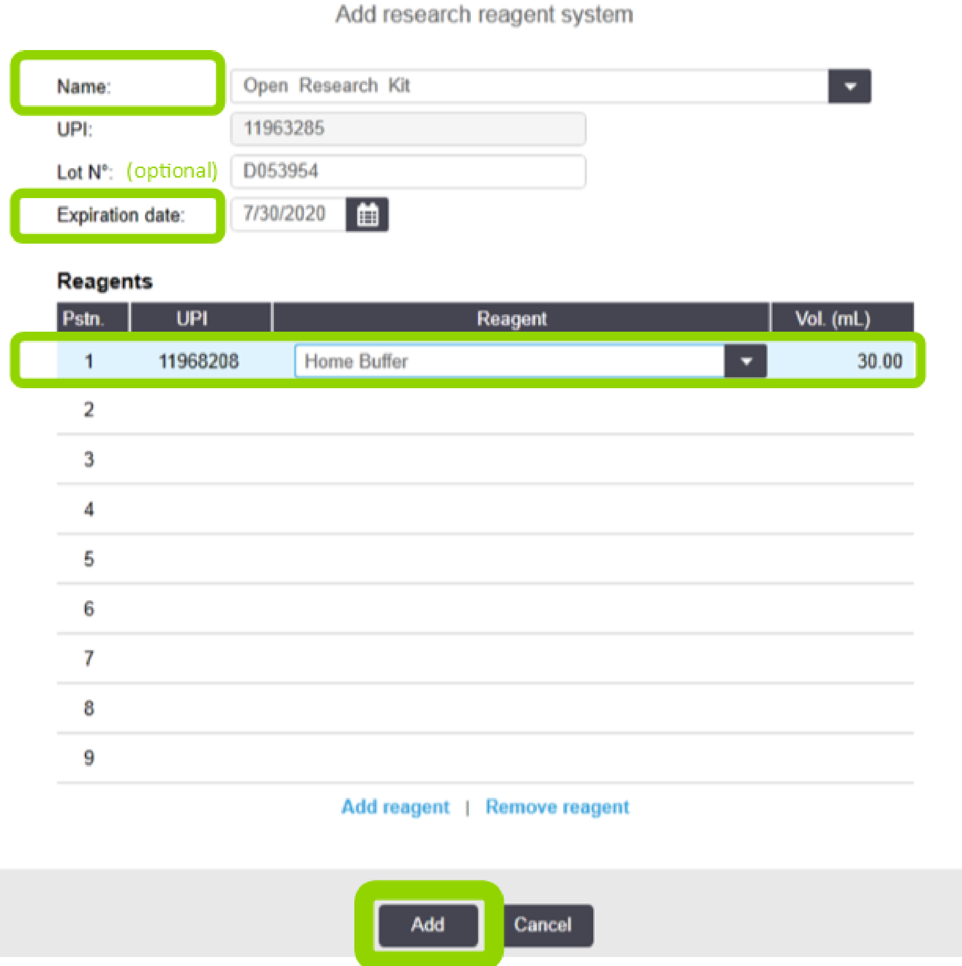

Figure 10: Add research reagent system window

4. Label the **BOND Research Detection System** reagent tray to match the name entered in the **Add research reagent system** window.
5. Label the **30 mL Open Container in position 1** as *Home Buffer*. The BOND Research Detection System is now registered.

Protein Protocol Setup

The protein protocol must be created the first time it is used on the BOND RX/RX[m]. Once it is established, it can be used for all GeoMx protein slides.

1. Click the **Protocol setup** icon (see Figure 11).

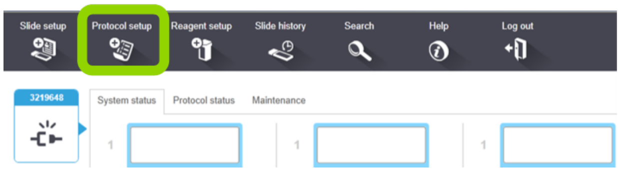

Figure 11: Protocol setup icon

2. Highlight the ***IHC Open Dispense Template** or ***IF Protocol** row and click **Copy** (see Figure 12) .

Protocol name	Protocol type	Description	Modified by
*AccuCyte CTC IF Protocol	IHC staining	AccuCyte CTC IF protocol	Leica
*AccuCyte CTC IHC Protocol	IHC staining	AccuCyte CTC IHC protocol	Leica
*IF Protocol	IHC staining	IF protocol	Leica
*IHC Open Dispense Template	IHC staining	IHC template with Open Ancillary and Chromogen dispenses	Leica

Figure 12: Protocol setup window

3. In the **Edit protocol properties** window (see Figure 13), enter the following information:
   - Enter *IHC DSP Protocol* in the **Name** field.
   - Enter *IHCDSP* in the **Abbreviated Name** field.
   - Enter *GeoMxProtein Leica Biosystems Protocol* in the **Description** field.
   - Check all of the boxes for **Staining method**.
   - Select your BOND Research Detection System from the **Preferred detection system** drop down menu.
   - Check the box for **Show wash** steps.

4. Change the protocol (using **Insert Wash**, **Insert Reagent**, and **Delete Step** buttons), ensuring the **Inc (min)** and **DispenseType** fields are modified to match those in Figure 13 and Table 5. (See **NOTE** below Table 5 for alternate instruction if BOND RX/RX^{m} is updated with data file BOND-RX-Ext-v29). Make sure the **Home Buffer** reagent matches the reagent which has been assigned to the first container in your BOND Research Detection System.

5. Click **Save**.

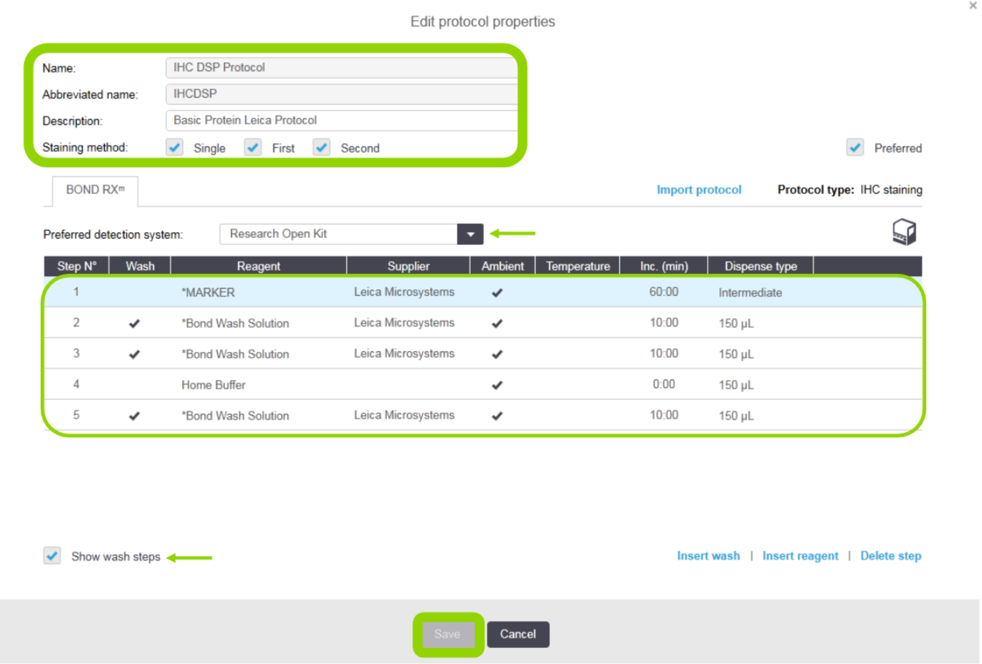

Figure 13: Edit protocol properties window (protein)

Table 5: Semi-atuomated protein slide preparation protocol

Step No.	Wash	Reagent	Supplier	Ambient Temp	Temp	Inc. (mins)	Dispense type
1		*Marker *(actually Buffer W)*	Leica Microsystems	✓		60:00	Intermediate
2	✓	*BOND Wash Solution	Leica Microsystems	✓		10:00	150 µL
3	✓	*BOND Wash Solution	Leica Microsystems	✓		10:00	150 µL
4		Home Buffer		✓		0:00	150 µL
5	✓	*BOND Wash Solution	Leica Microsystems	✓		10:00	150 µL

NOTE: If the BOND RX/RX^{m} is updated with file BXD-RX-Ext-v29, Buffer W is already programmed in as an ancillary reagent. Set Step 1 Reagent to ***GeoMx Buffer W** instead of *Marker.

6. Read the notification and click **Yes** (see Figure 14).

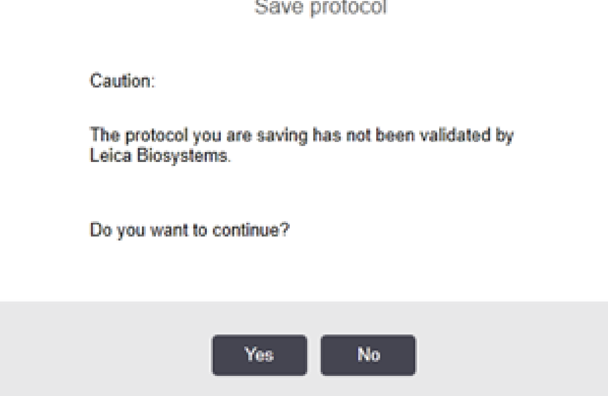

Figure 14: Save protocol notification

Register Additional Containers for Use

1. Label and add the **Buffer W reagent BOND titration container**.
   - Insert a **BOND titration container** with plastic insert in position 2 of the tray.
   - Label the **titration container in position 2** with *Buffer W*.
   - Scan the front barcode of the titration container.
   - In the **Add open container** window (see Figure 15), select **Buffer W** from the **Reagent name** drop-down menu, enter a **Lot No.** (if required), and enter an **Expiration date**.
   - Click **OK**.

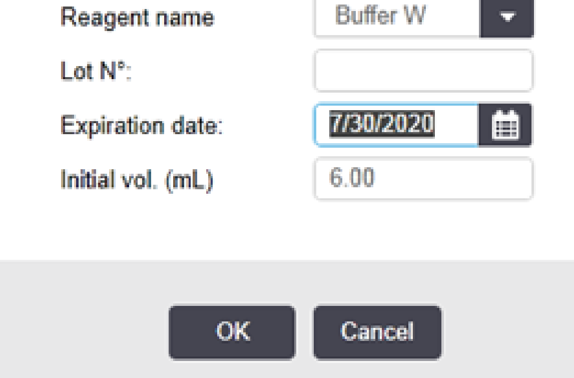

Figure 15: Add open container window

4 Start a run on the BOND RX

Fill Reagent Containers

1. **Fill the Home Buffer container in position 1 with 30 mL of 1X BOND Wash solution**. Ensure you have 150 µL per slide plus 5 mL of dead volume. Leave the lid open.
2. **Fill the titration container in position 2 with Buffer W**, allowing at least **150 µL per slide plus 350 µL of dead volume**. Ensure there is enough volume for your run.
3. **Load the BOND Research Detection System onto the BOND RX/RX^m^.** The instrument will recognize containers and check volumes. Once it has finished, you may click on the **Processing Module** icon (see Figure 16) to view the status of any of the containers in the **System Status** window. Locate the desired container in this window and right-click on it. Select **Inventory** to view the volume measured in the container. Refill if required.

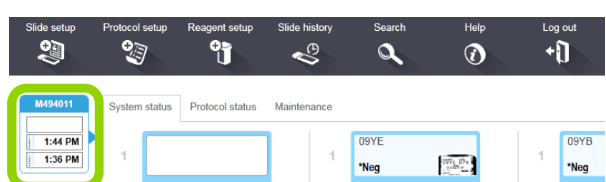

Figure 16: BOND RX screen with Processing Module icon indicated

Add Your Study and Slides

1. Click the **Slide setup** icon (see Figure 17).

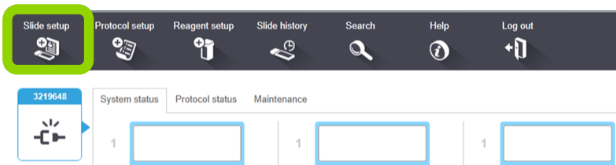

Figure 17: Slide setup icon

2. Click the **Add study** button (see Figure 18).

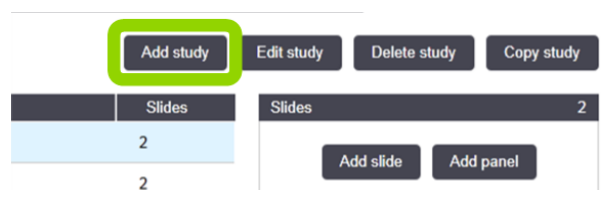

Figure 18: Add study button

3. In the **Add study** window (see Figure 19), enter your study information.
    - Enter your **Study ID** (required).
    - Enter your **Study name** (required).
    - Enter your **Study comments** (optional).
    - Choose a **Researcher** (optional, refer to BOND RX user manual to add new researchers).
    - Check **Dispense volume**: **150 µL**.
    - Select **Preparation protocol** : ***Bake and Dewax** from the drop down list.
    - Click **OK**.

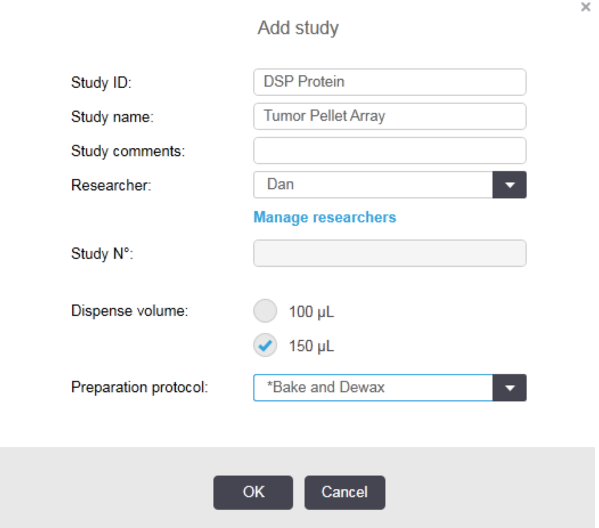

Figure 19: Add study window

4. Highlight your study and click **Add slide** (see Figure 20).

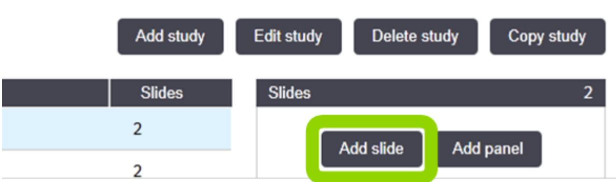

Figure 20: Add slide button

5. In the **Add slide** window (see Figure 21), enter your slide information.
   - Enter **Slide comments**.
   - Check **Test tissue** for **Tissue type**.
   - Check **150 µL** for **Dispense volume**.
   - Select **Single** and **Routine** from the **Staining mode** drop down lists.
   - Check **IHC** for **Process**.
   - Select the BOND Titration Container named **Buffer W** for the **Marker** from the drop down list.
6. On the **Protocols** tab:
   - Select **IHC DSP protocol** for **Staining**.
   - Select ***Bake and Dewax** for **Preparation**.
   - Select ***HIER 20 minutes with ER1** for **HIER**.
   - Select **Enzyme**: ***- - - -** (No selection).
7. Click **Add Slide**. Continue to click **Add slide** until you have sufficient labels for all of your slides. Change **Slide comments,** as needed.
8. Click **Close**.
9. Click **Print Labels** (see Figure 22).

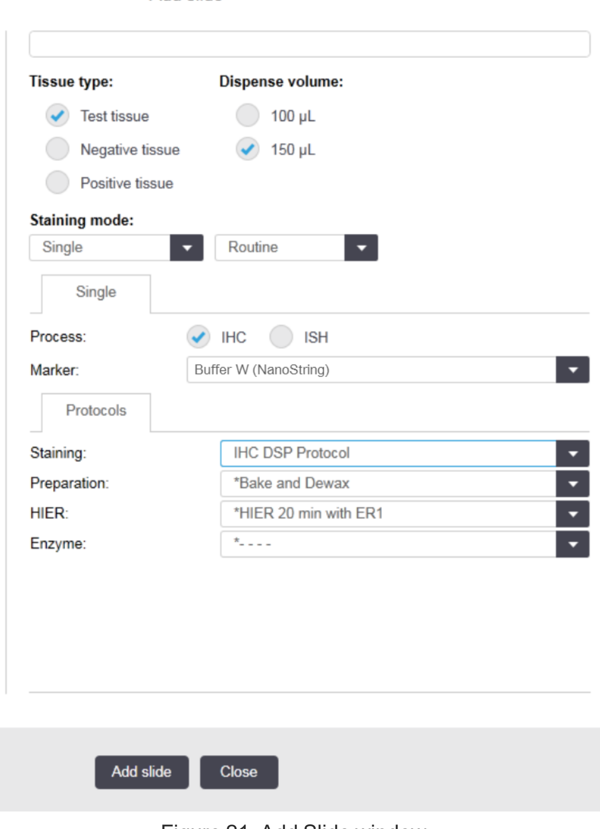

Figure 21: Add Slide window

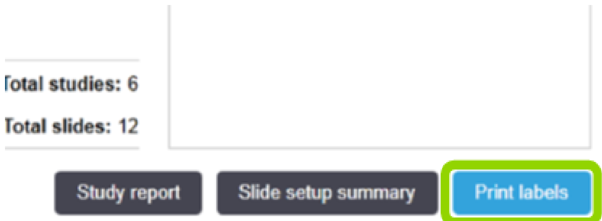

Figure 22: Print Labels button

10. In the **Print slide labels** window (see Figure 23), select **All slide labels not yet printed for current study** and click on **Print.**
11. Place the labels on the same side of the slides as the samples, aligning them at the top of the slide.

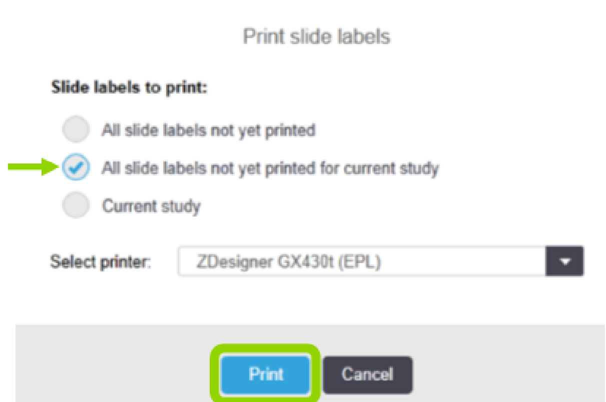

Figure 23: Print Slide Labels window

5 Load and run your slides

1. Check that all bulk containers are at least half full and that the bulk waste container is empty.
2. Referencing the embossed image on the slide tray, **load the slides onto the slide tray** in the correct orientation (see Figure 24). Ensure the sample and label are facing upwards, with the label at the top of the slide tray .
3. **Place covertiles** on top of the slides with the tab at the bottom and *Leica* facing upwards.

Figure 24: Slides in slide tray

4. Gently load the slide trays with slides and covertiles onto the BOND RX/RX^{m}; ensure that none of the slides or covertiles are dislodged.
5. Press the LED button on the front of the instrument to lower the slide tray. The instrument will scan the labels of the slides.
6. If a label is not recognized, hover over the missing slide on the screen, right-click, and choose **Select Manually**. Take care to select the correct information for the manually entered slide (refer to the BOND RX/RX^{m} User Manual for more information).

7. Once all slides and reagents have been reconciled, click the **Start** button under the slide tray image on the screen to begin the run.

Complete the Run

1. Once the run has finished, the LED button below the slide tray will blink green. Press the LED button to raise the slide tray.
2. Gently **slide the slide tray out** of the instrument and place on a flat, stable surface.
3. Handle slides one at a time so that they do not dry out. Remove each covertile by holding down the label of the slide, then carefully putting pressure downwards on the neck of the covertile to lift the end of the covertile off the slide. Do not slide the covertile across the surface of the slide, as you may damage the tissue. **Place slide in 1X PBS**.
4. Handling slides one a time, remove from PBS and wipe around tissue edge with a Kimwipe. Using a hydrophobic pen, **draw a closed hydrophobic barrier** around each tissue section. Minimize the size of the area to be stained. Dip slide into 1X PBS if needed to keep from drying out during this step. Return to 1X PBS after barrier is drawn.
5. Clean covertiles following the BOND RX/RX^{m} User Manual.

Prepare Materials for Next Step

1. **Thaw detection antibody (Ab) mix** on ice, keeping protected from light.
2. **Prepare the humidity chamber** by lining the tray with Kimwipes, then filling the tray with water just to cover the bottom. Do not overfill to avoid splashing while moving the chamber.

6 Primary antibody incubation (overnight)

You will need the following materials and reagents for this step: **prepared humidity chamber, thawed detection antibody mix** (core and optional modules)**, morphology markers,** and **Buffer W**. See the Equipment, Materials, and Reagents lists on page 13 for more details.

IMPORTANT: Probe mixes should be assembled in an area separate from nCounter work, NGS library prep, or other GeoMx workflows. GeoMx detection reagents can cross-contaminate probe mixes and give misleading or incorrect results. Areas should be cleaned thoroughly with RNase AWAY after probe mix formulation. Alternatively, probe mixes can be made in PCR workstations that are decontaminated with UV light. Gloves should be changed after handling probe mixes to avoid cross-contamination.

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

For nCounter readout: Not all protein modules are compatible with one another. Some combinations require Substitute Probe R for nCounter readout. Plan your modules by referring to Appendix IV: Substitute Probe R Guidance on page 104.

1. **Mix** the detection Ab mix by flicking then spin down. **Do not vortex.**

   Each tube of detection antibody (Ab) mix contains sufficient reagent for 12 slides (112 µL). If you are using the entire Ab mix in one week, then store at 4°C. If not, **aliquot the Ab mix** into 4-slide aliquots (37 µL + 37 µL + 38 µL) and freeze unused aliquots at -80°C. Do not exceed more than 2 freeze/thaw cycles and do not freeze diluted antibody.

   The morphology marker antibodies are stored at 4°C. Aliquoting is not required.

2. **Make a working Ab solution** by diluting detection antibodies and morphology markers into Buffer W (*n* = number of slides) (see Table 6). Adjust to reflect the number of core, module, and morphology reagents, and the number of slides to be prepared (**up to a total volume of 200 µL per slide**). Table 6 addresses both NGS assays and nCounter assays.

Table 6: Working antibody mix equation for protein slide prep for NGS or nCounter assays (*n* = number of slides)

Core Mix	Module 1	Module 2	Other Modules*	Morph Marker1**	Morph Marker2	Other Markers	Buffer W***	Final Volume
8 µL x *n*	8 µL x *n*	8 µL x *n*	...	5 µL x *n*	5 µL x *n*	...	(up to 200 µL) x *n*	200 µL x *n*

* If adding a custom-barcoded detection antibody, follow instructions in Appendix V: Adding Custom Barcoded Antibodies on page 105.

** If using non-NanoString morphology markers, optimal concentration in the working antibody mix must be determined by user testing.

*** If using a different number of detection or morphology reagents, Buffer W amount must be adjusted to bring total volume up to **200 µL/slide.**

3. **Remove slide from humidity chamber and remove Buffer W** by tapping the slide on a clean, absorbent surface, such as a paper towel, then using an absorbent wipe to carefully remove excess buffer from the slide, without touching the tissue.
4. **Place the slide back into the humidity chamber in a horizontal position. Cover the tissue with 200 µl of the diluted antibody solution**. Make sure the entire tissue is covered and no bubbles are present.

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

5. **Transfer the humidity chamber** to a 4°C refrigerator and **incubate overnight**. Ensure the humidity chamber stays level to avoid losing antibody solution.

7 Postfix (70 minutes)

You will need the following materials and reagents for this step: **staining jars, 1X TBS-T,** and **4% PFA**. See the Equipment, Materials, and Reagents lists on page 13 and Prepare Reagents steps on page 18.

> **IMPORTANT:** Everything that comes into contact with the antibody solution, such as containers for TBS-T, must be dedicated to this protocol and thoroughly cleaned with RNase AWAY, as probes may contaminate subsequent runs. Use separate staining jars for different probe mixes. Staining jars should be cleaned with RNase AWAY before each use.

1. **Removing one slide at a time from the humidity chamber, carefully tap off the antibody solution from each slide** on a clean, absorbent surface, such as several paper towels.
2. Wash the slides in **3 washes of 1X TBS-T for 10 minutes each**.

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

3. **Removing one slide at a time, carefully tap off each slide** on a clean, absorbent surface to remove excess wash solution.
4. Ensure that the hydrophobic barrier is still intact or draw a fresh barrier over the old one using the hydrophobic pen.
5. Cover the sample with up to **200 µL 4% PFA** and **incubate for 30 minutes** in the humidity chamber at room temperature.

6. (Optional) Remove SYTO 13 nuclear stain from -20°C and allow it to warm to room temperature for use in the next step.
7. Carefully tap each slide on clean, absorbent surface to remove excess 4% PFA. Wash slides in **two washes of 1X TBS-T for 5 minutes** each.

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Nuclei staining (20 minutes)

You will need the following materials and reagents for this step: **humidity chamber, staining jars, razor, SYTO 13 nuclear stain, 1X TBS, and 1X TBS-T**. See the Equipment, Materials, and Reagents lists on page 13 and Prepare Reagents steps on page 18.

> **IMPORTANT:** Before using the humidity chamber in the following steps, clean it with RNase AWAY. Prep the humidity chamber by lining with Kimwipes and adding just enough water to cover the bottom of the chamber.

1. Allow SYTO 13 to warm to room temperature.
2. Once thawed, **vortex then picofuge SYTO 13** for at least **1 minute** to bring the solution and insoluble particles to the bottom of the vial. When pipetting SYTO 13, pipette from the top of the vial to avoid insoluble particles.
3. **Dilute SYTO 13 1:10 in 1X TBS.** Prepare a sufficient volume per slide to completely cover tissue (~200 µL per slide). Mix by pipetting up and down. Close SYTO 13 stock vial tightly and store at -20°C.
4. **Remove one slide at a time from the 1X TBS-T,** remove excess liquid by tapping on a clean, absorbent surface, then place slides in humidity chamber in a horizontal position and **cover the tissue with diluted SYTO 13**.

5. **Stain for 15 minutes** at room temperature in the humidity chamber.
6. **Wash** slides by dipping in a staining jar with **1X TBS-T**.
7. **Transfer** to another staining jar with fresh **1X TBS-T**.

8. Working with one slide at a time, and dipping back into 1X TBS-T to avoid drying out, carefully **scrape off the hydrophobic pen** with a razor blade. Be sure to remove all of the wax without damaging or removing any of the tissue.
9. **Store stained slides in 1X TBS-T**. If it is necessary to re-stain with SYTO 13, re-draw the hydrophobic barrier, then repeat steps 1-8.

Once the slides are prepared, load them onto the GeoMx DSP (see the GeoMx DSP Instrument User Manual (MAN-10152)) or store slides according to guidelines on next page. **DO NOT let slides dry out.**

Safe storage guidelines for protein slides

- **Storage for up to 1 day:** submerge in 1X TBS-T and store at 4°C, protected from light to maintain the integrity of the photocleavable barcodes.
- **Storage for 1 day to 3 months:**
    1. **Rinse slide** to be mounted with TBS-T or PBS-T. Touch the slide edge to a paper towel to remove excess liquid. Place slide on a flat surface.
    2. Using a pipette tip (200 µL tip works well), **add one drop (~50 µL) of Fluoromount-G** to the slide; add more as necessary to ensure the slide does not dry out and tissue is adequately covered.
    3. **Mount coverslip** by aligning one edge of the coverslip then slowly lowering from one side to the other. Remove excess mounting medium.
    4. **Allow slide to dry** at room temperature overnight, protected from light (e.g. in a bench drawer).
    5. Store slide at 4°C, protected from light, for up to 3 months.

Slide unmounting procedure

1. Submerge mounted slide in 1X TBS-T or PBS-T until coverslip is loose or has fallen off. With gentle agitation, the coverslip typically falls off within 15 minutes.
2. If coverslip has fallen off, slide is ready for use. If coverslip is still attached but loose, gently remove it using tweezers.
3. Wash slide with fresh 1X TBS-T for 5 minutes to ensure removal of mounting media.

Stripping and re-probing procedure for protein slides

GeoMx Protein assay slides are reusable and can be restained with a different commercial or custom panel following this protocol. This protocol requires a UV light box or transilluminator capable of emitting 302/312 nm UV light (example) and 1X TBS-T.

1. Place the slide flat on the surface of a UV transilluminator.
2. Apply enough **1X TBS-T** to completely cover the tissue (50–200 µL depending on the size of the tissue).
3. **Expose to UV light** for **3 minutes** to cleave tags from bound antibodies.
4. Carefully tap each slide on a clean, absorbent surface (e.g. paper towel) to remove liquid and avoid oligo contamination.
5. **Wash slides** by dipping in a staining jar with **1X TBS-T.**
6. Transfer to another staining jar with fresh **1X TBS-T**.
7. To apply new probes, the semi-automated protein slide preparation protocol can be repeated starting from the antigen retrieval step, by modifying the standard protocol as follows. Please note that these steps have not yet been validated by NanoString and are provided only as guidance. Alternatively, perform manual probe re- application following GeoMx DSP Manual Slide Preparation User Manual (MAN-10150).
   - On the BOND RX/RXm, **Add a Study** and set **Dispense volume** to 150 µL and **Preparation protocol** to *----.
   - Make a copy of the standard GeoMx protein slide preparation protocol, give it a unique name, then delete all steps except the Home Buffer and one *BOND Wash **(see Table 7)**:

Table 7: Semi-automated protein slide preparation protocol for reprobing

Step No.	Wash	Reagent	Supplier	Ambient Temp	Temp	Inc. (mins)	Dispense type
1		Home Buffer		✓		0:00	150 µL
2	✓	*BOND Wash Solution	Leica Microsystems	✓		10:00	150 µL

   - When you **Add Slide**, set parameters to **Staining mode:** Single, Routine; **Process:** IHC; **Marker:** *Negative; **Staining:** HIER Only; **Preparation:** *----; **HIER:** *HIER 20 min with ER1; **Enzyme:** *----.

Semi-Automated RNA Slide Preparation Protocol (FFPE)

This protocol requires the BOND RX/RXm Fully Automated IHC/ISH Stainer from Leica Biosystems running BOND RX Version 6.0 software (or later).

This is not a fully automated protocol. The BOND RX/RXm performs the dewax, epitope retrieval, proteinase K digestion, and post-fixation steps. After processing on the BOND RX/RXm, probe application, stringent washes, and morphology marker application are performed manually.

To run the fully automated RNA slide preparation protocol, refer to page **64**.

IMPORTANT: NanoString recommends testing your system by performing GeoMx DSP with your BOND RX/RXm on well-characterized samples prior to use on experimental samples.

IMPORTANT: For any given study, NanoString recommends using only one sample type preparation method (e.g., FFPE or fresh frozen samples, but not a combination of sample types).

1 Equipment, Materials, and Reagents

The following tables list equipment, materials, and reagents that are required for this protocol but are **not supplied by NanoString**.

Table 8 : Equipment for RNA slide prep not provided by NanoString.

Equipment	Source	Part No.
BOND RX Fully Automated IHC/ISH Stainer **or** BOND RXm Compact Fully Automated IHC/ISH Stainer	Leica Biosystems	Contact Leica Biosystems
BOND RX Controller, running BOND RX software version 6.0 and above	Leica Biosystems	Contact Leica Biosystems
Baking oven	Quincy Lab, Inc. (or comparable)	Various GC models
Hybridization oven including hybridization chamber*:		
HybEZ II Hybridization System **or**	ACDBio	321710/321720
RapidFISH Slide Hybridizer	Boekel Scientific	240200 for 120V
Water bath (up to at least 37°C)	Various	Various

*NanoString recommends the listed hybridization ovens for this protocol. The following alternatives are acceptable, but have not been validated by NanoString. Test to ensure slides remain hydrated overnight. Abbott ThermoBrite, Leica ThermoBrite

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Table 9: Materials for RNA slide prep not provided by NanoString.

Materials	Source	Part No.
BOND Research Detection System (includes 6 x 30 mL Open Containers)	Leica Biosystems	DS9455
BOND Titration Kit (includes 50 inserts)	Leica Biosystems	OPT9049
BOND Universal Covertiles	Leica Biosystems	S21.2001
BOND Open Containers 30 mL (if additional needed)	Leica Biosystems	OP309700
BOND Titration Container Inserts (if additional needed)	Leica Biosystems	OPT9719
Pipettes for 5–1,000 µL	Various	Various
Filter tips (DNase/RNase free)	Various	Various
Microcentrifuge tubes (DNase/RNase free)	Various	Various
Apex BOND Adhesive slides	Leica Biosystems	3800040
Slide staining jars (Coplin jars) (recommended number: 12) and slide holder inserts	VWR (or comparable)	25608-904, 25608-868
Humidity chamber	Simport	M920-2
Benchtop protector sheet (fits inside the hybridization oven, optional)	Fisher Scientific (or comparable)	14-206-62
HybriSlip hybridization covers (22 mm x 40 mm x 0.25 mm) NOTE: Other products have not been validated by NanoString.	Grace Bio-Labs	714022
RNase AWAY® or 10% Bleach (RNaseZap® is not a substitute)	Thermo Fisher	7003PK
USB drive v3.0, 64 GB or higher (ability to be NTFS formatted)	SanDisk (or comparable)	SDCZ800-128G-G46

Table 10: Reagents for RNA slide prep not provided by NanoString. RT = room temperature

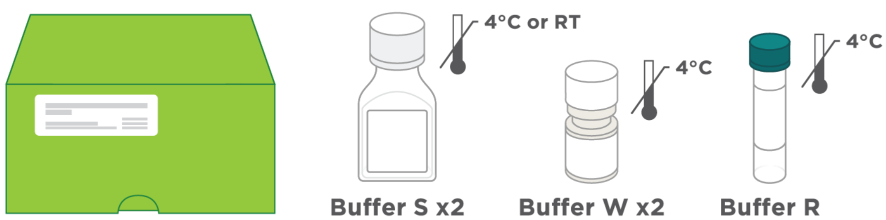
Reagents	Source, Part No.	Storage
BOND Dewax Solution, 1 L	Leica Biosystems, AR9222	4°C or RT
BOND Epitope Retrieval 2, 1 L	Leica Biosystems, AR9961	4°C
BOND Wash Solution 10X Concentrate, 1 L	Leica Biosystems, AR9590	4°C
Reagent-grade ethanol	Various	RT
DEPC-treated water	Thermo Fisher, AM9922 (or comparable) NOTE: As an alternative to commercial DEPC-treated water, prepare your own following standard protocols.	RT
10X phosphate buffered saline pH 7.4 (PBS)	Sigma-Aldrich, P5368-10PAK, P5368-5X10PAK (or comparable)	RT
10% neutral buffered formalin (NBF)	EMS Diasum, 15740-04 (or comparable)	RT
100% deionized formamide	Thermo Fisher, AM9342 **or** VWR, VWRV0606 (or comparable) NOTE: If deionized formamide is unavailable, molecular grade formamide may be substituted.	4°C (bring to RT before opening)
20X SSC (DNase/RNase free)	Sigma-Aldrich, S6639	RT
Proteinase K (recommended) **or** Leica Biosystems Enzyme Pretreat Kit	Ambion, 2546 Leica Biosystems, AR9551 Thermo Fisher, AM2548 or 25530049 NOTE: Using proteinase K from any other supplier will require optimization of timing and concentration.	See manufacturer's instructions
Tris base	Sigma-Aldrich, 10708976001 (or comparable)	RT
Glycine	Sigma-Aldrich, G7126 (or comparable)	RT

NanoString Reagents

The following reagents are **supplied by NanoString.** Contact your NanoString Sales Representative to use our reagent planning tools to calculate required quantities.

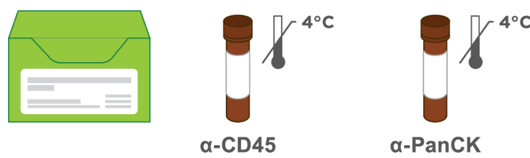
GeoMx RNA Slide Prep Kit (standard kit for manual or semi-automated method)

This semi-automated RNA slide preparation protocol requires only the standard RNA Slide Prep Kit and not the GeoMx Automated RNA Slide Prep Kit.

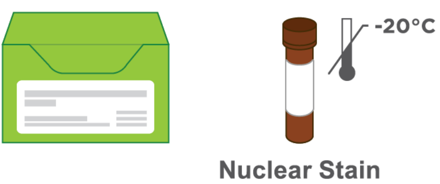


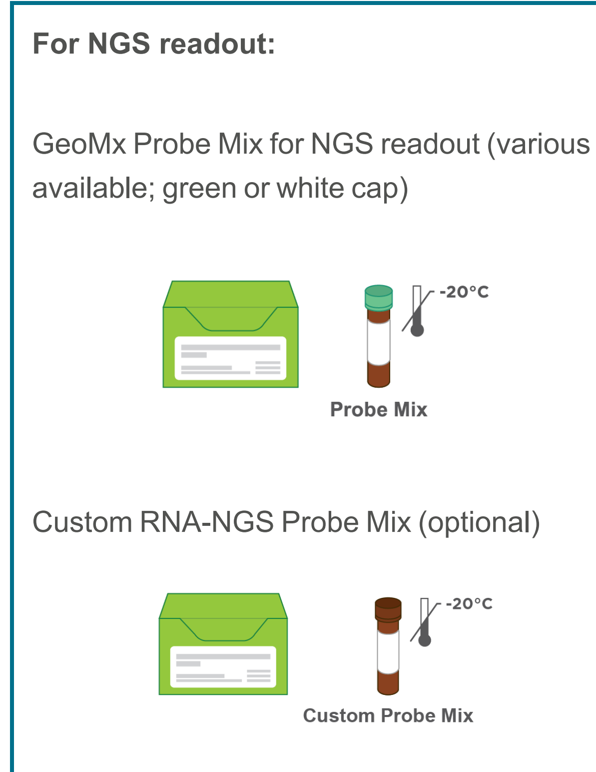
GeoMx Morphology Kit - Human or Mouse RNA compatible (various available)

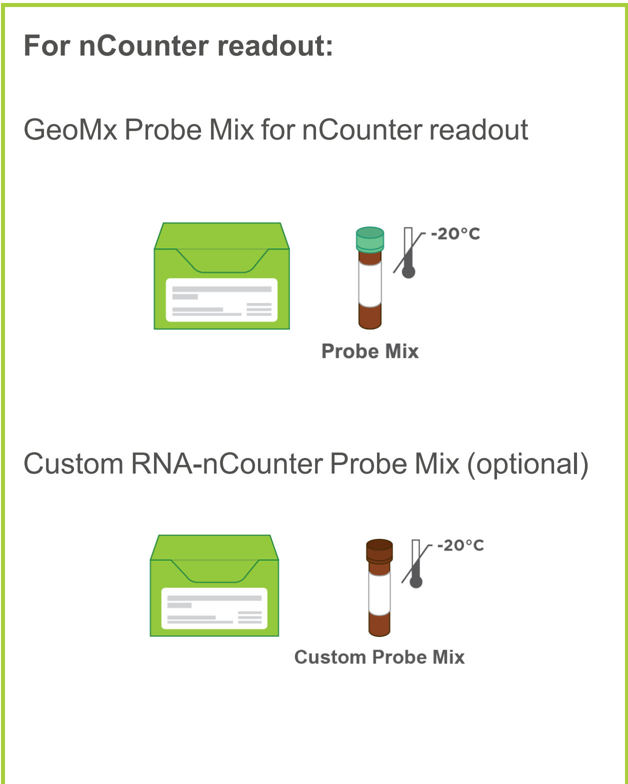
Example morphology kit (Solid Tumor TME)



GeoMx Nuclear Stain Morphology Kit







DO NOT use RNA probe mixes for fully automated slide preparation (yellow label, red cap) with this semi-automated protocol. Use RNA probe mixes designated for manual/semi-automated RNA slide preparation (white label, green/white/amber cap). DO NOT use RNA probe mixes for fully automated slide preparation (yellow label, red cap) with this semi- automated protocol. Use RNA probe mixes designated for manual/semi- automated RNA slide preparation (white label, green/white/amber cap).

Prepare reagents

Prepare the reagents using the dilution instructions **(see Table 11)**. **Use DEPC-treated water for all dilutions** (including 1X BOND Wash Solution in bulk reservoir) and in DI water reservoir. Buffers can be stored in BOND Open containers at 4°C unless otherwise noted.

IMPORTANT: Take care to maintain nuclease-free conditions. The greatest risk of contamination comes from GeoMx probes and other oligos. We recommend the use of RNase AWAY (Thermo Fisher 7002) for cleaning of all surfaces and equipment, as it will limit contamination from oligos, GeoMx probes, and nucleases. After using RNase AWAY, allow area/items to air dry completely, or rinse with DEPC-treated water. See manufacturer's instructions for details.

Table 11: Reagent prep for RNA semi-automated slide preparation

Reagent	Dilution	Storage
NBF stop buffer	Combine **6.06 g Tris base** and **3.75 g glycine** in **500 mL** DEPC-treated water to yield 0.1M Tris, 0.1M Glycine. Can be stored at RT for 1 month.	RT
Proteinase K	Default concentration (**1 µg/mL**) is made by adding **2 µL** of **20 mg/mL** Proteinase K to **40 mL** of **1X PBS** made with DEPC-treated water. See Table 14 for alternative concentrations by tissue type. If using Proteinase K from the BOND Enzyme Pretreatment Kit, dilute stock (17 mg/ml) to 1 µg/mL, rather than concentrations recommended by Leica Biosystems. NOTE: Prepare fresh and do not reuse. Accurate dilution of proteinase K is critical for proper assay performance.A serial dilution is recommended for any volume smaller less than 40 mL or if using 0.1 µg/mL proteinase K.	n/a
1X BOND Wash Solution	For the first run, prepare **30 mL** 1X by combining **3 mL** of 10X BOND Wash Solution with **27 mL** DEPC-treated water. Prepare more as needed.	RT
1X PBS pH 7.4	Prepare **1 L** of 1X PBS by combining **100 mL** of 10X PBS and **900 mL** of DEPC-treated water.	RT
2X SSC	Prepare **1 L** of 2X SSC by combining **100 mL** of 20X SSC and **900 mL** of DEPC-treated water. Do not reuse.	RT
2X SSC-T	Prepare **250 mL** of 2X SSC-T by combining **25 mL** of 20X SSC, **2.5 mL** of 10% Tween-20, and **222.5 mL** of DEPC-treated water. Do not reuse.	RT
4X SSC	Prepare **1 L** of 4X SSC by combining **200 mL** of 20X SSC and **800 mL** of DEPC-treated water. Do not reuse.	RT

2 Prepare tissue samples

Appendix I: Selecting and Sectioning FFPE Samples on page 87 covers FFPE block selection and sectioning in detail. Review it prior to beginning the Slide Preparation protocol.

GeoMx has been validated for sample blocks up to 3 years old prepared from tissues with a cold ischemic time of less than 1 hour using 10% NBF or similar fixative. For best results, do not use FFPE blocks 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

Slide Preparation

1. Tissue sections should be **5 µm** thick and mounted on **Apex BOND Adhesive slides**. Tissue sections must **fit within the white dots** marking the boundary on the Apex BOND slides, and be within the **Scan Area** (shown in green) (see Figure 25). Sections can be no larger than 35.3 mm long by 14.1 mm wide and should not overlap the slide gasket (shown in blue) or the Tip Calibration area (shown in red). If mounting multiple sections per slide, ensure that tissues are at least 2–3 mm apart and still fit within the Scan Area.

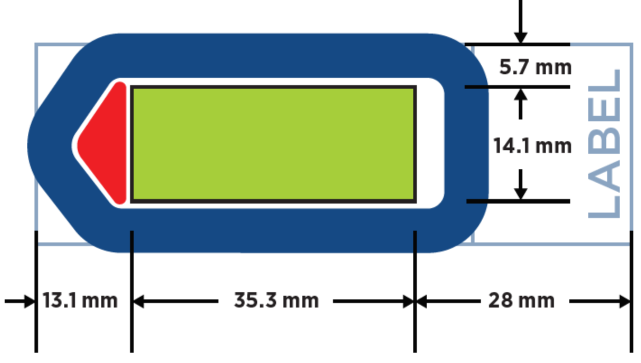


Figure 25: Slide dimensions

If sections are too large and/or placed off-center, continue with slide preparation as usual. Just before loading the slide in the instrument slide tray, scrape off parts of the tissue exceeding the scan area, making sure the slide gasket and tip calibration area are free of tissue. Scraping off tissue before slide preparation could generate tissue folds that may result in staining or binding artifacts.

IMPORTANT: The GeoMx DSP instrument will only image the area inside the Scan Area. **Tissue outside of the Scan Area will not be imaged and may cause problems in tissue detection by the optical system.**

2. **Bake sections on slides** in a 60°C drying oven for 30 minutes to 3 hours prior to deparaffinization. Stand slides vertically during baking to allow excess paraffin to flow off. Longer baking times may be necessary for some tissues to adhere to the slide; this should be empirically tested.

3 Initial set up of the BOND RX

See [Figure 26](#) for an overview of the BOND RX/RX^m user interface. Refer to the **BOND RX/RX^m User Manual**, accessible from the **Help** icon in the instrument software, for additional information.

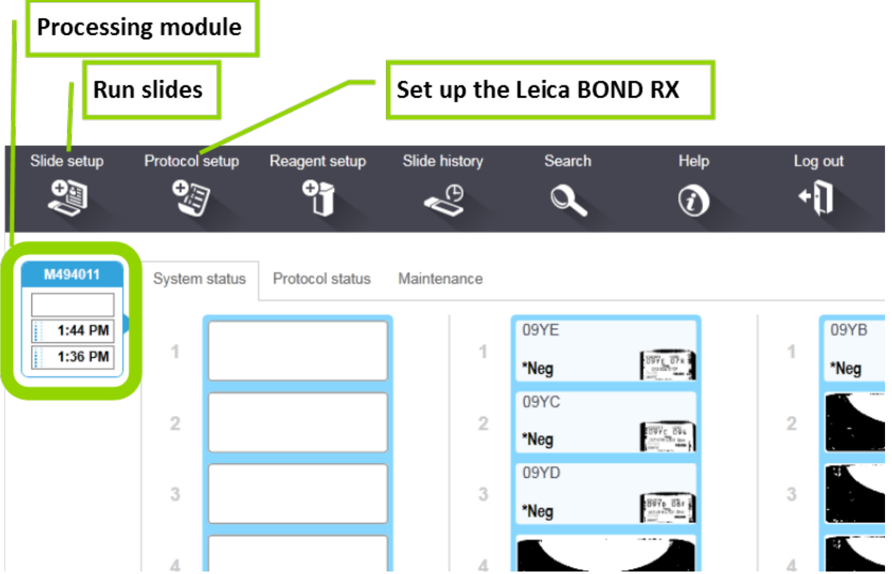


Figure 26: BOND RX/RX^m user interface

To set up the BOND RX/RX^m, you must setup the reagents, register the Detection System, set up the protocols to be used, and register the reagents and the containers in which they will be loaded.

Reagent Setup

If these reagents are not registered in the system, you will need to add them: **NBF**, **NBF Stop Buffer**, and **Home Buffer**.

1. Select the **Reagent Setup** icon [(see Figure 27)](#).

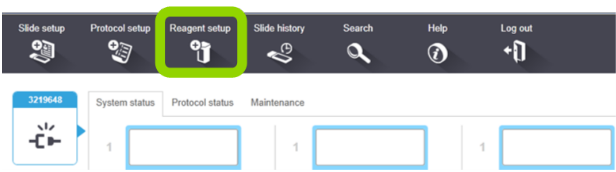


Figure 27: Reagent setup icon

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2. From the **Setup** tab, click the **Add** button (see Figure 28).

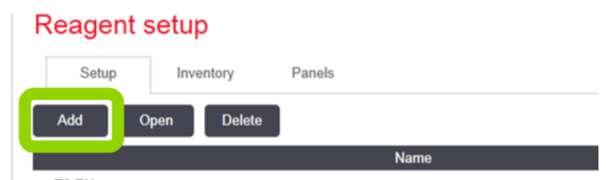

Figure 28: Add reagent button

3. In the **Add Reagent** window (see Figure 29), enter a unique name in the **Name** field.
    - *10% NBF* to register NBF.
    - When registering NBF Stop Buffer and Home Buffer, enter their respective names.
4. Enter a unique abbreviated name in the **Abbreviated Name** field (up to eight characters).
    - For example, enter *NBF* as the abbreviated name for NBF.
5. For NBF, NBF Stop Buffer, and Home Buffer, select **Ancillary**.
6. Check the **Hazardous** check box for NBF.
7. Click **Save**.
8. Repeat steps 2–7 to register NBF Stop Buffer and Home Buffer.

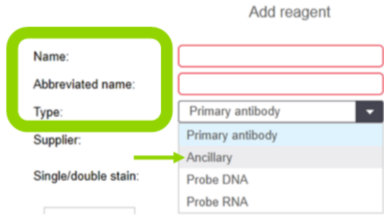

Figure 29: Add reagent window

Register the BOND Research Detection System

The BOND Research Detection System is a barcoded tray that holds containers of reagents.

1. **Remove any containers** from the BOND Research Detection System.
2. **Scan the barcode** on the side of the system (see Figure 30).
3. In the **Add research reagent system** window (see Figure 31), enter the information for your reagent system.
    - Enter a **Name** for your BOND Research Detection System (if one has been previously registered, select the name

Figure 30: Research detection system barcode

from the drop down menu).

- Enter the **Lot No.** (optional).
- Enter an **Expiration Date**.
- Highlight **Row 1** and **scan the front barcode of a new 30 mL BOND Open Container** (the barcode will appear in the field for Row 1). Place the container in position 1 of the BOND Research Detection System tray.
- Select **Home Buffer** from the drop down menu in the Reagent column.
- Click **Add**.

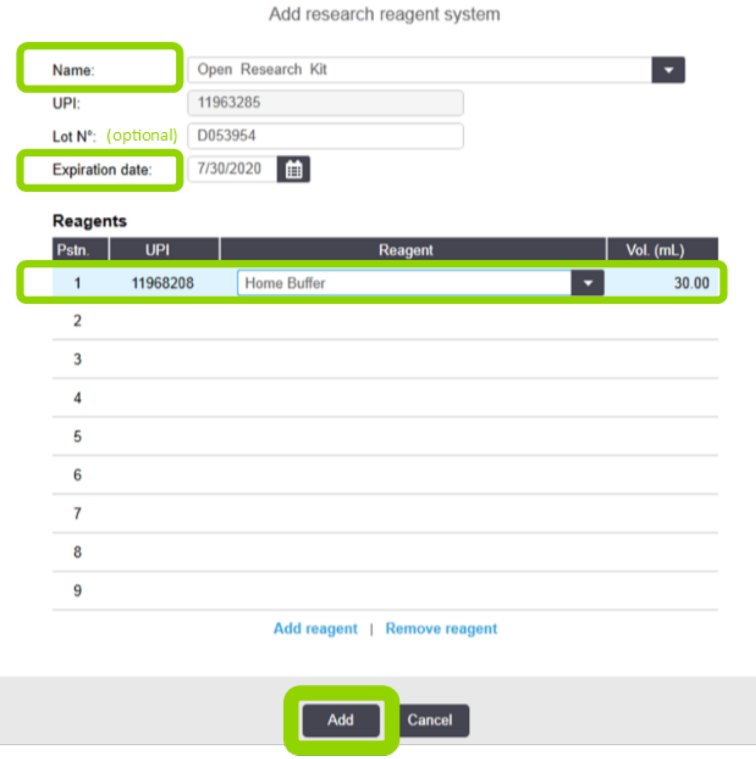

Figure 31: Add research reagent system window

4. Label the **BOND Research Detection System** reagent tray to match the name entered in the **Add research reagent system** window.
5. Label the **30 mL Open Container in position 1** as *Home Buffer*. The BOND Research Detection System is now registered.

RNA Protocol Setup

The RNA protocol must be created the first time it is used on the BOND RX/RX[m]. Once it is established, it can be used for all GeoMx RNA slides prepared with this semi-automated workflow.

1. Click the **Protocol setup** icon (see Figure 32).

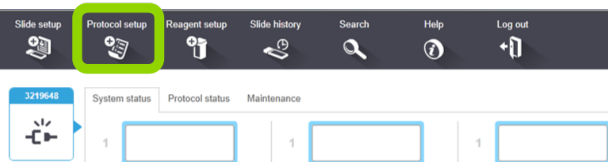

Figure 32: Protocol setup icon

2. ighlight the ***IHC Open Dispense Template** row and click **Copy** (see Figure 33).

Protocol setup

Copy

Protocol name	Protocol type	Description	Modified by
*AccuCyte CTC IF Protocol	IHC staining	AccuCyte CTC IF protocol	Leica
*AccuCyte CTC IHC Protocol	IHC staining	AccuCyte CTC IHC protocol	Leica
*IF Protocol	IHC staining	IF protocol	Leica
*IHC Open Dispense Template	IHC staining	IHC template with Open Ancillary and Chromogen dispenses	Leica

Figure 33: Protocol setup window

3. In the **Edit protocol properties** window (see Figure 34), enter the following information:
   - Enter *GeoMx RNA slide prep* in the **Name** field.
   - Enter *RNAprep* in the **Abbreviated Name** field.
   - Fill in the **Description** field, e.g. **Bake and Dewax, set ER and PK steps*.
   - Check the **Single** and **First** (or **Preliminary**) boxes for **Staining method**.
   - Select your BOND Research Detection System from the **Preferred detection system** drop down menu.
   - Check the box for **Show wash** steps.

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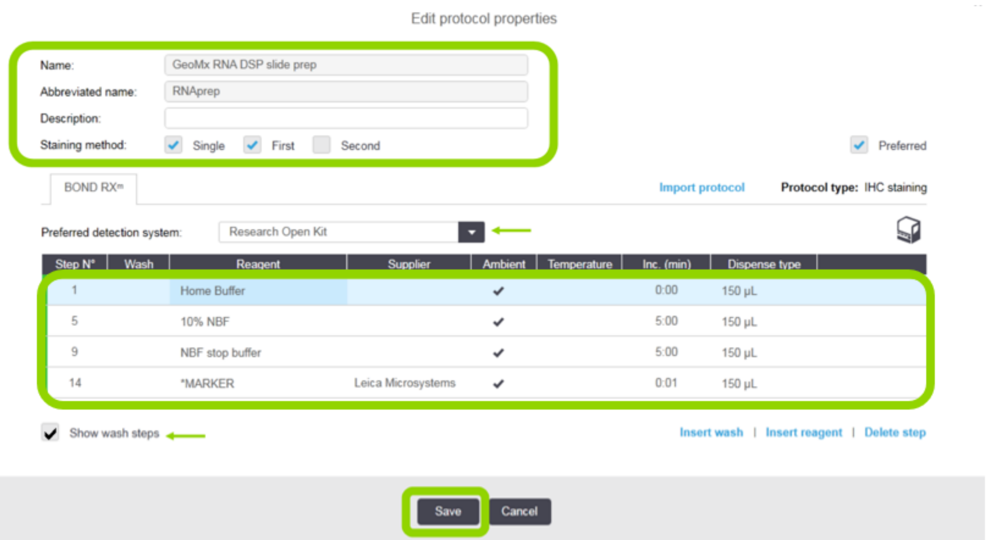

Figure 34: Edit protocol properties window (RNA)

4. Change the protocol (using **Insert Wash**, **Insert Reagent**, and **Delete Step** buttons) ensuring the **Inc (min)** and **DispenseType** fields are modified to match those in the table **(see Table 12)**. Make sure the **Home Buffer** reagent matches the reagent which has been assigned to the first container in your BOND Research Detection System.

Table 12: Semi-automated RNA slide preparation protocol

Step No.	Wash	Reagent	Supplier	Ambient Temperature	Temp	Inc. (minutes)	Dispense type
1		Home Buffer		✓		0:00	150 µL
2	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	150 µL
3	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	150 µL
4	✓	*BOND Wash Solution	Leica Microsystems	✓		5:00	150 µL
5		10% NBF		✓		5:00	150 µL
6	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	150 µL
7	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	150 µL
8	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	150 µL
9		NBF Stop Buffer		✓		5:00	150 µL
10	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	150 µL
11	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	150 µL
12	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	150 µL
13		*BOND Wash Solution	Leica Microsystems	✓		5:00	150 µL
14	✓	*Marker	Leica Microsystems	✓		0:01	150 µL
15	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	150 µL
16	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	150 µL
17	✓	*BOND Wash Solution	Leica Microsystems	✓		5:00	150 µL

5. Click **Save**.

6. Read the notification and click **Yes** (see Figure 35).

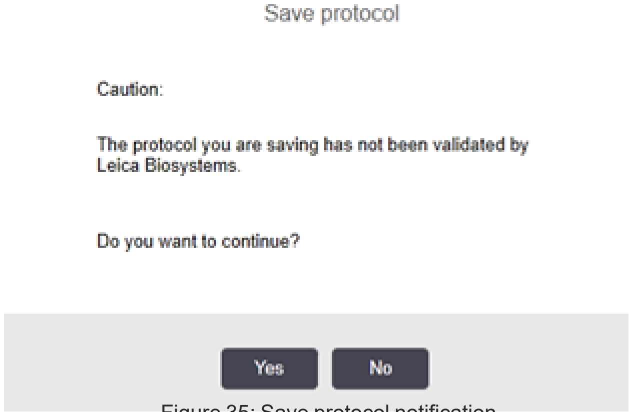

Figure 35: Save protocol notification

Register Additional Containers for Use

1. Label and add the **10% NBF Open container**.
   - Insert a **BOND 30 mL Open container** in position 2 of the tray.
   - Label the **Open container in position 2** with *10% NBF*.
   - Scan the front barcode of the Open container.
   - In the **Add open container** window (see Figure 36), select **10% NBF** from the **Reagent name** drop down menu, enter a **Lot No.** (if required), and enter an **Expiration date**.
   - Click **OK**.

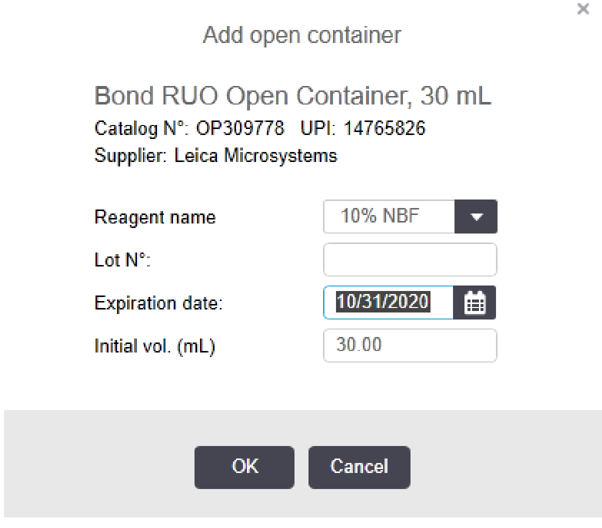

Figure 36: Add open container window

2. Repeat these steps for **NBF Stop Buffer**. Use a BOND 30 mL Open Container and place in position 3 of the tray.

3. Repeat these steps for ***Enzyme 1**. Use a 6 mL Titration Container with Insert and place in position 4 of the tray.

4 Start a run on the BOND RX

Fill Reagent Containers

1. **Fill the Home Buffer container in position 1 with 30 mL of 1X BOND Wash solution.** Ensure you have at least 150 µL per slide plus 5 mL of dead volume. Leave the lid open.
2. **Working in a fume hood, fill the Open Container in position 2 with up to 30 mL 10% NBF** (minimum 500 µL per slide plus 5 mL of dead volume).

> **WARNING:** NBF is hazardous. Handle with care and minimize inhalation risks. Please handle and dispose of according to your institution's EHS guidelines.

3. **Fill the open container in position 3 with up to 30 mL NBF Stop Buffer** (minimum 500 µL per slide plus 5 mL for dead volume).
4. **Fill the titration container in position 4 with up to 6 mL Proteinase K** (default 1 µg/mL) (minimum 500 µL per slide plus 500 µL dead volume).
5. **Load the BOND Research Detection System onto the BOND RX/RXm.** The instrument will recognize containers and, depending on your Dip Test settings, may check container volumes. Once finished, you may click on the **Processing Module** icon (see Figure 37) to view the status of any of the containers in the **System Status** window. Right-click on the desired container and select **Inventory** to view the volume measured in the container. Refill if required.

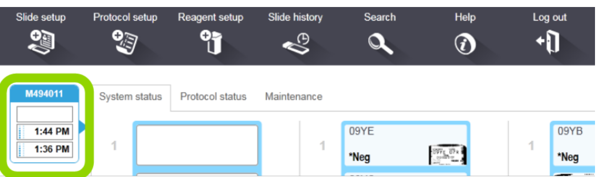

Figure 37: BOND RX screen with Processing Module icon indicated

> The BOND RX/RXm may display an alert for low volumes in the Titration Containers. Unload and reload the reagent tray to allow a second volume check. Ensure that there are no bubbles in the solutions, which can trigger the alert.

Add Your Study and Slides

1. Click the **Slide setup** icon (see Figure 38).

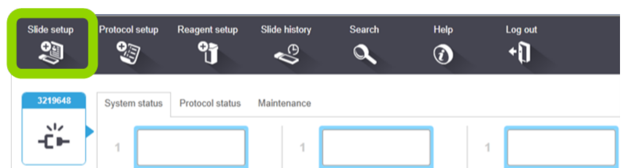

Figure 38: Slide setup icon

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2. Click the **Add study** button (see Figure 39).

Figure 39: Add study button

3. In the **Add study** window (see Figure 40), enter your study information.
   - Enter your **Study ID** (required).
   - Enter your **Study name** (required).
   - Enter your **Study comments** (optional).
   - **Researcher** (optional, refer to BOND RX/RX m user manual to add new researchers).
   - Check **Dispense volume**: **150 µL**.
   - Select **Preparation protocol** : ***Bake and Dewax** from the drop down list. (For fresh frozen samples, select *Frozen Slide Delay. See Appendix II: Modifications to Automated Protocol for Fresh Frozen Samples on page 89).
   - Click **OK**.

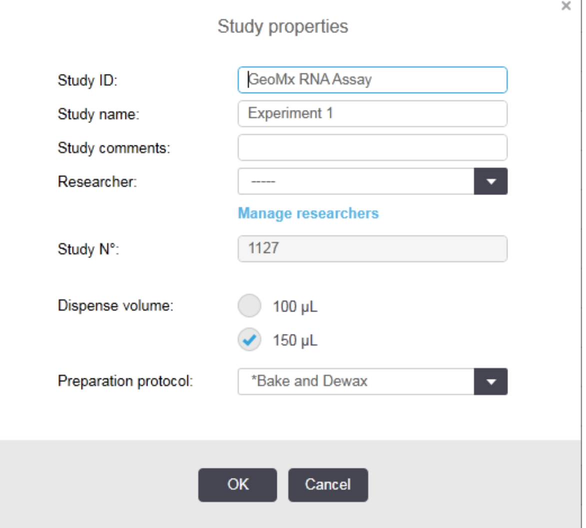

Figure 40: Add study window

4. Highlight your study and click **Add slide** (see Figure 41).

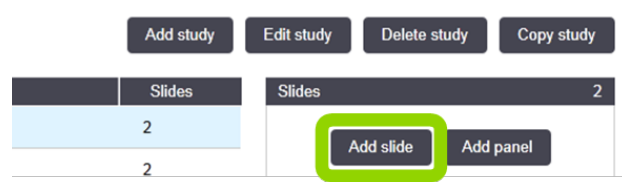

Figure 41: Add slide button

5. In the **Add slide** window (see Figure 42), enter your slide information.
   - Enter **Slide Comments**.
   - Check **Test tissue** for **Tissue type**.
   - Check **150 µL** for **Dispense volume**.
   - Select **Single** and **Routine** from the staining mode drop down lists.
   - Check **IHC** for **Process**.
   - Select ***Negative** from the **Marker** drop down list.

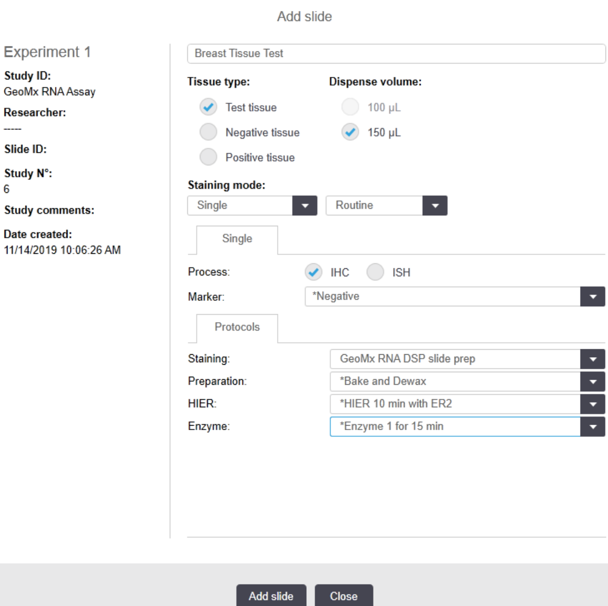

Figure 42: Add slide window

6. On the **Protocols** tab:

   - Select **GeoMx RNA slide prep** for **Staining**.
   - Select ***Bake and Dewax** for **Preparation**. (For fresh frozen samples, select *Frozen Slide Delay. See Appendix II: Modifications to Automated Protocol for Fresh Frozen Samples on page 89).
   - **HIER**: Select the appropriate incubation time based on the tissue type (see Table 13). Optimal incubation time and temperatures may differ by tissue and may need to be empirically determined. To create a new protocol, see NOTE below Table 13.

Table 13: Target retrieval times and temperatures by tissue type

Tissue Type	Target Retrieval
Breast	*HIER 10 min with ER2 @ 100°C
Cell pellets	*HIER 10 min with ER2 @ 85°C
Colorectal	*HIER 20 min with ER2 @ 100°C
Melanoma	*HIER 20 min with ER2 @ 100°C
Mouse tissue	*HIER 10 min with ER2 @ 100°C
NSCLC	*HIER 20 min with ER2 @ 100°C
Prostate tumor	*HIER 20 min with ER2 @ 100°C
Tonsil	*HIER 10 min with ER2 @ 100°C

NOTE: To create a new protocol with different HIER conditions, click the Protocol Setup icon (see Figure 32). Find the existing *HIER protocol, copy it, and modify the conditions as needed. Save with a unique name and abbreviated name. Check the checkbox for “Preferred” protocol.

- **Enzyme**: Select *Enzyme1 and the appropriate incubation time based on the tissue type (see Table 14). The default is **1 µg/mL** Proteinase K for 15 minutes at 37°C, however, optimal Proteinase K concentration and incubation time differ by tissue and may need to be empirically determined. To create a new protocol, see NOTE below **Table 14**.

Table 14: Proteinase K digest concentrations and times by tissue type

Tissue Type	Proteinase K Digest
Breast	0.1 µg/mL for 15 min
Cell pellets	1 µg/mL for 5 min
Colorectal	1 µg/mL for 15 min
Melanoma	1 µg/mL for 15 min
Mouse tissue	1 µg/mL for 15 min
NSCLC	1 µg/mL for 15 min
Prostate tumor	1 µg/mL for 15 min
Tonsil	1 µg/mL for 15 min

NOTE: To create a new Proteinase K protocol, click the Protocol Setup icon (see Figure 32). Find the existing *Enzyme 1 for 15 minutes protocol, copy it, and modify the conditions as needed. Save with a unique name and abbreviated name. Check the checkbox for “Preferred” protocol.

7. Click **Add Slide**. Continue to click **Add slide** until you have sufficient labels for all of your slides. Change **Slide Comments,** as needed. Click **Close**.

8. Click **Print Labels** (see Figure 43).

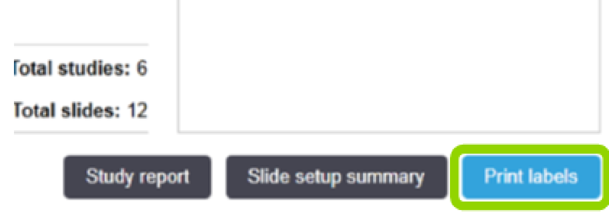

Figure 43: Print labels button

9. In the **Print slide labels** window (see Figure 44), select **All slide labels not yet printed for current study** and click on **Print.**

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10. Place the labels on same side of the slides as the samples, aligning them at the top of the slide.

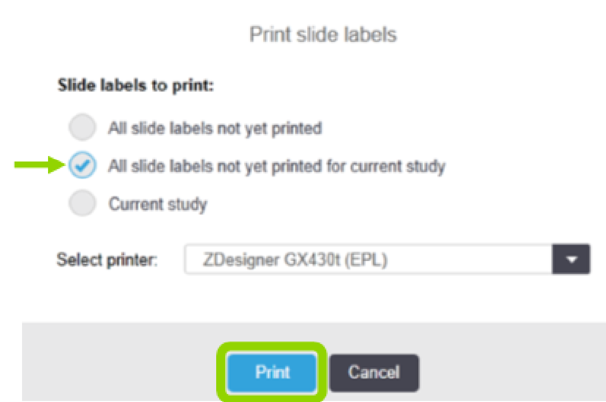

Figure 44: Print slide labels window

5 Load and run your slides

1. Check that all bulk containers are at least half-full and that the bulk waste container is empty.
2. Referencing the embossed image on the slide tray, **load the slides onto the slide tray** in the correct orientation (see Figure 45). Ensure the sample and label are facing upwards, with the label at the top of the slide tray.
3. **Place covertiles** on top of the slides with the tab at the bottom and *Leica* facing upwards.

Figure 45: Slides in slide rack

4. Gently load the slide tray(s) with slides and covertiles onto the BOND RX/RX^{m}; ensure that none of the slides or covertiles are dislodged.
5. Press the corresponding LED button below the slide tray. The instrument will scan the labels of the slides.
6. If a label is not recognized, hover over the missing slide on the screen, right-click, and choose **Select Manually**. Take care to select the correct information for the manually entered slide (refer to the BOND RX/RX^{m} User Manual for more information).

7. Once all slides and reagents have been reconciled, click the **Start** button under the slide tray image on the screen to begin the run.

Complete the run

1. Once the run has finished, the LED button below the slide tray will blink green. Press the LED button to raise the slide tray.
2. Gently **slide the slide tray out** of the instrument and place on a flat, stable surface.
3. Handle slides one at a time so that they do not dry out. Remove each covertile by holding down the label of the slide, then carefully putting pressure downwards on the neck of the covertile to lift the end of the covertile off the slide. Do not slide the covertile across the surface of the slide, as you may damage the tissue. **Place slide in 1X PBS** then proceed to In situ hybridization (overnight) on page 57.
4. Clean covertiles following the BOND RX/RX^{m} User Manual.

In situ hybridization (overnight)

You will need the following materials and reagents for this step: **hybridization chamber, hybridization oven, Buffer R, RNA Probe Mix,** and **2X SSC** or **DEPC-treated water**. See the Equipment, Materials, and Reagents lists on page 38 for more details.

> **IMPORTANT:** Probe mixes should be handled in an area separate from nCounter work, NGS library prep, or other GeoMx workflows. GeoMx detection reagents can cross-contaminate probe mixes and give misleading or incorrect results. Areas should be cleaned thoroughly with RNase AWAY after probe mix formulation. Alternatively, handle probe mixes in PCR workstations that are decontaminated with UV light. Gloves should be changed after handling any probe mixes to avoid cross-contamination.

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

1. **Prepare reagents**: Warm Buffer R and RNA detection probes to room temperature before opening. (Warming RNA detection probes reduces viscosity, improving pipetting accuracy.) Before use, briefly vortex, then spin down. Store unused RNA detection probes at 4°C for up to 6 months or re-freeze.
2. **Clean the hybridization chamber and other equipment with RNase AWAY** and allow to dry, or rinse with DEPC-treated water. The hybridization chamber can be a key source of contamination by oligos. Arrange fresh Kimwipes on the bottom of the chamber and wet with 2X SSC or DEPC-treated water. Kimwipes should be thoroughly damp, but liquid should not pool. If your chamber is light-permeable, wrap the lid in aluminum foil to minimize light exposure.

   > If your hybridization chamber does not seal with a gasket, place the chamber in a zip-lock bag to simulate a sealed chamber. Chambers sealed in this manner should be tested prior to use to ensure they maintain humidity (i.e. that slides do not dry out) for 24 hours. Unsealed chambers can result in evaporation of the hybridization solution.

3. **Make hybridization solution** following Table 15 for NGS assays or Table 16 for nCounter assays. Confirm that you are using probe mix for manual/semi-automated slide preparation (white label, green/white/amber cap) and not probe mix for fully automated slide prep (yellow label, red cap).

For NGS readout:

Table 15: Hybridization solution for assays with NGS readout (Whole Transcriptome Atlas (WTA), Cancer Transcriptome Atlas (CTA)). *n* = number of slides

Panel Configuration	Buffer R	Atlas Probe Mix	Custom assay 1	Custom assay 2	DEPC-treated H_2O	Final Volume
CTA or WTA	200 µL x *n*	25 µL x *n*	0	0	25 µL x *n*	250 µL x *n*
CTA or WTA + 1 custom assay	200 µL x *n*	25 µL x *n*	12.5 µL x *n*	0	12.5 µL x *n*	250 µL x *n*
CTA or WTA + 2 custom assays	200 µL x *n*	25 µL x *n*	12.5 µL x *n*	12.5 µL x *n*	0	250 µL x *n*
1 custom assay, standalone (no CTA or WTA)	200 µL x *n*	0	12.5 µL x *n*	0	37.5 µL x *n*	250 µL x *n*
2 custom assays, standalone (no CTA or WTA)	200 µL x *n*	0	12.5 µL x *n*	12.5 µL x *n*	25 µL x *n*	250 µL x *n*

For nCounter readout:

Table 16: Hybridization solution for assays with nCounter readout . *n* = number of slides

Panel Configuration	Buffer R	Immune Pathways Panel	Custom assay*	DEPC-treated H_2O	Final Volume
RNA Immune Pathways Panel	200 µL x *n*	37.5 µL x *n*	0	12.5 µL x *n*	250 µL x *n*
RNA Immune Pathways Panel + 1 custom assay	200 µL x *n*	37.5 µL x *n*	12.5 µL x *n*	0	250 µL x *n*

*Only one custom assay may be added to the Immune Pathways Panel.

4. **One at a time, remove slides from 1X PBS,** wipe away excess liquid, and set in hybridization chamber in a horizontal position. **Take care not to let the slides dry out.**
5. Ensure that the Kimwipes and liquid do not contact the slides. Hybridization solution can wick off of the slides if it comes into contact with Kimwipes or liquid.
6. **Add 200 µL hybridization solution** to each slide. Take care not to introduce any bubbles.

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

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

7. **Gently apply a Grace Bio-Labs HybriSlip**. Start by setting one edge of the coverslip down in solution on the slide, then gradually laying down the rest of the coverslip to avoid the formation of air bubbles (see Figure 46).

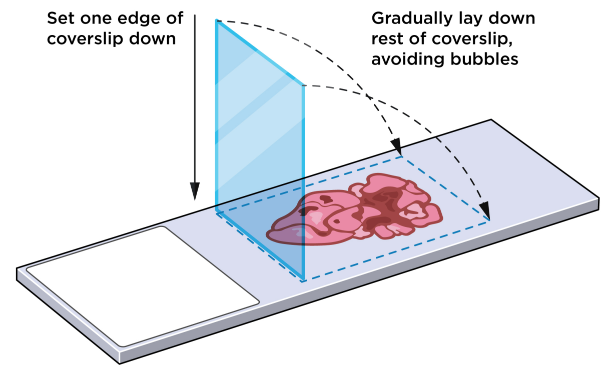

Figure 46: Applying coverslip

8. Repeat steps 5–8 for each slide.

9. Close hybridization chamber, insert into hybridization oven, and clamp into place (see Figure 47). **Incubate at 37°C overnight** (16–24 hr).

Figure 47: Placing chamber in oven

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

You will need the following materials and reagents for this step: **water bath, 4X SSC, 100% formamide**, **2X SSC**, and (optional) **2X SSC-T**. See the Equipment, Materials, and Reagents lists on page 38 and Prepare Reagents steps on page 43.

WARNING: Use of appropriate personal protective equipment is advised.

IMPORTANT: Everything that comes into contact with the hybridization solution, such as containers for SSC, must be dedicated to this protocol and thoroughly cleaned with RNase AWAY, as probes may contaminate subsequent runs. Use separate staining jars for different probe mixes. Staining jars should be cleaned with RNase AWAY before each use.

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

IMPORTANT: The stringent wash buffer **must** be at 37°C before washing the slides.

3. **Dip** slides in **2X SSC** allowing coverslips to slide off. Continue to wash steps within **5 minutes**.

If coverslips do not come off immediately, move them to 2X SSC-T for a maximum of 5 minutes. If coverslips have not fallen off in 5 minutes, proceed to the first stringent wash.

IMPORTANT: Forcibly removing coverslips will damage the tissue. Allow the coverslips to slide off freely.

4. Perform **2 washes in Stringent Wash at 37°C for 25 minutes each**, then **2 washes in 2X SSC for 2 minutes each** (see Figure 48). After the last wash, slides can be stored in 2X SSC for up to 1 hour.

Figure 48: Stringent washes to remove off-target probes

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Add morphology markers (100 minutes)

You will need the following materials and reagents for this step: **humidity chamber, Buffer W, SYTO 13 nuclear stain, morphology markers,** and **2X SSC**. See the Equipment, Materials, and Reagents lists on page 38 and Prepare Reagents steps on page 43.

IMPORTANT: Before using the humidity chamber in the following steps, clean it with RNase AWAY. Prep the humidity chamber by lining with Kimwipes wetted with 2X SSC or DEPC-treated water. Add just enough liquid to cover the bottom of the chamber.

1. **Remove SYTO 13 nuclear stain from -20°C** and allow it to warm to room temperature.
2. **Block with Buffer W:** Remove one slide at a time from 2X SSC and tap slide on clean, absorbent surface to remove excess liquid. Place slide in the humidity chamber. Cover tissue with up to **200 µL Buffer W** and leave at room temperature for 30 minutes, protected from light.

   Ensure adequate Buffer W surrounds the edges of the tissues so they don't dry out. Use a pipette tip to gently move the solution so there is a 2–3 mm border around the tissue. A hydrophobic barrier can be used at this step if desired, but must be carefully removed with a razor blade before loading on the instrument.

3. **Once thawed, vortex then picofuge SYTO 13 for at least 1 minute** to bring the solution and insoluble particles to the bottom of the vial. When pipetting SYTO 13, pipette from the top of the vial.

4. **Prepare 220 µL of morphology marker solution per slide** (see Table 17) (*n* = number of slides).

Table 17: Morphology marker solution equation

Nuclear stain (SYTO 13)	Morphology Marker 1	Morphology Marker 2	Other Markers*	Buffer W**	Final Volume
22 µL x *n*	5.5 µL x *n*	5.5 µL x *n*	...	187 µL x *n*	220 µL x *n*

* If using non-NanoString morphology markers, optimal concentration in the morphology marker solution must be determined by user testing.

**If using a different number of morphology markers, Buffer W amount needs to be adjusted to bring total volume up to 220 µL per slide.

5. **Mix morphology marker solution** by flicking and briefly picofuging.
6. **Remove Buffer W** from one slide at a time by tapping slide onto a Kimwipe, then return the slide to the humidity chamber.

7. **Cover tissue with morphology marker solution (up to 200 µL)**. Repeat steps 6 and 7 for each slide.
8. Stain for **1 hour** in the humidity chamber at room temperature, protected from light.

9. After staining, **remove solution** by tapping slide on a clean, absorbent surface. **Wash in 2X SSC**, 2 times for 5 min each.

Slides can remain in 2X SSC until loading on the GeoMx DSP (see safe storage guidelines, below). **DO NOT let slides dry out.** If a hydrophobic barrier was used, carefully remove it before loading on the instrument (see instructions **on page 35**).

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

Safe storage guidelines for RNA slides

- Storage up to 6 hr: submerge in **2X SSC and store at room temperature, protected from light.**
- Storage from 6 hr to 7 days: submerge in **2X SSC and store at 4°C, protected from light.**
- Slides may be stored longer than 7 days, but counts will decrease as a function of days stored. For best results, minimize storage time between slide preparation and loading on the GeoMx DSP.

Stripping and re-probing procedure for RNA slides

GeoMx RNA assay slides are reusable and can be restained with a different commercial or custom panel following this protocol. This protocol requires a UV light box or transilluminator capable of emitting 302/312 nm UV light (example), 2X SSC-T, 1X Tris-EDTA, and 2X SSC.

1. Place the slide flat on the surface of a UV transilluminator.
2. Apply enough **2X SSC-T** to completely cover the tissue (50–200 µL depending on the size of the tissue).
3. **Expose to UV light** for **3 minutes** to cleave tags from bound probes.
4. Carefully tap each slide on a clean, disposable surface (e.g., paper towel) to remove liquid and avoid oligo contamination.
5. **Wash slides** by dipping in a staining jar with **2X SSC-T.**
6. Transfer to another staining jar with fresh **2X SSC-T**.
7. Incubate the slides in **1X Tris-EDTA at 85°C** using either a hotplate or a pressure cooker on a low pressure setting for 15 minutes.
8. Wash 3 times in **2X SSC**.
9. Proceed to **In situ hybridization (overnight) on page 57**.

Fully Automated RNA Slide Preparation Protocol (FFPE)

This is a fully automated RNA slide preparation protocol for FFPE samples using the BOND RX/RX^m. Following this protocol, up to 30 slides can be prepared at once. Once initiated, the run time on the BOND RX/RX^m is approximately 22 hours.

The BOND RX/RX^m must be running BOND RX Version 6.0 software (or later) and have file BOND-RX-Ext-v29 (or later) installed. Download the file from the Leica BOND RX/RX^m Resources webpage and upload to your BOND RX/RX^m using a USB drive. Installation must be performed by an Admin user and takes <10 minutes. Once the BOND RX/RX^m application has been restarted, the instrument's database will include new reagents and protocol templates. Refer to your BOND RX/RX^m User Manual, accessible from the Help icon in the instrument software, for additional information.

> **IMPORTANT:** Ensure that you are performing this assay within the BOND RX/RX^m specifications for humidity and elevation. If you are outside the specifications, please contact your NanoString Application Scientist.

> **IMPORTANT:** NanoString recommends testing your system by performing GeoMx DSP with your BOND RX/RX^m on well-characterized samples prior to use on experimental samples.

> **IMPORTANT:** For any given study, NanoString recommends using only one sample type preparation method (e.g., FFPE or fresh frozen samples, but not a combination of sample types).

1 Equipment, Materials, and Reagents

The following tables list equipment, materials, and reagents that are required for this protocol but are **not supplied by NanoString**.

Table 18 : Equipment for RNA slide prep not provided by NanoString.

Equipment	Source	Part No.
BOND RX Fully Automated IHC/ISH Stainer **or** BOND RX^m Compact Fully Automated IHC/ISH Stainer	Leica Biosystems	Contact Leica Biosystems
BOND RX Controller, running BOND RX software version 6.0 and above	Leica Biosystems	Contact Leica Biosystems
Baking oven	Quincy Lab, Inc. (or comparable)	Various GC models

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Table 19: Materials for RNA slide prep not provided by NanoString.

Materials	Source	Part No.
BOND Research Detection System (includes 6 x 30 mL Open Containers - 4 are required for 4-slide run)	Leica Biosystems	DS9455
BOND Titration Kit (includes 50 inserts - 5 are required for 4-slide run)	Leica Biosystems	OPT9049
BOND Universal Covertiles	Leica Biosystems	S21.2001
Pipettes for 5–1,000 µL	Various	Various
Filter tips (DNase/RNase free)	Various	Various
Microcentrifuge tubes (DNase/RNase free)	Various	Various
Apex BOND Adhesive slides	Leica Biosystems	3800040
Slide staining jars (Coplin jars) and slide holder insert	VWR (or comparable)	25608-904, 25608-868
RNase AWAY® or 10% Bleach (RNaseZap® is not a substitute)	Thermo Fisher	7003PK
USB drive v3.0, 64 GB or higher (ability to be NTFS formatted)	SanDisk (or comparable)	SDCZ800-128G-G46

Table 20: Reagents for RNA slide prep not provided by NanoString. RT = room temperature

Reagents	Source, Part No.	Storage
BOND Dewax Solution, 1 L	Leica Biosystems, AR9222	4°C or RT
BOND Epitope Retrieval 2, 1 L	Leica Biosystems, AR9961	4°C
BOND Wash Solution 10X Concentrate, 1 L	Leica Biosystems, AR9590	4°C
BOND Aspirating Probe Cleaning Kit	Leica Biosystems, CS9100	4°C
Reagent-grade ethanol	Various	RT
DEPC-treated water, 5 L NOTE: Approx. 5 L required for initial setup to fill BOND DI water reservoir and make all dilutions.	Thermo Fisher, AM9922 (or comparable) NOTE: As an alternative to commercial DEPC-treated water, prepare your own following standard protocols.	RT
10X phosphate buffered saline pH 7.4 (PBS)	Sigma-Aldrich, P5368-10PAK, P5368-5X10PAK (or comparable)	RT
10% neutral buffered formalin (NBF)	EMS Diasum, 15740-04 (or comparable)	RT
100% deionized formamide	Thermo Fisher, AM9342 **or** VWR, VWRV0606 (or comparable) NOTE: If deionized formamide is unavailable, molecular grade formamide may be substituted.	4°C (bring to RT before opening)
20X SSC (DNase/RNase free)	Sigma-Aldrich, S6639	RT
Proteinase K (recommended) **or** Leica Biosystems Enzyme Pretreat Kit	Ambion, 2546 Leica Biosystems, AR9551 Thermo Fisher, AM2548 or 25530049 NOTE: Using proteinase K from any other supplier will require optimization of timing and concentration.	See manufacturer's instructions
Tris base	Sigma-Aldrich, 10708976001 (or comparable)	RT
Glycine	Sigma-Aldrich, G7126 (or comparable)	RT

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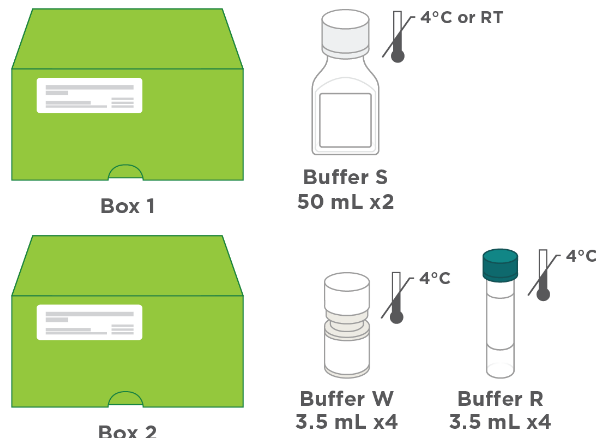
NanoString Reagents

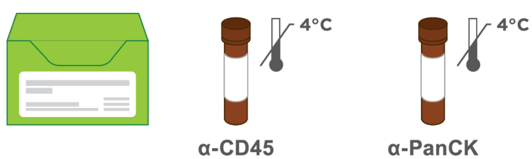
The following reagents are **supplied by NanoString**. Contact your NanoString Sales Representative to use our reagent planning tools to calculate required quantities.

GeoMx RNA Automated Slide Prep Kit (for preparation of 12 slides)

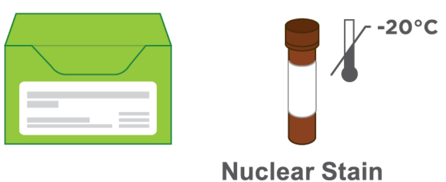


GeoMx Morphology Kit - Human or Mouse RNA compatible (various available)

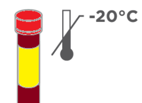
Example morphology kit (Solid Tumor TME)



GeoMx Nuclear Stain Morphology Kit

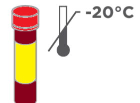


For NGS readout:
GeoMx Atlas for NGS readout (various available) - **formulated for automation**



Custom RNA-NGS Probe Mixes formulated for automation have the same appearance.

For nCounter readout:
GeoMx Immune Pathways Panel for nCounter readout - **formulated for automation**



Custom RNA-nCounter Probe Mixes formulated for automation have the same appearance.

DO NOT use RNA probe mixes for manual/semi- automated slide preparation (white label, green/white/amber cap) with this fully automated protocol. These reagents are not cross-compatible.

Prepare reagents

Prepare the reagents using the dilution instructions **(see Table 21)**. Refer to **Table 25** for minimum volumes required for a 4-, 8-, or 12-slide run. If running <4 slides, use the volumes for a 4-slide run. Once reagents have been added to BOND containers, store at 4°C unless otherwise noted. **Use DEPC-treated water for all dilutions** (including 1X BOND Wash Solution in bulk reservoir) and in DI water reservoir.

IMPORTANT: Take care to maintain nuclease-free conditions. The greatest risk of contamination comes from GeoMx probes and other oligos. We recommend the use of RNase AWAY (Thermo Fisher 7002), as it will limit contamination from oligos, GeoMx probes, and nucleases. After using RNase AWAY, allow to air dry completely, or rinse with DEPC-treated water. See manufacturer's instructions for details.

Table 21: Reagent prep for RNA semi-automated slide preparation

Reagent	Dilution	Storage
NBF stop buffer	Combine **6.06 g Tris base** and **3.75 g glycine** in **500 mL** DEPC-treated water to yield 0.1M Tris, 0.1M Glycine. Can be stored at RT for 1 month.	RT
Proteinase K	Default concentration (**1 µg/mL**) is made by adding **2 µL** of **20 mg/mL** Proteinase K to **40 mL** of **1X PBS** made with DEPC-treated water. See **Table 27** for alternative concentrations by tissue type. If using Proteinase K from the BOND Enzyme Pretreatment Kit, dilute stock (17 mg/ml) to 1 µg/mL, rather than concentrations recommended by Leica Biosystems. NOTE: Prepare fresh and do not reuse. Accurate dilution of proteinase K is critical for proper assay performance.A serial dilution is recommended for any volume smaller less than 40 mL or if using 0.1 µg/mL proteinase K.	n/a
1X BOND Wash Solution	For the first run, prepare **30 mL** 1X by combining **3 mL** of 10X BOND Wash Solution with **27 mL** DEPC-treated water. Prepare more as needed.	RT
1X PBS pH 7.4	Prepare **1 L** of 1X PBS by combining **100 mL** of 10X PBS and **900 mL** of DEPC-treated water.	RT
50% Formamide, 2X SSC	Warm 100% formamide to RT before opening. Prepare solution by mixing equal volumes of 100% formamide and 4X SSC. NOTE: Prepare fresh for each use.	n/a
2X SSC	Prepare **1 L** of 2X SSC by combining **100 mL** of 20X SSC and **900 mL** of DEPC-treated water.	RT

2 Prepare tissue samples

Appendix I: Selecting and Sectioning FFPE Samples on page 87 covers FFPE block selection and sectioning in detail. Review it prior to beginning the Slide Preparation protocol.

GeoMx has been validated for sample blocks up to 3 years old prepared from tissues with a cold ischemic time of less than 1 hour using 10% NBF or similar fixative. For best results, do not use FFPE blocks 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

Slide Preparation

1. Tissue sections should be **5 µm** thick and mounted on **Apex BOND Adhesive slides**. Tissue sections must **fit within the white dots** marking the boundary on the Apex BOND slides, and be within the **Scan Area** (shown in green) (see Figure 49). Sections can be no larger than 35.3 mm long by 14.1 mm wide and should not overlap the slide gasket (shown in blue) or the Tip Calibration area (shown in red). If mounting multiple sections per slide, ensure that tissues are at least 2–3 mm apart and still fit within the Scan Area.

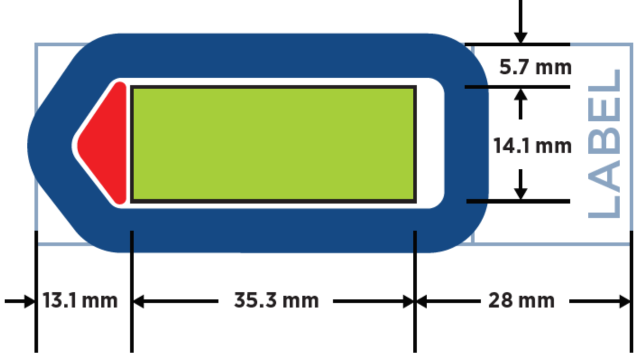


Figure 49: Slide dimensions

If sections are too large and/or placed off-center, continue with slide preparation as usual. Just before loading the slide in the instrument slide tray, scrape off parts of the tissue exceeding the scan area, making sure the slide gasket and tip calibration area are free of tissue. Scraping off tissue before slide preparation could generate tissue folds that may result in staining or binding artifacts.

IMPORTANT: The GeoMx DSP instrument will only image the area inside the Scan Area. **Tissue outside of the Scan Area will not be imaged and may cause problems in tissue detection by the optical system.**

2. **Bake sections on slides** in a 60°C drying oven for 30 minutes to 3 hours prior to deparaffinization. Stand slides vertically during baking to allow excess paraffin to flow off. Longer baking times may be necessary for some tissues to adhere to the slide; this should be empirically tested.

3 Initial set up of the BOND RX

See Figure 50 for an overview of the BOND RX/RX^m user interface. Refer to the **BOND RX/RX^m User Manual**, accessible from the **Help** icon in the instrument software, for additional information.

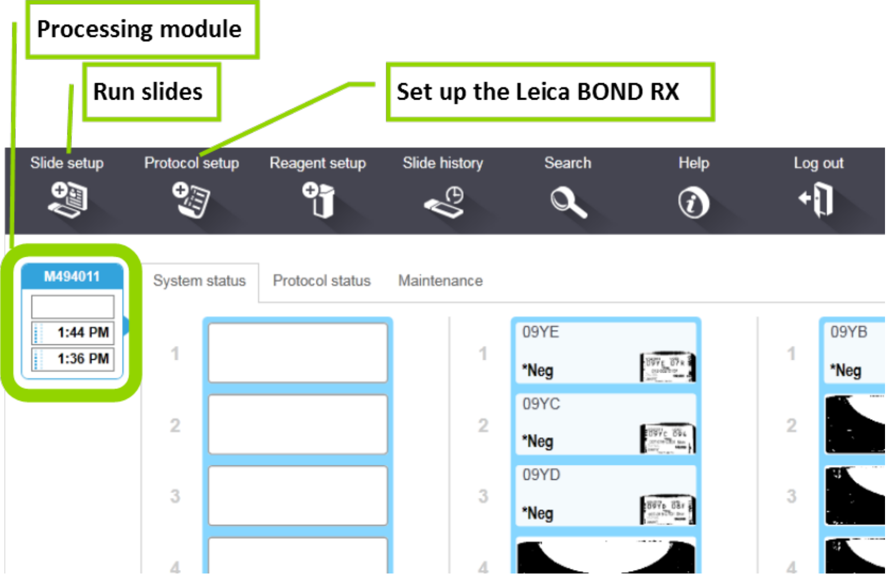


Figure 50: BOND RX/RX^m user interface

To set up the BOND RX/RX^m, you must setup the reagents, register the Detection System, set up the protocols to be used, and register the reagents and the containers in which they will be loaded.

BOND RX/RX^m data file BOND-RX-Ext-v29 includes pre-programmed protocols and reagents, marked with an asterisk (*). These are placeholders that will be assigned, adopted, and in some cases adapted for your slide preparation protocol.

To ensure that you can locate the applicable protocols and reagents from the drop-down menus in subsequent steps:

1. Click on Reagent Setup. At the bottom of the menu, change Staining Status and Preferred Status to ALL. In the Name column, click to Sort in alphabetical order. Locate each reagent beginning with *GeoMx [asterisk GeoMx], open, and mark reagent as Preferred.
2. Click on Protocol Setup and mark two protocols with Preferred status: *GeoMx RNA Protocol and *GeoMx Probe Application.

Register the BOND Research Detection System

The BOND Research Detection System is a barcoded tray that holds containers of reagents. It is preferable to use a new or not-yet-assigned Research Detection System for this protocol. If using a previously assigned Research Detection System, modifications to downstream steps may be required, as previous data for the Research Detection System may carry forward.

1. **Remove any containers** from the BOND Research Detection System.
2. **Scan the barcode** on the side of the system (see Figure 51).
3. In the **Add research reagent system** window (see Figure 52), enter the information for your reagent system.
    - Enter a **Name** for your BOND Research Detection System (suggested: GeoMx FA RNA). If the system was previously registered, select the name from the drop-down menu.
    - Enter the **Lot No.** (optional).
    - Enter an **Expiration Date**.
    - Highlight **Row 1** and **scan the front barcode of a new BOND 30 mL Open Container** (the barcode will appear in the field for Row 1). Place the container in position 1 of the BOND Research Detection System tray.
    - Select ***GeoMx Home Buffer** from the drop down menu in the Reagent column.
    - Click **Add**.

Figure 51: Research detection system barcode

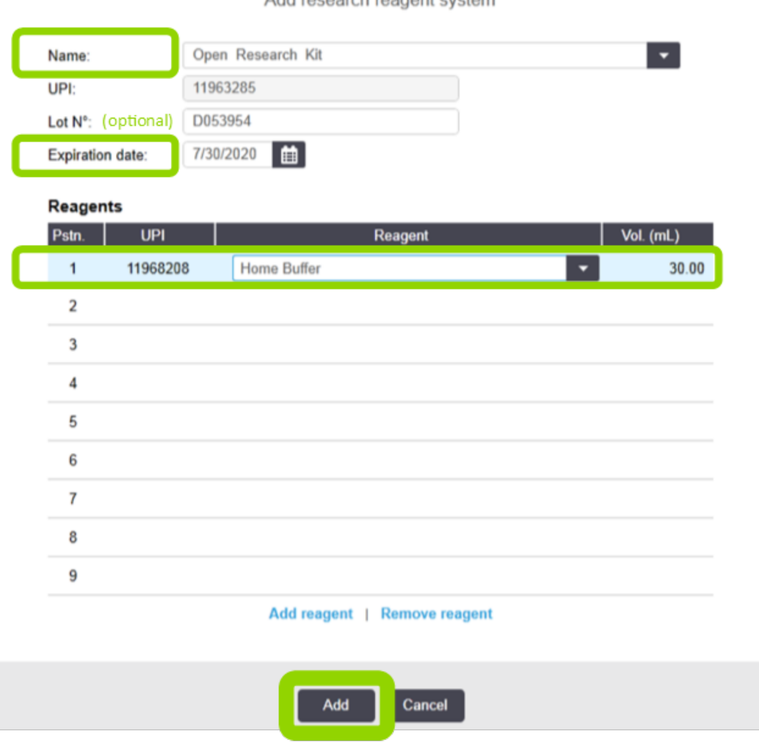


Figure 52: Add research reagent system window

4. Label the **BOND Research Detection System** reagent tray to match the name entered in the **Add research reagent system** window.
5. Label the **30 mL Open Container in position 1** as *Home Buffer*. The BOND Research Detection System is now registered.

RNA Protocol Setup

The fully automated RNA protocol must be created the first time it is used on the BOND RX/RX^m. Once it is established, it can be used for all GeoMx RNA slides prepared with this fully automated workflow.

1. Click the **Protocol setup** icon (see Figure 53).

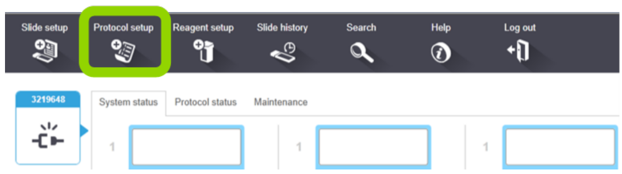


Figure 53: Protocol setup icon

2. Highlight the ***GeoMx RNA Protocol** row and click **Copy** (see Figure 54).

Figure 54: Protocol setup window

3. In the **Edit protocol properties** window (see Figure 55), enter the following information:
   - Enter *GeoMx RNA Protocol* in the **Name** field.
   - Enter *GeoMxRNA* in the **Abbreviated Name** field.
   - Fill in the **Description** field, e.g. *Fully Automated GeoMx RNA Protocol.*
   - Check the **Single** box for **Staining method**.
   - Select your BOND Research Detection System from the **Preferred detection system** drop down menu.
4. Click **Save**.

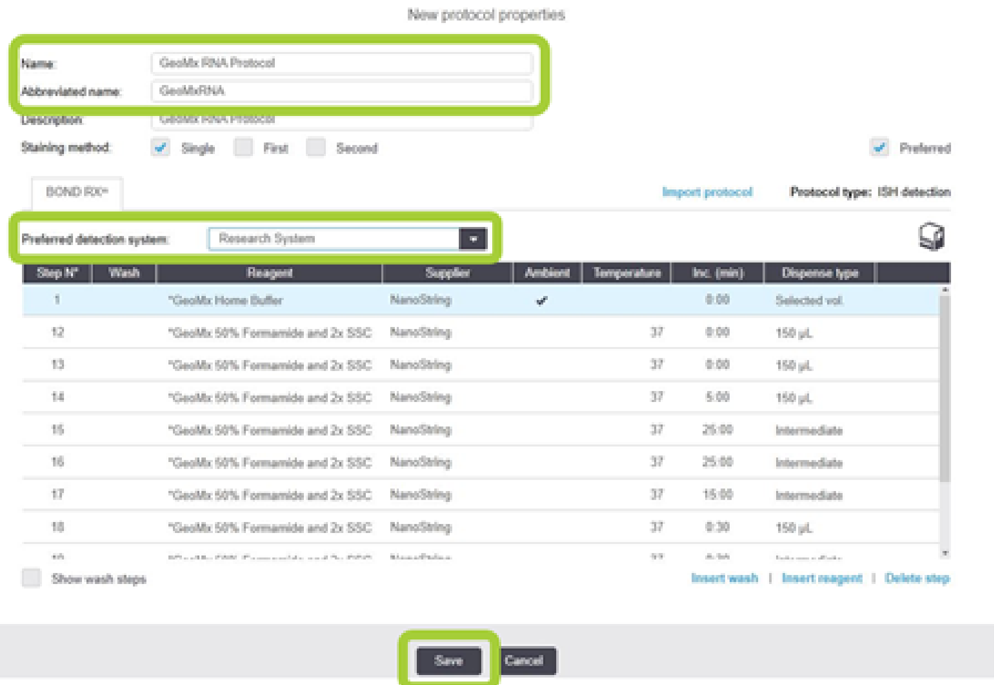


Figure 55: Edit protocol properties window (RNA)

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5. Read the notification and click **Yes** (see Figure 56).

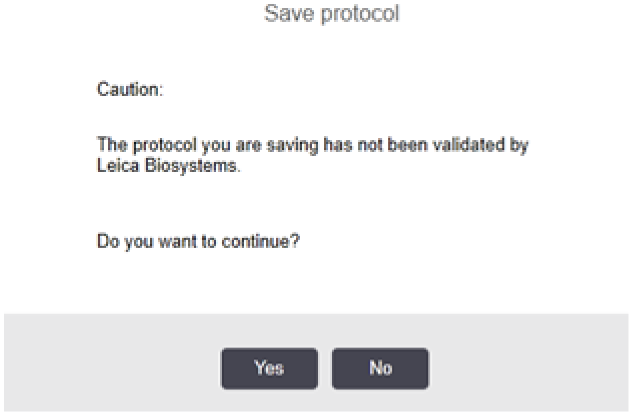

Figure 56: Save protocol notification

Register the RNA Probe Mix

Each GeoMx RNA Probe Mix (for example, Whole Transcriptome Atlas, Cancer Transcriptome Atlas, Custom Panel 1, Custom Panel 2) should be registered to its own RNA Probe reagent. To create a new RNA Probe reagent:

1. Select the **Reagent Setup** icon (see Figure 57).

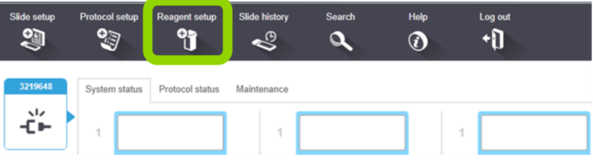

Figure 57: Reagent setup icon

2. From the **Setup** tab, click the **Add** button (see Figure 58).

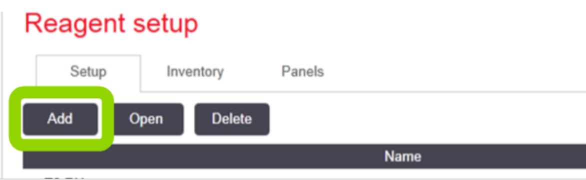

Figure 58: Add reagent button

3. Enter in a **Name** and an **Abbreviated Name**, unique to that GeoMx RNA Probe Mix.
4. From the **Type** drop-down menu, select **Probe RNA**.

5. Enter the following information in the **Single** tab:
   - **Single/double stain:** Single/Sequential DS
   - **Default staining protocol:** GeoMx RNA Protocol
   - **Default HIER protocol:** *HIER 10min with ER2
   - **Default enzyme protocol:** *Enzyme 1 for 15 min
   - **Default denaturation protocol:** (leave blank)
   - **Default hybridization protocol:** *ISH Hybridization (16Hr)

5. For **Compatible bulks**, enter *BWash.

6. Ensure the 'Preferred' checkbox is selected. Click **Save**.

Register Additional Containers for Use

1. Label and add the ***GeoMx 10% NBF Open container**.
   - Insert a **BOND 30 mL Open container** in position 2 of the tray.
   - Label the **Open container in position 2** with *GeoMx 10% NBF*.
   - Scan the front barcode of the Open Container.
   - In the **Add open container** window (see Figure 59), select ***GeoMx 10% NBF** from the **Reagent name** drop- down menu, enter a **Lot No.** (if required), and enter an **Expiration date**.
   - Click **OK**.

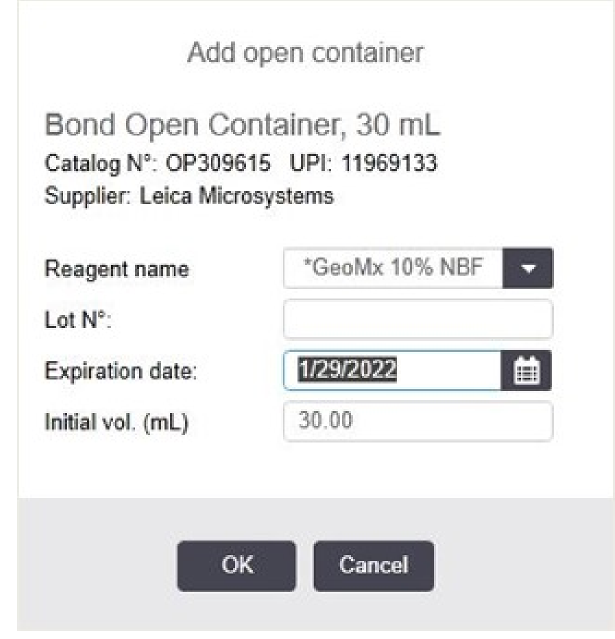

Figure 59: Add open container window

2. Repeat these steps for ***GeoMx NBF Stop Buffer** in position 3 and ***GeoMx 50% Formamide and 2X SSC** in position 4 of the tray. Use BOND 30 mL Open Containers for both.

3. Repeat these steps for ***Enzyme 1, *GeoMx Buffer R, GeoMx RNA Probes** (selecting the RNA Probe Mix reagent you previously set up), ***GeoMx Buffer W, and *GeoMx RNA Morphology Markers**. Use 6 mL Titration Containers with Inserts and place in positions 5-9 of the tray.

4 Start a run on the BOND RX

IMPORTANT: It is recommended that only the NanoString GeoMx assay is run on the BOND RX/RXᵐ at any one time. This is to then be followed by an Aspirating Probe Clean, ensuring a comprehensive clean of the aspirating probe prior to running other assays.

IMPORTANT: Probe formulations for fully automated slide preparation are different from probe formulations for manual and semi- automated slide prearation. They may not be used interchangeably or modified to substitute one for the other.

IMPORTANT: RNA probe mixes should be assembled in an area separate from any nCounter work, NGS library prep, or other GeoMx workflow areas. GeoMx detection reagents contaminated by RNA probe mixes can give misleading or incorrect results. Areas should be cleaned thoroughly with RNase AWAY after probe mix preparation. Alternatively, mixes can be prepared in PCR workstations and decontaminated with UV light. Gloves should be changed after handling any probe mixes to avoid cross-contamination.

Prepare Probe Mix and Morphology Marker Mix

1. Thaw RNA detection probes and let warm to room temperature. (Warming RNA detection probes reduces viscosity, improving pipetting accuracy.) Before use, briefly vortex, then spin down. Store unused RNA detection probes at 4°C for up to 6 months or re-freeze.
2. Remove SYTO 13 nuclear stain from -20°C and let warm to room temperature. Once thawed, picofuge for at least 1 minute to precipitate insoluble particles.
3. Warm Buffer R to room temperature before opening.
4. Make RNA Probe Mix following the instructions in Table 22 for NGS assays or Table 23 for nCounter assays. **Confirm that you are using probe mix for fully automated slide preparation (yellow label, red cap)** and not probe mix for manual/semi-automated slide prep (white label, green/white/amber cap). Avoid bubbles when pipetting reagents, especially Buffer R.

For NGS readout:

Table 22: RNA Probe Mix for fully automated protocol - NGS assays

Panel Configuration	Buffer R	Atlas Probe Mix	Custom assay 1	Custom assay 2	DEPC-treated water	Final Volume
Whole or Cancer Transcriptome Atlas alone	1240 µL per 4 slides	155 µL per 4 slides	n/a	n/a	155 µL per 4 slides	1550 µL per 4 slides
Atlas with 1 custom assay	1240 µL per 4 slides	155 µL per 4 slides	77.5 µL per 4 slides	n/a	77.5 µL per 4 slides	1550 µL per 4 slides
Atlas with 2 custom assays	1240 µL per 4 slides	155 µL per 4 slides	77.5 µL per 4 slides	77.5 µL per 4 slides	n/a	1550 µL per 4 slides
1 custom assay (no Atlas)	1240 µL per 4 slides	n/a	77.5 µL per 4 slides	n/a	232.5 µL per 4 slides	1550 µL per 4 slides
2 custom assays (no Atlas)	1240 µL per 4 slides	n/a	77.5 µL per 4 slides	77.5 µL per 4 slides	155 µL per 4 slides	1550 µL per 4 slides

For nCounter readout:

Table 23: RNA Probe Mix for fully automated protocol - nCounter assays

Panel Configuration	Buffer R	Immune Pathways Panel	Custom assay	Final Volume
RNA Immune Pathways alone	1317.5 µL	232.5 µL	n/a	1550 µL per 4 slides
RNA Immune Pathways plus 1 custom assay	1240 µL	232.5 µL	77.5 µL	1550 µL per 4 slides

5. Make GeoMx Morphology Marker mix following the instructions in Table 24. Use SYTO 13 nuclear stain which is fully thawed and spun down. Do not pipette from the bottom of the SYTO 13 vial. After making the Morphology Marker mix, flick tube and picofuge briefly.

Table 24: Morphology Marker mix for fully automated protocol

Nuclear stain (SYTO 13)	Morphology Marker 1*	Morphology Marker 2	Morphology Marker 3	Buffer W**	Final Volume
185 µL per 4 slides	46.5 µL per 4 slides	46.5 µL per 4 slides	46.5 µL per 4 slides	1525.5 µL per 4slides	1850 µL per 4 slides

* If using non-NanoString morphology markers, optimal concentration in the morphology marker solution must be determined by user testing.

**If using a different number of morphology markers, Buffer W amount needs to be adjusted to bring total volume up to 1850 µL per 4 slides.

Fill Reagent Containers

Minimum volumes are shown in **Table 25**; if running less than 4 slides, use the volumes for a 4-slide run. Maximum volume for Open Containers is 30 mL and for Titration Inserts, 6 mL. Leave the container lids open.

1. **Fill the *GeoMx Home Buffer** container in position 1 with 1X BOND Wash solution.
2. **Working in a fume hood, fill the *GeoMx 10% NBF container** in position 2 with 10% NBF.

WARNING: NBF is hazardous. Handle with care and minimize inhalation risks. Please handle and dispose of according to your institution's EHS guidelines.

3. Fill the Open Containers in positions 3–4, and the Titration Containers in positions 5-9, according to **Table 25**:

Table 25: Filling Reagent Tray Containers

			Minimum volume needed for:		
Tray Position	**Reagent**	**Container**	**4 slides**	**8 slides**	**12 slides**
1	Wash Buffer	BOND 30 mL Open Container assigned *GeoMx Home Buffer	5.75 mL	6.5 mL	7.25 mL
2	10% NBF	BOND 30 mL Open Container assigned *GeoMx 10% NBF	5.75 mL	6.5 mL	7.25 mL
3	NBF Stop Buffer	BOND 30 mL Open Container assigned *GeoMx NBF stop buffer	5.75 mL	6.5 mL	7.25 mL
4	50% Formamide, 2X SSC	BOND 30 mL Open Container assigned *GeoMx 50% Formamide, 2X SSC	11.75 mL	18.5 mL	25.2 mL
5	Proteinase K	BOND 6 mL Titration Container assigned *Enzyme 1	1.35 mL	2.35 mL	3.35 mL
6	Buffer R	BOND 6 mL Titration Container assigned *GeoMx Buffer R	1.85 mL	3.35 mL	4.85 mL
7	GeoMx RNA Probes	BOND 6 mL Titration Container assigned GeoMx RNA Probes	1.55 mL	2.75 mL	3.95 mL
8	Buffer W	BOND 6 mL Titration Container assigned *GeoMx Buffer W	1.85 mL	3.35 mL	4.85 mL
9	GeoMx Morphology Markers	BOND 6 mL Titration Container assigned *GeoMx RNA Morphology Markers	1.85 mL	3.35 mL	4.85 mL

4. **Load the BOND Research Detection System onto the BOND RX/RX^{m}.** The instrument will recognize containers and, depending on your Dip Test settings, may check container volumes. Once finished, you may click on the **Processing Module** icon (see Figure 60) to view the status of any of the containers in the **System Status** window. Right-click on the desired container and select **Inventory** to view the volume measured in the container. Refill if required.

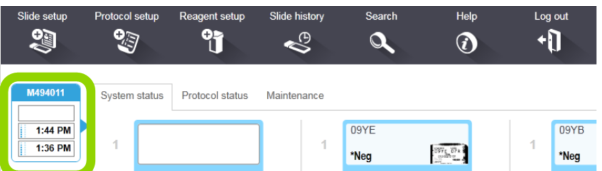

Figure 60: BOND RX screen with Processing Module icon indicated

> The BOND RX/RX^{m} may display an alert for low volumes in the Titration Containers. Unload and reload the reagent tray to allow a second volume check. Ensure that there are no bubbles in the solutions, which can trigger the alert. If the alert persists, you may need to add to the reagent volume.

Add Your Study and Slides and Print Slide Labels

1. Click the **Slide setup** icon (see Figure 61).

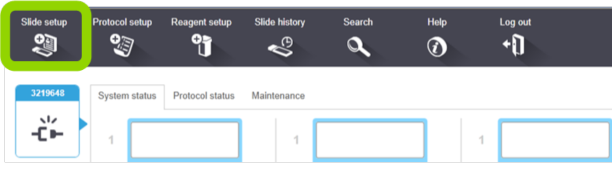

Figure 61: Slide setup icon

2. Click the **Add study** button (see Figure 62).

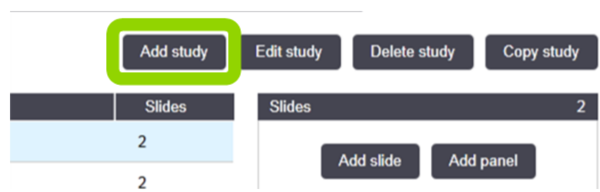

Figure 62: Add study button

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3. In the **Add study** window (see Figure 63), enter your study information.
   - Enter your **Study ID** (required).
   - Enter your **Study name** (required).
   - Enter your **Study comments** (optional).
   - **Researcher** (optional, refer to BOND RX/RX m user manual to add new researchers).
   - Check **Dispense volume**: **150 µL**.
   - Select **Preparation protocol** : ***Bake and Dewax** from the drop down list.
   - Click **OK**.

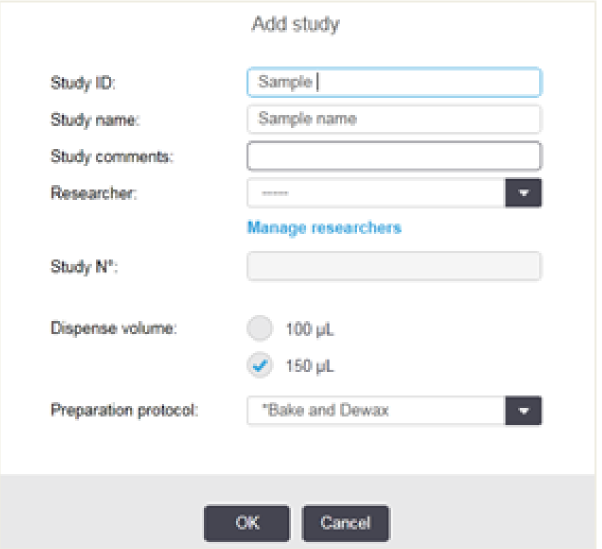

Figure 63: Add study window

4. Highlight your study and click **Add slide** (see Figure 64).

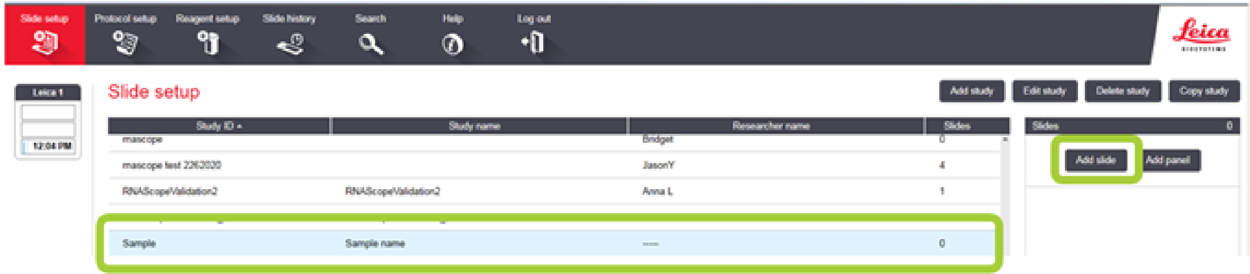

Figure 64: Add slide button

5. In the **Add slide** window (see Figure 65), enter your slide information.
   - Enter **Slide Comments**.
   - Check **Test tissue** for **Tissue type**.
   - Check **150 µL** for **Dispense volume**.
   - Select **Single** and **Routine** from the staining mode drop-down lists.
   - Check **ISH** for **Process**.
   - From the Marker drop-down list, select your registered Probe Mix (the name you assigned in Register the RNA Probe Mix on page 75.

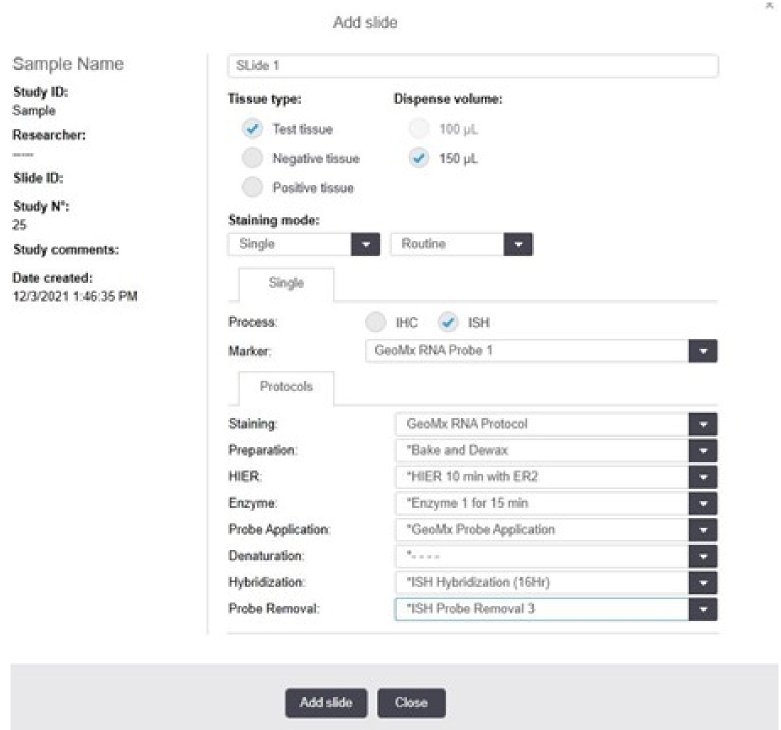

Figure 65: Add slide window for fully automated protocol

6. On the **Protocols** tab:
   - Select **GeoMx RNA Protocol** for **Staining**.
   - Select ***Bake and Dewax** for **Preparation**.
   - **HIER**: Select the appropriate incubation time based on the tissue type (see Table 26). Optimal incubation time and temperatures may differ by tissue and may need to be empirically determined. To create a new protocol, see NOTE below Table 26.

Table 26: Target retrieval times and temperatures by tissue type

Tissue Type	Target Retrieval
Breast	*HIER 10 min with ER2 @ 100°C
Cell pellets	*HIER 10 min with ER2 @ 85°C
Colorectal	*HIER 20 min with ER2 @ 100°C
Melanoma	*HIER 20 min with ER2 @ 100°C
Mouse tissue	*HIER 10 min with ER2 @ 100°C
NSCLC	*HIER 20 min with ER2 @ 100°C
Prostate tumor	*HIER 20 min with ER2 @ 100°C
Tonsil	*HIER 10 min with ER2 @ 100°C

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NOTE: To create a new protocol with different HIER conditions, click the Protocol Setup icon (see Figure 53). Find the existing *HIER protocol, copy it, and modify the conditions as needed. Save with a unique name and abbreviated name. Check the checkbox for “Preferred” protocol.

- **Enzyme**: Select *Enzyme1 and the appropriate incubation time based on the tissue type (see Table 27). The default is **1 µg/mL** Proteinase K for 15 minutes at 37°C, however, optimal Proteinase K concentration and incubation time differ by tissue and may need to be empirically determined. To create a new protocol, see NOTE below .

Table 27: Proteinase K digest concentrations and times by tissue type

Tissue Type	Proteinase K Digest
Breast	0.1 µg/mL for 15 min
Cell pellets	1 µg/mL for 5 min
Colorectal	1 µg/mL for 15 min
Melanoma	1 µg/mL for 15 min
Mouse tissue	1 µg/mL for 15 min
NSCLC	1 µg/mL for 15 min
Prostate tumor	1 µg/mL for 15 min
Tonsil	1 µg/mL for 15 min

NOTE: To create a new Proteinase K protocol, click the Protocol Setup icon (see Figure 53). Find the existing *Enzyme 1 for 15 minutes protocol, copy it, and modify the conditions as needed. Save with a unique name and abbreviated name. Check the checkbox for “Preferred” protocol.

- **Probe application:** Select *GeoMx Probe Application.
- **Denaturation**: Leave as *----.
- **Hybridization**: Select *ISH Hybridization (16Hr).
- **Probe Removal**: Select *ISH Probe Removal 3.

7. Click **Add Slide**. Continue to click **Add Slide** until you have sufficient labels for all your slides. Change **Slide Comments** as needed.
8. Click **Close**.
9. Click **Print Labels** (see Figure 66).

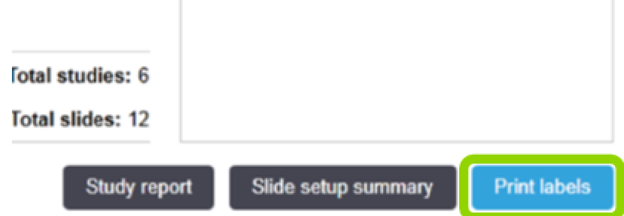

Figure 66: Print labels button

10. In the **Print slide labels** window (see Figure 67), select **All slide labels not yet printed for current study** and click on **Print.**

11. Place the labels on same side of the slides as the samples, aligning them at the top of the slide.

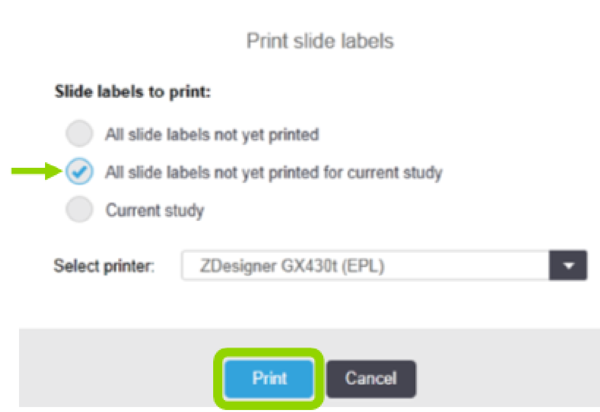

Figure 67: Print slide labels window

5 Load and run your slides

1. Check that all bulk containers are at least half-full and that the bulk waste container is empty.
2. Referencing the embossed image on the slide tray, **load the slides onto the slide tray** in the correct orientation (see Figure 68). Ensure the sample and label are facing upwards, with the label at the top of the slide tray.
3. **Place covertiles** on top of the slides with the tab at the bottom and *Leica* facing upwards.

Figure 68: Slides in slide rack

4. Gently load the slide tray(s) with slides and covertiles onto the BOND RX/RX^{m}; ensure that none of the slides or covertiles are dislodged.
5. Press the corresponding LED button below the slide tray. The instrument will scan the labels of the slides.
6. If a label is not recognized, hover over the missing slide on the screen, right-click, and choose **Select Manually**. Take care to select the correct information for the manually entered slide (refer to the BOND RX/RX^{m} User Manual for more information).
7. Once all slides and reagents have been reconciled, click the **Start** button under the slide tray image on the screen to begin the run.

> **IMPORTANT:** The run will take approximately 22 hours to complete. **Delay the start of the BOND RX/RX^{m} slide preparation run, if needed, to allow you to unload the slides immediately upon completion of the protocol.** Unloading the slides without delay avoids undesired background signal from probes that have not been washed away completely.

Complete the run

1. Once the run has finished, the LED button below the slide tray will blink green. Press the LED button to raise the slide tray.
2. Gently **slide the slide tray out** of the instrument and place on a flat, stable surface.
3. Handle slides one at a time so that they do not dry out. Remove each covertile by holding down the label of the slide, then carefully putting pressure downwards on the neck of the covertile to lift the end of the covertile off the slide. Do not slide the covertile across the surface of the slide, as you may damage the tissue.
4. **Using a Kimwipe, wipe away the residue from each long edge,** where the covertile met the slide. Fold or turn the Kimwipe between wiping motions to apply a clean area to each edge.
5. **Place slide in 2X SSC until loading on the GeoMx DSP** (refer to GeoMx DSP Instrument User Manual (MAN-10152)).
   - NanoString recommends **washing slides individually two times** (e.g. submerging quickly, then submerging for 5 min, in 2X SSC) **before combining for storage in 2X SSC**, to avoid any increase in background from residual probe contamination left over from multiple slides begin stored together.
   - Store protected from light in 2X SSC at RT for up to 6 hr, or in 2X SSC at 4°C for up to 7 days. Slides may be stored longer than 7 days, but counts will decrease as a function of days stored. For best results, minimize storage time between slide preparation and loading on the GeoMx DSP.

5 Clean up the BOND RX

1. Clean covertiles following BOND RX/RXm User Manual.
2. Load the **Aspirating Probe Cleaning kit**. From the Maintenance tab, run **Clean aspirating probe** cycle **(see Figure 69)** before any other users begin work on the BOND RX/RXm.

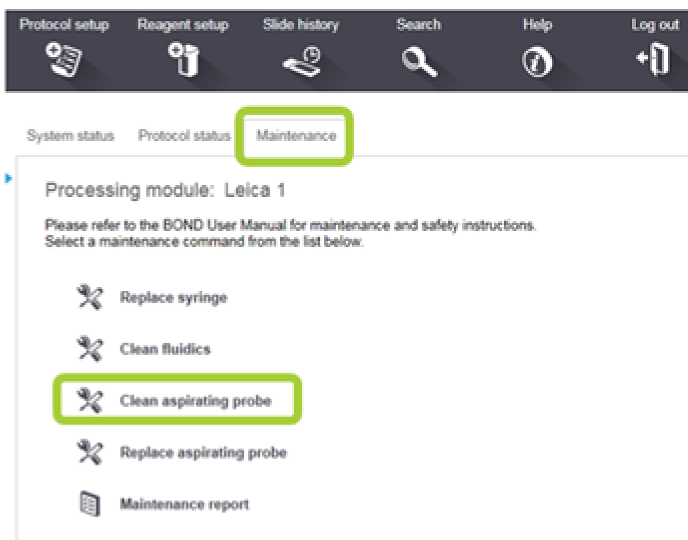

Figure 69: Run Aspirating probe cleaning cycle

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Appendix I: Selecting and Sectioning FFPE Samples

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

> GeoMx has been validated for sample blocks up to 3 years old prepared from tissues with a cold ischemic time of less than 1 hour using 10% NBF or similar fixative. For best results, do not use FFPE blocks 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.

Selecting FFPE blocks

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

1. Blocks should be fixed in 10% neutral buffered formalin for 18 to 24 hours at room temperature. This applies to tissues less than 0.5 cm in thickness. Larger tissues have not been tested by NanoString and may require longer fixation times.
2. Tissues should be fixed immediately after excision for best results. 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 GeoMx DSP assay performance. This is not meant to be an all-inclusive guide on sectioning. Please refer to your local pathologist or core facility for training on sectioning.

- Always discard the first few sections from the block face.
- NanoString recommends SuperFrost™ Plus slides (for manual slide preparation) or Apex BOND® slides (for BOND automated slide preparation or manual slide preparation for tissues that are prone to falling off the slide).
- Tissue sections should be cut at 5 µm thickness on a calibrated microtome and mounted on the slide immediately, without scratches or folds.
- Tissue sections must be placed in the **Scan Area** (shown in green) (see Figure 70) in the center of the slide and be **no larger than 35.3 mm long by 14.1 mm wide**. They should not overlap the slide gasket (shown in blue) or the Tip Calibration area (shown in red). Tissues that will be prepared using the BOND RX/RX^m must also fit within the white dots on the Apex BOND slides. If mounting multiple sections per slide, ensure that tissues are at least 2–3 mm apart and still fit within the Scan Area.

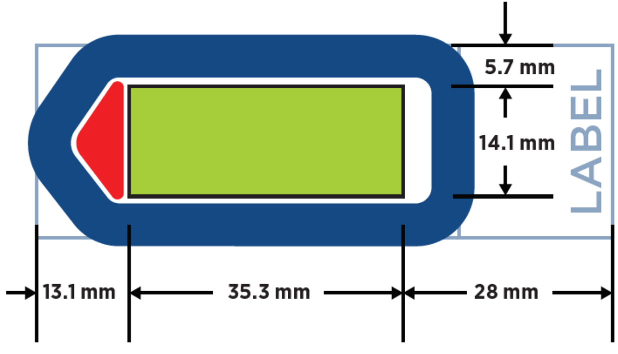

Figure 70: Slide dimensions

> **IMPORTANT:** The GeoMx DSP instrument will only image the area inside the Scan Area. **Tissue outside of the Scan Area will not be imaged and may cause problems in tissue detection by the optical system.**

- 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.
- Mounted slides should be allowed to air dry overnight prior to use. Store slides in a vertical position such that any remaining water can drain away from the tissue section.
- Slides stored in a dessicator (or in a sealed container with a dessicant pouch) at 4°C yield quality results for up to 3 months. Quality of results is tissue and block dependent and should be tested empirically.

Appendix II: Modifications to Automated Protocol for Fresh Frozen Samples

Disclaimers

- Human fresh frozen tissues can carry pathogens and should be handled according to your institution's lab safety guidelines.
- It is essential to minimize RNases when processing fresh frozen tissues for RNA assays. Ensure all buffers are made with DEPC-treated water and all equipment is RNase-free.
- NanoString has not optimized the assays for fresh frozen tissues and recommends that empirical optimization be performed for your samples.

IMPORTANT: For any given study, NanoString recommends that you use only one sample type preparation method (e.g., use only FFPE or only fresh frozen samples).

Selecting fresh frozen blocks

- Select tissues that are known to have been snap frozen in liquid nitrogen as quickly as possible after dissection. 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 (OCT) compound before sectioning.
- Blocks embedded in OCT should be stored at -80°C.

Sectioning fresh frozen blocks

The following are general guidelines for sectioning fresh frozen blocks for optimal GeoMx DSP assay performance. See also Cryosectioning technique on page 92.

- Always discard the first few sections from the block face.
- NanoString recommends SuperFrost Plus slides (for manual slide preparation) or Apex BOND Adhesive slides (for BOND automated slide preparation, or manual slide preparation for tissues that are prone to falling off the slide).
- Sections should be cut at 5–10 µm thickness on a calibrated cryostat and mounted immediately on the slide, without scratches or folds.

- Tissue sections must be placed in the **Scan Area** (shown in green) (see Figure 71) in the center of the slide and be **no larger than 35.3 mm long by 14.1 mm wide**. They should not overlap the slide gasket or the Tip Calibration area (shown in red). Tissues that will be prepared using the BOND RX/RX^{m} must also fit within the white dots on the Apex BOND slides. If mounting multiple sections per slide, ensure that tissues are at least 2–3 mm apart and still fit within the Scan Area.

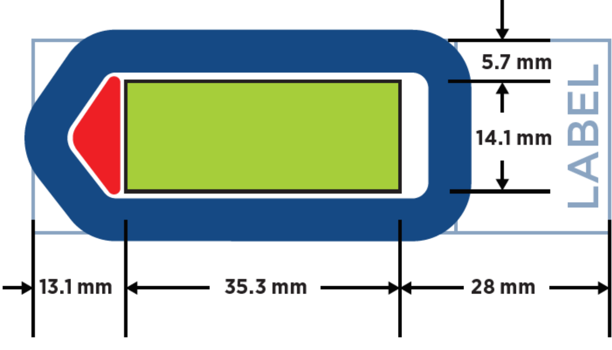

Figure 71: Slide dimensions

IMPORTANT: The GeoMx DSP instrument will only image the area inside the Scan Area. **Tissue outside of the Scan Area will not be imaged and may cause problems in tissue detection by the optical system.**

- After sectioning, the exposed block face should be covered with OCT to avoid desiccation of the sample.
- Slides can be stored at -80°C for several weeks before use.

Fresh frozen sample preparation for protein assays

Please refer to the manual slide preparation methods in GeoMx DSP Manual Slide Preparation User Manual (MAN-10150) for instructions to prepare fresh frozen samples for GeoMx protein assays.

Fresh frozen sample preparation for RNA assays

Reagents required for this protocol: 10% NBF, 1X PBS, 50% ethanol, 70% ethanol, 100% ethanol.

1. **In a BSL2 room, handle fresh frozen slides in a biosafety cabinet.** Remove the slides from -80°C. **Submerge in** 10% NBF overnight (12–16 hours) at room temperature to thaw and fix tissue. Thorough fixation is required to maintain tissue integrity.
2. Transfer slides from 10% NBF to 1X PBS. Wash the slides 3 times, for 2 minutes each wash.
3. Transfer slides from 1X PBS to a slide rack and bake at 60°C for 30 min.
4. Wash the slides in 50% ethanol for 5 minutes.
5. Wash the slides in 70% ethanol for 5 minutes.
6. Wash the slides in 100% ethanol 2 times for 5 minutes each.
7. Let slides air dry for at least 5 minutes (but not more than 1 hour).

Proceed to the semi-automated RNA slide preparation protocol on page 45. **The following modifications are required for fresh frozen samples:**

- When adding your study, select **Preparation protocol: *Frozen Slide Delay** instead of *Bake and Dewax.
- When adding your slides, select **Preparation protocol: *Frozen Slide Delay** instead of *Bake and Dewax.
- **Default target retrieval conditions are 100°C for 15 minutes**. 85°C for 15 minutes may improve tissue integrity in more delicate tissues, such as brain and cell pellets. It may also improve some morphology markers' performance, at the cost of a moderate reduction in the RNA assay's efficiency. Timing and temperature may need to be empirically determined for different tissue types and samples.
- **Default Proteinase K digestion conditions are 1 µg/mL Proteinase K for 15 minutes**. Proteinase K concentration and incubation time may need to be empirically determined for different tissue types and samples.

Cryosectioning technique

This is not meant to be an all inclusive guide on cryosectioning. Please refer to your local pathologist or core facility for training on cryosectioning.

Prior to cryosectioning, read the user manual for the cryostat model being used, making sure to review the safety guidelines. Follow that manual's temperature recommendations for different tissue types. Recommendations should be used as a starting point but may require adjustments for your samples.

1. Set the specimen head temperature following the recommendation noted above. If the cryostat allows the temperature of the blade to be set separately from that of the specimen head, set the blade temperature to be 2°C warmer than the specimen head.
2. Equilibrate the frozen tissue block to the intended cryosectioning temperature, either in the cryostat chamber or in a -20°C freezer (not frost-free).
3. Use OCT to mount the frozen tissue block to the cryostat chuck: dispense a small amount of OCT onto a room temperature chuck and place the tissue block onto the liquid OCT. Keep in mind the desired sectioning plane when mounting the frozen tissue block onto the chuck. To freeze the OCT, place in an environment that is equivalent to or colder than that of the cryosectioning temperature for ~5 minutes. It may be beneficial to first trim the block to facilitate the mounting of cryosections onto the microscope slides. One preferred method of trimming a square or rectangular block is to cut the corners.
4. Cut 5–10 µm-thick sections and mount on Superfrost Plus or BOND Plus microscope slides. Personal preference plays into the technique used for generating sections, and there are many tutorials and resources on the web that discuss how to cryosection. Make sure to mount tissue sections in the GeoMx DSP Scan Area (see diagram on page 90). After the tissue section has been mounted onto the slide, inspect the section, and make sure it does not contain visible wrinkles, which will negatively impact the performance in the workflow. Once mounted, move slide to dry ice and let sit for at least one hour and then store sections in a -80°C freezer for at least 12 hours.

If sections rip or fail to stay intact during sectioning, try adjusting the sectioning temperature by a few degrees. Softer or fat-containing tissue may benefit from colder sectioning temperatures, while tissues with a high water content may benefit from warmer sectioning temperatures.

Appendix III: Automated Protocol for Fixed Frozen Samples

This protocol is for the semi-automated preparation of fixed frozen (FxF) samples for **RNA assays** only.

Tissue that has undergone an initial perfusion-based fixation is preferred, as that process rapidly inactivates endogenous RNases and preserves tissues in a uniform manner. Tissue immersed in fixative post-harvest is also acceptable.

Preparing fixed frozen tissue block from harvested tissue

Reagents required for this protocol: 10% NBF, 10X PBS, DEPC-treated water, 10% Tween-20, sucrose, OCT compound.

Prepare reagents (1 hour)

All buffers should be made with DEPC-treated water to minimize RNase contamination.

- Prepare **30% Sucrose** as follows:
  1. Weigh out 15 g sucrose and place in a 50 mL conical tube.
  2. With a tissue culture-grade disposable pipette, add DEPC-treated water up to the 50 mL mark of the tube.
  3. Centrifuge conical tube.
- Prepare **20% Sucrose** as follows:
  1. Weigh out 10 g sucrose and place in a 50 mL conical tube.
  2. With a tissue culture-grade serological pipette, add DEPC-treated water up to the 50 mL mark of the tube.
  3. Centrifuge conical tube.
- Prepare **10% Sucrose** as follows:
  1. Weigh out 5 g sucrose and place in 50 mL conical tube.
  2. With a tissue culture-grade disposable pipette, add DEPC-treated water up to the 50 mL mark of the tube.
  3. Centrifuge conical tube.

- Prepare **50:50 solution of OCT:30% sucrose** as follows:
    1. As OCT compound is too viscous to pipette, dispense 5 mL of OCT by pouring into a 15 mL conical tube up to the 5 mL mark.
    2. Add 5 mL of 30% sucrose (prepared above) using a tissue culture grade serological pipette.
    3. Mix by inverting several times, then mix using a serological pipette until the two components have formed a homogenous mixture. Be careful not to introduce bubbles.

Prepare fixed frozen tissue block (2 days)

Incubations should be performed in a 50 mL conical tube. The volume of solution for each incubation should exceed the volume of the tissue by a factor of 15. Agitation should be gentle, and the tube should be positioned at an angle such that the entire solution volume is swirled over the tissue with each rotation.

1. After the mouse has been anesthetized (and ideally perfused with fixative) and euthanized according to an IACUC-approved protocol, harvest tissue. Tissue should be harvested quickly but gently using clean dissection tools. It is important to perform this step as quickly as possible to minimize RNA degradation, especially if the tissue has not undergone a perfusion-based fixation step.
2. Cut tissue such that the maximum size in one dimension does not exceed 10 mm. This size restriction enables adequate fixative penetration into the center of the tissue.
3. Fix tissues in 10% NBF at room temperature for 24 hours with gentle shaking.
4. The next day, wash 2 times in 1X PBS for 30 minutes each wash.
5. Incubate in each of the following solutions, in order. For each incubation, shake on ice for 1.5 to 2 hours, or until the tissue sinks to bottom of tube (indicating tissue is adequately equilibrated).
    a. 10% sucrose
    b. 20% sucrose
    c. 30% sucrose
6. While processing tissue in 30% sucrose, prepare a dry ice/ethanol bath. Place an aluminum block with its top exposed in the center of the bath and let chill for 30 minutes.
7. Place tissue in 50:50 OCT:30% sucrose for 30 minutes on ice.
8. Place a few drops of OCT into a Tissue-Tek Cryomold.
9. Place the tissue into the cryomold and cover with OCT, minimizing the formation of bubbles.

10. Incubate for 15 minutes at room temperature.
11. Freeze samples in OCT by placing on the aluminum block in the dry ice/ethanol bath for 10 minutes.
12. Tightly wrap frozen sample in foil and store at -80°C.

Sectioning fixed frozen blocks

The following are general guidelines for sectioning fixed frozen blocks for optimal GeoMx DSP assay performance. See also Cryosectioning technique on page 92

- Always discard the first few sections from the block face.
- NanoString recommends SuperFrost Plus slides (for manual slide preparation) or Apex BOND Adhesive slides (for BOND automated slide preparation, or for manual slide preparation for tissues that are prone to falling off the slide).
- Sections should be cut at 5–10 µm thickness on a calibrated cryostat and mounted immediately on the slide, without scratches or folds.
- Tissue sections must be placed in the **Scan Area** (shown in green) (see Figure 72) in the center of the slide and be **no larger than 35.3 mm long by 14.1 mm wide**. They should not overlap the slide gasket or the Tip Calibration area (shown in red). Tissues that will be prepared using the BOND RX/RXm must also fit within the white dots on the Apex BOND slides. If mounting multiple sections per slide, ensure that tissues are at least 2–3 mm apart and still fit within the Scan Area.

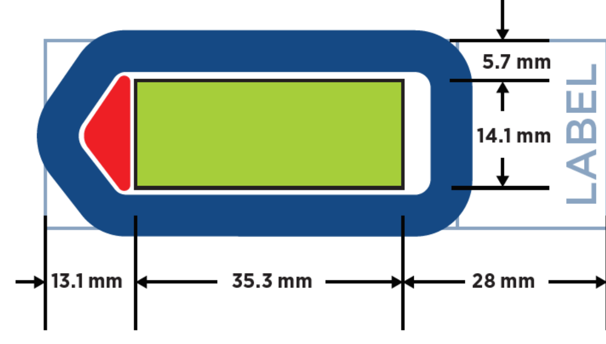

Figure 72: Slide dimensions

IMPORTANT: The GeoMx DSP instrument will only image the area inside the Scan Area. **Tissue outside of the Scan Area will not be imaged and may cause problems in tissue detection by the optical system.**

- After sectioning, the exposed block face should be covered with OCT to avoid desiccation of the sample.
- Slides can be stored at -80°C for several weeks before use.

Required fixed frozen sample preprocessing steps

Reagents required for this protocol: 1X PBS; 50%, 70%, and 100% ethanol solutions, prepared fresh.

All washes should be performed in staining jars treated with RNase Away and rinsed with DEPC-treated water.

1. Remove the fixed frozen tissue slides from -80°C and place face up on a clean surface.
2. Equilibrate to room temperature for 1–2 minutes. Condensation may form as slide warms.
3. Wash in 1X PBS for 5 minutes to remove OCT. Dip slide up and down every ~1 minute.
4. Remove from 1X PBS and use a Kimwipe to wick PBS from the edge of the slide. Place in a rack that orients mounted tissue vertically.

Optional: Bake at 60°C for 30 minutes. Perform this step if tissue is prone to detaching from the slide during slide prep. NanoString has not observed a negative effect from baking on the outcome of the GeoMx RNA assay.

5. Wash the slides in 50% ethanol for 5 minutes.
6. Wash the slides in 70% ethanol for 5 minutes.
7. Wash the slides in 100% ethanol 2 times for 5 minutes each.
8. Let slides air dry for at least 5 minutes (but not more than 1 hour).

Perform HIER and Proteinase K Digestion on the BOND RX

This section describes the steps to create a new protocol on the BOND RX/RX^m specifically for the preparation of fixed frozen samples.

Reagents required for this protocol: 1X BOND Wash Solution; Proteinase K solution (default 1 µg/mL, minimum volume equal to 500 µL per slide plus 500 µL dead volume) in a BOND 6 mL Titration Container.

Reagent setup on the BOND RX/RX^m

If Home Buffer not yet been registered in the system, register it now following the steps in Reagent Setup on page 45.

Register the BOND Research Detection System

1. Remove any containers from the BOND Research Detection System tray.
2. Scan the barcode on the side of the tray.
3. In the **Add research reagent system** window (see Figure 73), enter the information for your reagent system:
   - Name: enter a name or select a previously registered name from the drop-down menu.
   - Lot No.: (optional).
   - Expiration Date: enter expiration date for reagents.
   - Highlight Row 1 and scan the front barcode of a new 30 mL BOND Open Container. Place the container in position 1 of the BOND Research Detection System tray.
   - Select **Home Buffer** from the drop-down menu in the Reagent column.

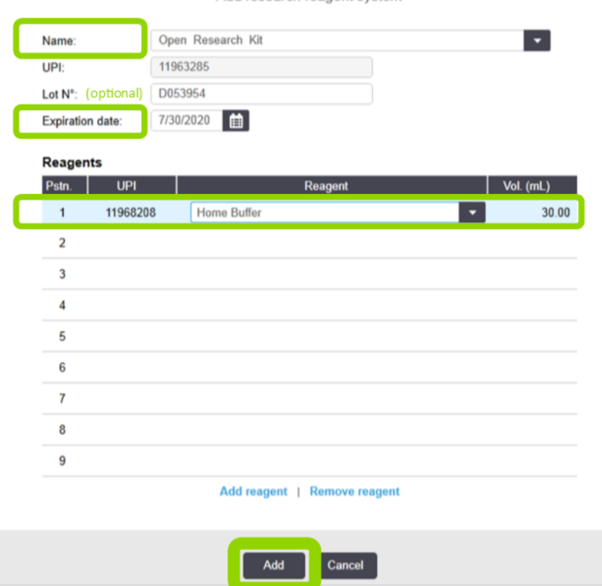

Figure 73: Add research reagent system window

   - Click **Add**.
4. Label the BOND Research Detection System reagent tray to match the name entered in the software. Label the 30 mL Open Container in position 1 with **Home Buffer**.

Create a Fixed Frozen Tissue Staining Protocol

The fixed frozen protocol must be created the first time it is to be used. Once established, it can be used for all fixed frozen samples for GeoMx RNA assays.

1. Click the **Protocol setup** icon (see Figure 74).
2. In the Protocol setup window (see Figure 75), highlight the ***IHC Open Dispense Template row** and click **Copy**.

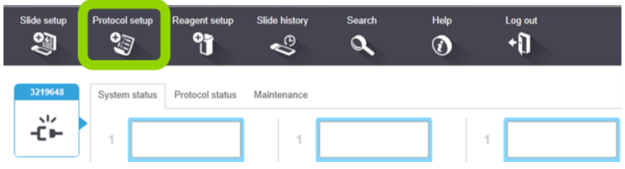

Figure 74: Protocol setup icon

Figure 75: Protocol setup window

3. In the **Edit protocol properties** window (see Figure 76), enter the following information:
   - **Name:** *FxF empty*
   - **Abbreviated Name**: *FxF empt*
   - **Description:** FxF home buffer
   - **Staining method:** Check the **Single**, **First** (or Preliminary), and **Second** boxes
   - **Preferred detection system:** Open Research Kit 1

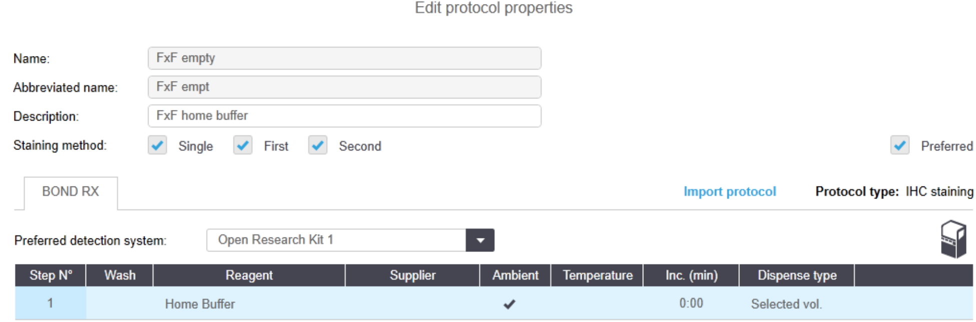

Figure 76: Edit protocol properties for fixed frozen tissue

4. Change the protocol (using **Insert Wash**, **Insert Reagent**, and **Delete Step** buttons), ensuring the **Inc (min)** and **DispenseType** fields are modified to match those in Table 28.

Table 28: Fixed Frozen RNA protocol on BOND RX

Step No.	Wash	Reagent	Supplier	Ambient Temperature	Temp	Inc. (minutes)	Dispense type
1		Home Buffer		✓		0:00	Selected volume

5. Click **Save**, Read the notification and click **Yes**.

Register additional containers for use

1. Label and add the ***Enzyme 1** container:
    1. Insert a BOND 6 mL Titration Container in position 4 of the tray.
    2. Label with **Enzyme 1.*
    3. Scan the front barcode of the Titration Container.
    4. In the **Add open container** window, select ***Enzyme 1** from the Reagent name drop-down menu, enter a **Lot No.** (if required), and enter an **Expiration date**. Click **OK**.

Fill reagent containers

1. **Fill the Home Buffer container in position 1 with 30 mL of 1X BOND Wash solution**. Ensure you have at least 150 µL per slide plus 5 mL of dead volume. Leave the lid open.
2. **Fill a BOND 6 mL Titration Container insert with Proteinase K solution** (default 1 µg/mL (see Table 30), minimum volume equal to 500 µL per slide plus 500 µL dead volume). Place in a BOND 6 mL Titration Container (*Enzyme 1 or other *Enzyme container). Add the container to the reagent tray in position 4.
3. **Load the BOND Research Detection System onto the BOND RX/RX^m^.** The instrument will recognize containers and, depending on your Dip Test settings, may check container volumes. Once finished, you may click on the **Processing Module** icon to view the status of any of the containers in the **System Status** window. Right-click on the desired container and select **Inventory** to view the volume measured in the container. Refill if required.

Add your study and slides

1. Click the **Slide setup** icon (see Figure 77).
2. Click **Add Study** (see Figure 78).

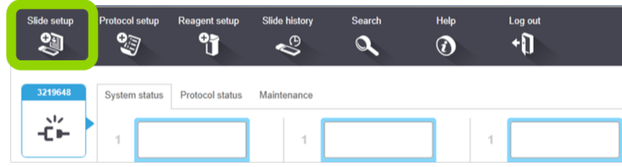

Figure 77: Slide setup icon

3. In the **Add Study** window (see Figure 79), enter your study information:
    - **Study ID:** (required).
    - **Study name:** (required).
    - **Study comments:** (optional).
    - **Researcher:** (optional, refer to the BOND RX user manual to add new researchers).
    - **Dispense volume:** 150 µL
    - **Preparation protocol:** Select ***- - - -** from the drop-down list.
    - Click **OK**.

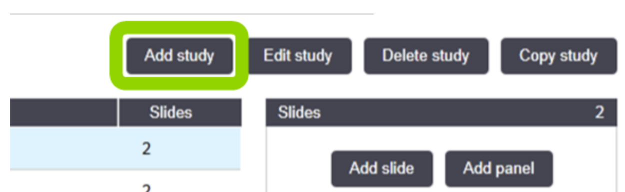

Figure 78: Add study button

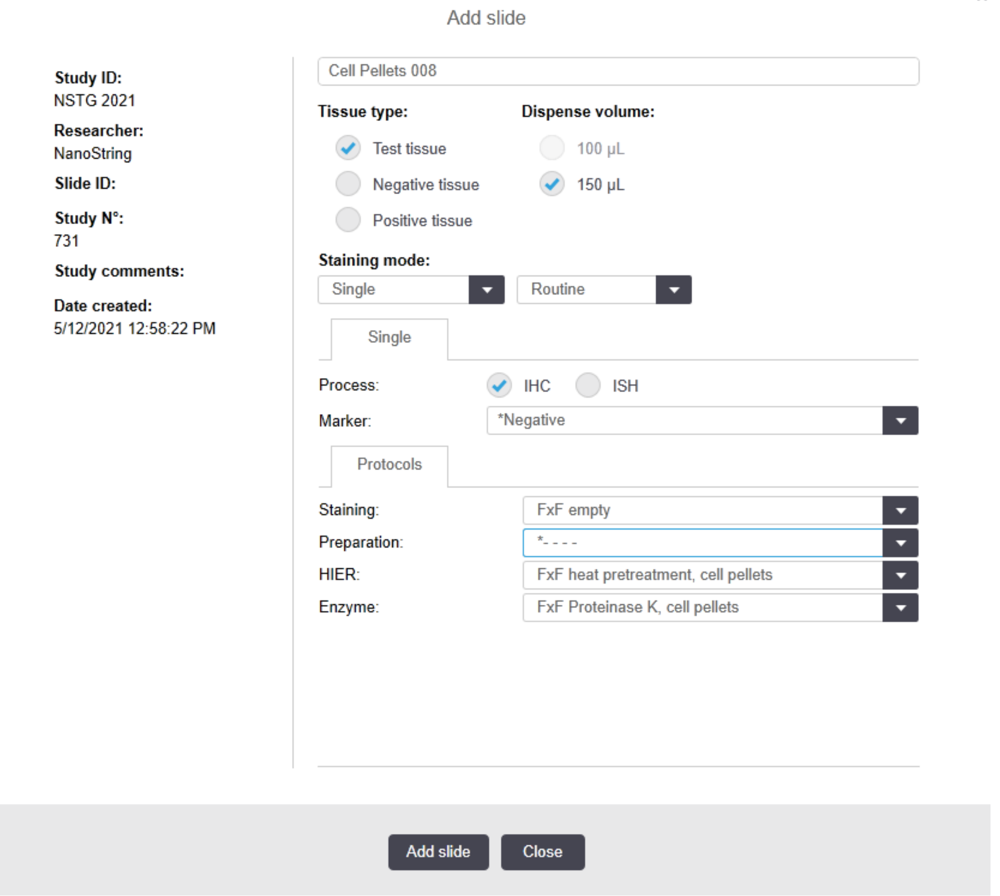

Figure 79: Add slide window - Fixed Frozen protocol

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4. Highlight your study and click Add slide.

   a. In the Add slide window, enter your slide information:

      - Enter **Slide Comments**.
      - **Tissue type:** Check **Test tissue**
      - **Dispense volume:** Check **150 µL**
      - **Staining mode:** Select **Single** and **Routine** from the drop-down lists
      - **Process:** Check **IHC**
      - **Marker:** Select ***Negative** from the drop down list

   b. On the Protocols tab:

      - **Staining:** Select **FxF empty**
      - **Preparation:** Select ***- - - -**
      - **HIER**: Select a pre-defined HIER protocol from the drop-down list or select the appropriate incubation time based on **Table 29**. Incubation times and temperatures may differ by tissue and may need to be empirically determined. To create a new protocol, see NOTE below **Table 29**.

Table 29: Target retrieval times and temps by FxF tissue type—BOND slide prep

Tissue Type	Target Retrieval
Brain	*HIER 15 min with ER2 @ 95°C
Cell pellets	*HIER 10 min with ER2 @ 85°C
Heart	*HIER 10 min with ER2 @ 100°C
Kidney	*HIER 10 min with ER2 @ 100°C
Liver	*HIER 10 min with ER2 @ 100°C
Lung	*HIER 10 min with ER2 @ 100°C
Spleen	*HIER 10 min with ER2 @ 100°C

NOTE: To create a new protocol with different HIER conditions, click the Protocol Setup icon (see Figure 74). Find an existing *HIER protocol, copy it, and modify the conditions as needed. Save with a unique name and abbreviated name. Check the checkbox for “Preferred” protocol.

      - **Enzyme:**Select a pre-defined enzyme protocol from the drop-down list or select *Enzyme1 and the appropriate incubation time based on **Table 30**. Proteinase K concentration and incubation times differ by tissue and may need to be empirically determined. To create a new protocol, see NOTE below **Table 30**.

Table 30: Proteinase K digest concentrations and times by FxF tissue type

Tissue Type	Proteinase K Digest
Cell pellets	1 µg/mL for 5 min
All others	1 µg/mL for 15 min

NOTE: To create a new Proteinase K protocol, click the Protocol Setup icon (see Figure 74). Find the existing *Enzyme 1 for 15 minutes protocol, copy it, and modify the conditions as needed. Save with a unique name and abbreviated name. Check the checkbox for "Preferred" protocol.

c. Click **Add Slide**. Continue to click Add slide until you have sufficient labels for all of your slides. Change **Slide Comments** as needed.

d. Click **Close**.

e. Click **Print Labels**.

f. In the **Print slide labels** window, select **All slides not yet printed for current study** and click **Print**.

g. Place the labels on the same side of the slides as the samples, aligning them at the top of the slide.

Load and run your slides

1. Check that all bulk containers are at least half-full and that the bulk waste container is empty.
2. Referencing the embossed image on the slide tray, **load the slides onto the slide tray** in the correct orientation (see Figure 80). Ensure the sample and label are facing upwards, with the label at the top of the slide tray.
3. **Place covertiles** on top of the slides with the tab at the bottom and *Leica* facing upwards.

Figure 80: Slides in slide rack

4. Gently load the slide tray(s) with slides and covertiles onto the BOND RX/RX^{m}; ensure that none of the slides or covertiles are dislodged.

Complete the run

1. Once the run has finished, the LED button below the slide tray will blink green. Press the LED button to raise the slide tray.
2. Gently **slide the slide tray out** of the instrument and place on a flat, stable surface.
3. Handle slides one at a time so that they do not dry out. Remove each covertile by holding down the label of the slide, then carefully putting pressure downwards on the neck of the covertile to lift the end of the covertile off the slide. Do not slide the covertile across the surface of the slide, as you may damage the tissue. **Place slide in 1X PBS** then proceed to **In situ hybridization (overnight) on page 57**.
4. Clean covertiles following the BOND RX/RX^{m} User Manual.

Appendix IV: Substitute Probe R Guidance

For nCounter readout: This information applies only to GeoMx Protein assays with nCounter readout.

A Core protein panel can be run with up to 6 Modules at once. Core and Modules must all fall within the same group (e.g., Human IO, Mouse IO, or Human Neuroscience).

Each panel is assigned a Probe R space for nCounter readout (see Table 31). **Do not combine two modules with a common Probe R space (i.e. same Probe R_number) in the same experimental run, or the data cannot be interpreted.** Substitute Probe Rs are available from NanoString to allow the combination of modules that share Probe R space, such as MAPK Signaling and Immune Cell Typing.

IO Core and Modules

Table 31: Protein panels and their corresponding Probe R space

Panel	Probe R space	Substitute Probe Rs available
Immune Cell Profiling Core	IO R_1	
IO Drug Target	IO R_2	
Immune Activation Status	IO R_3	
Immune Cell Typing	IO R_4	
MAPK Signaling	IO R_4	IO R_2 IO R_3 IO R_5 IO R_6 IO R_7
Pan-Tumor	IO R_5	
Cell Death	IO R_6	
PI3K/AKT Signaling	IO R_7	
Custom	IO R_8 IO R_9	

Neuroscience Core and Modules

As of March 2022, all Human Neuroscience modules are compatible with one another, and all Mouse Neuroscience modules are compatible with one another, without Substitute Probe R.

Appendix V: Adding Custom Barcoded Antibodies

These instructions were developed for custom barcoded antibodies obtained through **NanoString's Protein Barcoding Service** or prepared with the NanoString **GeoMx Protein Barcoding Kit**. For custom barcoded antibodies obtained from Abcam, skip steps 1–2 and begin at step 3. These instructions do not apply to morphology marker antibodies.

NOTE: For users of the GeoMx Protein Barcoding Kit, do not add the kit's control antibody to the GeoMx DSP assay.

1. Dilute the custom-barcoded antibody from its stock solution by adding **2 µL antibody** to **6 µL** of **Buffer W**.
2. **Pipette up and down** at least 10 times to mix thoroughly.
3. **Add custom-barcoded antibody to the working antibody solution** prepared in **Table 6 on page 33****:**

   Volume to add to working antibody solution = (1.1 µL x n), where n = number of slides

> For barcoded antibodies that required a higher concentration to pass the post-conjugation IHC QC test, add (2.2 µL x n), where n = number of slides, to the working antibody solution. See the NanoString whitepaper **Selection and Validation of GeoMx Custom Antibody Spike-ins (MK3600)** for details.

4. Store diluted antibody at 4°C and discard after 2 weeks.

Appendix VI: RNAscope® and GeoMx RNA Assays using BOND RX

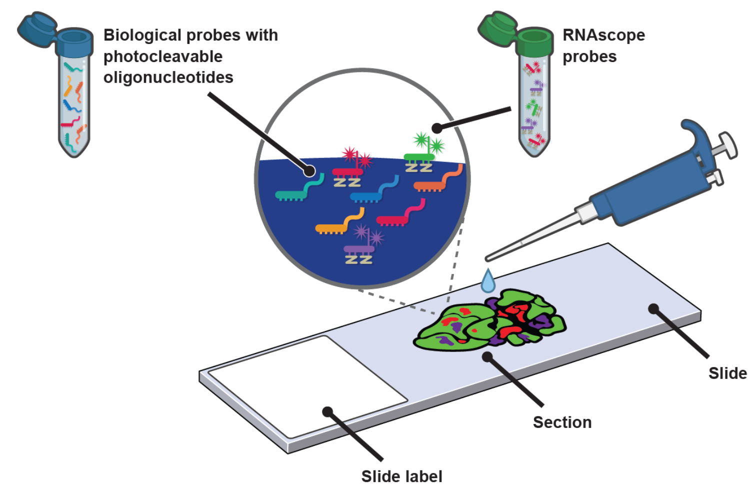

Figure 81: RNAscope probes applied to RNA slide

IMPORTANT: RNAscope can be used to help set the location of ROIs (i.e., place ROIs where a particular RNA is enriched). However, the punctate signals from **most** RNAscope probes are not compatible with the segmentation algorithm on the GeoMx, meaning that the GeoMx may not be able to segment based on the RNAscope signal. Segmentation may be possible with highly abundant RNAscope targets, so it is recommended to test the segmentation tools with an RNAscope probe of interest in advance of your GeoMx experiments. If segmenting is not supported, consider using RNAscope in 1-2 channels and fluorescent antibody morphology markers in the other channel(s) to define the segments. Alternatively, prepare serial sections, one with RNAscope and one with fluorescent antibody morphology markers. During ROI selection on the antibody-labeled slide, overlay the image of the RNAscope slide to guide ROI selection. Proceed with segmentation and collection from the antibody-labeled slide.

The following protocol applies only to slide preparation for GeoMx **RNA assays** using BOND RX/RX^{m}. To use RNAscope with **protein** assays, prepare serial sections and use the Image Overlay feature during ROI selection (see **GeoMx DSP Instrument User Manual** (MAN-10152)). Overlay the RNAscope image over the slide labeled with protein assay reagents to guide ROI selection.

1. Follow the RNAscope automated assay protocol provided by ACD (322800-USM), with these modifications and considerations:

- Do not use DAPI for DNA staining. Register a new BOND 30 mL Open Container as 'DAPI' but fill it with DEPC-treated water; llabel the container as "fake DAPI" or similar and place it in the DAPI slot of the reagent tray. This allows the BOND RX/RXm to run the RNAscope protocol without staining your slides with DAPI. (DAPI is not used in GeoMx workflows since the UV light needed to visualize DAPI would cleave GeoMx barcodes).
- Refer to Table 32 for recommended TSA fluorophore dilutions.

Table 32: TSA fluorophore dilutions

Fluorescence	Product number (PerkinElmer)	Recommended dilution
PerkinElmer TSA Plus Cyanine 3 System	NEL744001KT	1:1500
PerkinElmer TSA Plus Cyanine 5 System	NEL745001KT	1:3000

Dilution of TSA fluorophores may require optimization by target and tissue type. Refer to manufacturer documentation for additional details and guidance.

- Total run time for the automated RNAscope assay is 10–14 hr depending on the number of fluorophores and slides. Therefore, it may be ideal to run the BOND RX/RXm RNAscope protocol overnight.

2. Upon completion of the BOND RX/RXm RNAscope protocol, carefully remove slides from tray and remove covertiles from slides (see unloading instructions on page 56). Place slides in deionized water.

If your BOND RX/RXm RNAscope protocol did not include post-fixation steps (due to the maximum number of steps allowed on a single protocol), your samples may benefit from a post-fixation treatment at this point. Incubate slides as follows (at room temperature): 5 min in 10% NBF, 2 quick washes in 1X PBS, 5 min in 1X PBS, 5 min in NBF Stop Buffer, 2 quick washes in 1X PBS, then 5 min in 1X PBS. This is offered as guidance and has not yet been validated.

3. Proceed to step 6 of the Semi-Automated RNA Slide Preparation protocol, ***In situ* hybridization** on page 57 . After preparing your reagents and the hybridization chamber, you will prepare the hybridization solution for overnight incubation. Follow the remainder of the Semi-Automated RNA Slide Preparation protocol with these considerations:
   - If additional morphology markers are used in addition to RNAscope, ensure that RNAscope dyes and morphology marker fluorophores do not overlap in emission spectra.
   - When scanning slides labeled with RNAscope on the GeoMx DSP, optimal exposure times may need to be empirically determined. NanoString recommends setting 200 ms exposure time as a starting point.

Appendix VII: Secondary Antibody Immunofluorescence Staining for RNA Assays

Secondary antibody immunofluorescence staining is a viable alternative to using fluorescently conjugated primary antibodies in GeoMx RNA assays. It is **not viable** for protein assays since the secondary antibody may target the GeoMx DSP detection probe antibodies.

This protocol incorporates secondary antibody immunofluorescence staining to the **semi-automated** RNA slide preparation (FFPE) protocol. It may require optimization to minimize cross-reactivity between primary and secondary antibodies. NanoString recommends running a pilot experiment before completing this protocol with your experimental samples and probe mix.

The following secondary antibodies are recommended for this protocol:

Table 33: Recommended secondary antibodies

Target	Channel	Host	Source	Part No.
Mouse	647	Goat	Thermo Fisher	A21236
Mouse	594	Goat	Thermo Fisher	A11032
Rabbit	647	Goat	Thermo Fisher	A21245
Rabbit	594	Goat	Thermo Fisher	A11037

This protocol extends Day 2 of Slide Preparation for RNA Assays by about 3 hours, prior to loading on the GeoMx DSP instrument.

1. Follow the Semi-Automated RNA FFPE Slide Preparation protocol up to and including Blocking with Buffer W on page 61.
2. During blocking, prepare solution of **primary antibody (without fluorescent label) diluted in Buffer W**. Follow the vendor IHC recommended concentration (typically 1–10 µg/mL) or rely on empirical testing with the sample tissue and antibody of choice. Prepare 220 µL per slide.
3. Incubate slide(s) for **1 hr** (minimum) with **200 µL** of diluted primary antibody solution made in step 2, in humidity chamber at room temp. If your fluorescently conjugated primary antibodies are of a different host species than the unconjugated primary antibody, they can be added at this step along with the nuclear stain, and you can skip Step 8 below.
4. Wash slide(s) in **2X SSC** for 1 min, then wash in **2X SSC** 4 times for 3 min each.
5. Prepare solution of secondary antibody (with fluorescent label) diluted to 5 µg/mL (1:400 for secondary antibodies listed in Table 33) in Buffer W. Secondary antibody target must match host of primary antibody.

6. Incubate slide(s) for 30 min with **200 µL** of diluted secondary antibody solution made in step 5, in humidity chamber at room temp.
7. Wash slide(s) in fresh **2X SSC** for 1 min, then wash in **2X SSC** 4 times for 3 min each. Wash steps are critical to avoid high background staining.
8. If your fluorescently conjugated primary antibodies are of the same host species as the unconjugated primary antibody:
   a. Perform an additional **Blocking** step using goat- or other host species–specific serum for 30 min.
   b. Wash slide(s) in fresh **2X SSC** for 1 min, then wash in **2X SSC** 4 times for 3 min each.
   c. Incubate for **1 hr** (minimum) with your fluorescently conjugated primary antibodies and nuclear stain diluted in Buffer W, as described in step 4 on page 61.
   d. Wash slide(s) in fresh **2X SSC** for 1 min, then wash in **2X SSC** 4 times for 3 min each.
9. The slides are ready to be loaded on the GeoMx DSP instrument or stored (see Safe storage guidelines for RNA slides on page 62).

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Appendix VIII: Tyramide Signal Amplification (TSA) of Morphology Markers using BOND RX

TSA can be used to enhance a weak immunolabeling signal and provides an alternative to directly conjugated fluorescent antibody morphology markers in the GeoMx DSP workflow. It may also be preferable if the epitope recognized by a directly conjugated fluorescent antibody is protease sensitive and doesn't perform well with GeoMx RNA assays. (This protocol moves the Proteinase K digestion step to follow immunolabeling, in contrast to the standard RNA assay workflow).

This protocol applies to TSA in combination with **GeoMx RNA assays** prepared with the **semi-automated protocol using BOND RX/RX^{m}**.

For TSA in combination with GeoMx protein assays, please refer to the manual method in GeoMx DSP Manual Slide Preparation User Manual (MAN-10150).

Overview

FFPE tissue sections are baked, dewaxed, and subjected to HIER (but not Proteinase K digestion). Endogenous fluorescence and HRP activity is quenched with hydrogen peroxide, and immunolabeling is performed with a primary, unconjugated antibody. The primary antibody is then labeled with an HRP-conjugated secondary antibody, which in turn deposits an amplified signal on the tissue using a Cy3 or Cy5 amplification reagent. After HRP inactivation, the tissue is permeabilized with Proteinase K and the sample is ready for *in situ* hybridization.

Fluorophore selection

Both Cy3 and Cy5 are compatible with this protocol. Cy5 is preferred to minimize the potential for signal bleedthrough into other visualization channels of the GeoMx DSP, especially if other morphology markers labeled with Alexa Fluor 594 are used.

Primary antibody selection

NanoString has only tested primary antibodies raised in mouse and rabbit.

Additional reagents required for this protocol

Table 34: Reagents for TSA and RNA Assays on BOND RX/RXm

Reagent	Source/Part Number	Storage
Primary IgG antibody from rabbit or mouse	Various	-20°C
Anti-rabbit or anti-mouse HRP conjugated secondary antibody (choose the antibody appropriate for the selected primary)	Invitrogen Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody HRP (G-21234) Invitrogen Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, HRP (G-21040)	-20°C
TSA Plus Cyanine 3 System **OR** TSA Plus Cyanine 5 System Amplification Reagent (AR)	Akoya Cat No. NEL744001KT Akoya Cat No. NEL745001KT	4°C in dark
30% H_2O_2 stabilized with stannate and organophosphate	Sigma H1009	4°C

Prepare Reagents

Prepare the reagents using the dilution instructions **(see Table 35)**. Use DEPC-treated water for all dilutions and reconstitutions. Buffers can be stored in BOND Open containers at 4°C unless otherwise noted.

> **IMPORTANT:** Take care to maintain nuclease-free conditions. The greatest risk of contamination comes from GeoMx probes and other oligos. We recommend the use of RNase AWAY (Thermo Fisher 7002), as it will limit contamination from oligos, GeoMx probes, and nucleases. After using RNase AWAY, allow to air dry completely, or rinse with DEPC-treated water. See manufacturer's instructions for details.

Table 35: Prepare reagents for TSA for RNA on BOND RX/RXm protocol

Reagent	Dilution	Storage
Anti-rabbit or anti-mouse HRP-conjugated secondary antibody	Reconstitute to 1 mg/ml in 1X PBS (**in single-use aliquots**, do not freeze-thaw)	4°C (anti-rabbit) -20°C (anti-mouse)
Cyanine 3 or Cyanine 5 amplification reagent (AR)	Reconstitute as specified by the manufacturer	4°C

Reagent setup on the BOND RX/RXm

If the reagents in Table 36 are not yet registered in the system, you will need to add them.

Table 36. BOND reagents for TSA with RNA assays

Reagent	BOND Container type	BOND Container Designation
Buffer W	BOND 6mL Titration Container	Buffer W
Primary Ab	BOND 6mL Titration Container	Tube 2
Secondary Ab	BOND 6mL Titration Container	Tube 3
Cyanine dye AR	BOND 6mL Titration Container	Tube 4
0.3% H_2O_2	BOND 6mL Titration Container	Tube 5
Proteinase K	BOND 6mL Titration Container	Tube 6
3% H_2O_2	BOND 30 mL Open Container	Open Container 2

1. Select the **Reagent Setup** icon (see Figure 82).

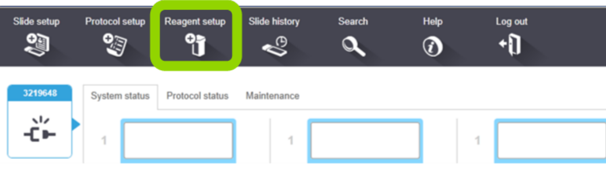

Figure 82: Reagent setup icon

2. From the **Setup** tab, click the **Add** button (see Figure 83).

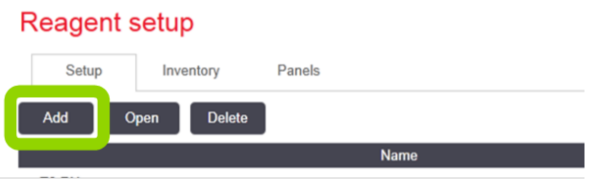

Figure 83: Add reagent button

3. In the **Add Reagent** window, enter a unique name in the **Name** field.
4. Enter a unique abbreviated name in the **Abbreviated Name** field (up to eight characters).
5. If applicable, select the **Hazardous** check box.
6. Click **Save**, then **OK**.
7. Repeat these steps to register all of the reagents listed in Table 36.

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Register the BOND Research Detection System

1. **Remove any containers** from the BOND Research Detection System tray.
2. **Scan the barcode** on the side of the tray (see Figure 84).
3. In the **Add research reagent system** window (see Figure 85), enter the information for your reagent system.
   - Enter a **Name** for your BOND Research Detection System (if one has been previously registered, select the name from the drop down menu).

Figure 84: Research detection system barcode

   - Enter the **Lot No.** (optional) and an **Expiration Date**.
   - Highlight **Row 1** and **scan the front barcode of a new 30 mL BOND Open Container**. Place the container in position 1 of the BOND Research Detection System tray.
   - Select **Home Buffer** from the drop down menu in the Reagent column.
   - Click **Add**.

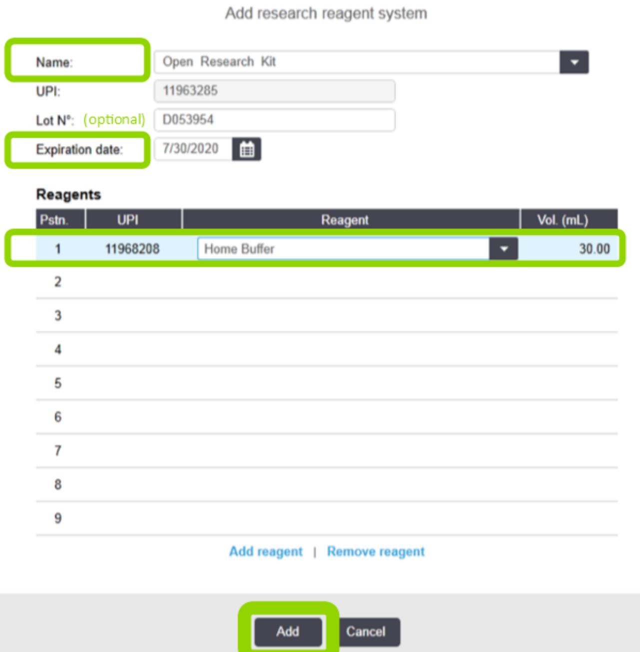

Figure 85: Add research reagent system window

4. Label the BOND Research Detection System reagent tray to match the name entered in the software. Label the 30 mL Open Container in position 1 with **Home Buffer**.

BOND Single Antibody IF/TSA protocol setup

The IF/TSA protocol must be created the first time it is to be used. Once established, it can be used for all RNA slides that are prepared with IF/TSA.

1. Click the **Protocol setup** icon (see Figure 86).
2. In the Protocol setup window, highlight the ***IHC Open Dispense Template row** and click **Copy**.

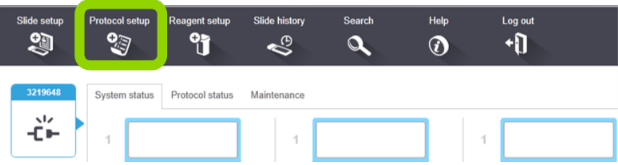

Figure 86: Protocol setup icon

3. In the **Edit protocol properties** window (see Figure 87), enter the following information:
   - **Name:** *IFTSA*
   - **Abbreviated Name**: *IFTSA*
   - **Description:** TSA compatible with ISH
   - **Staining method:** Check the **Single** and **First** (or Preliminary) boxes
   - **Preferred detection system:** Open Research Kit 1

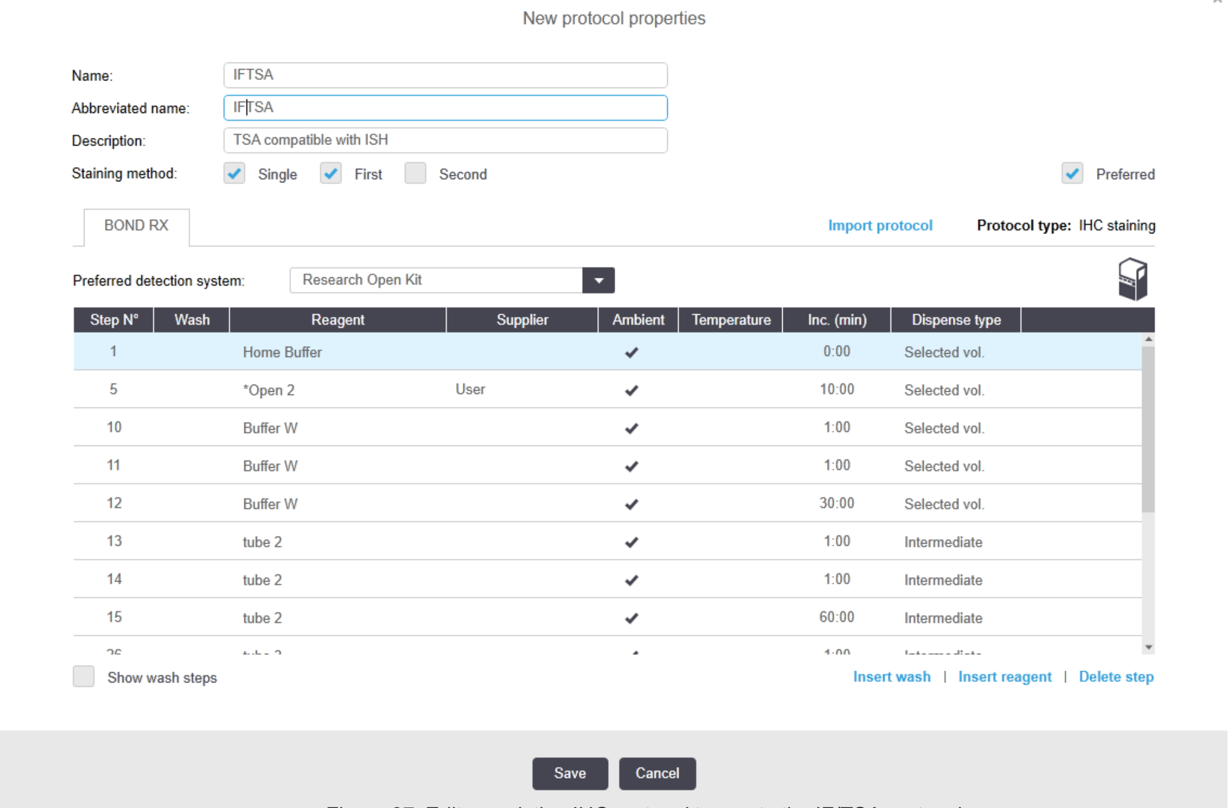

Figure 87. Edit an existing IHC protocol to create the IF/TSA protocol

4. Change the protocol (using **Insert Wash**, **Insert Reagent**, and **Delete Step** buttons), ensuring the **Inc (min)** and **DisenseType** fields are modified to match those in Table 37.

5. Click **Save**, Read the notification and click **Yes**.

Table 37: BOND RNA IF/TSA protocol

Step No.	Wash	Reagent	Supplier	Ambient Temperature	Temp.	Inc. (min)	Dispense type
1		Home Buffer	Leica Microsystems	✓		0:00	sel volume
2	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	sel volume
3	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	sel volume
4	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	sel volume
5		*Open 2 (3% H_2O_2)	User	✓		10:00	sel volume
6	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	sel volume
7	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	open
8	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	sel volume
9	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	sel volume
10		Buffer W	NanoString	✓		1:00	sel volume
11		Buffer W	NanoString	✓		1:00	sel volume
12		Buffer W	NanoString	✓		30:00	sel volume

Step No.	Wash	Reagent	Supplier	Ambient Temperature	Temp.	Inc. (min)	Dispense type
13		Tube 2 (Primary Ab)	NanoString	✓		1:00	intermed.
14		Tube 2 (Primary Ab)	NanoString	✓		1:00	intermed.
15		Tube 2 (Primary Ab)	NanoString	✓		60:00	intermed.
16	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	sel volume
17	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	sel volume
18	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	sel volume
19	✓	*BOND Wash Solution	Leica Microsystems	✓		1:00	sel volume
20	✓	*BOND Wash Solution	Leica Microsystems	✓		1:00	sel volume
21	✓	*BOND Wash Solution	Leica Microsystems	✓		1:00	sel volume
22	✓	*BOND Wash Solution	Leica Microsystems	✓		1:00	sel volume
23	✓	*BOND Wash Solution	Leica Microsystems	✓		1:00	sel volume
24	✓	*BOND Wash Solution	Leica Microsystems	✓		1:00	open
25	✓	*BOND Wash Solution	Leica Microsystems	✓		1:00	sel volume
26		Tube 3 (Secondary Ab)	NanoString	✓		1:00	intermed.
27		Tube 3 (Secondary Ab)	NanoString	✓		1:00	intermed.
28		Tube 3 (Secondary Ab)	NanoString	✓		30:00	intermed.
29	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	sel volume
30	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	sel volume
31	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	sel volume
32	✓	*BOND Wash Solution	Leica Microsystems	✓		1:00	sel volume
33	✓	*BOND Wash Solution	Leica Microsystems	✓		1:00	sel volume
34	✓	*BOND Wash Solution	Leica Microsystems	✓		1:00	sel volume
35	✓	*BOND Wash Solution	Leica Microsystems	✓		1:00	open
36	✓	*BOND Wash Solution	Leica Microsystems	✓		1:00	sel volume
37		Tube 4 (Cyanine dye AR)		✓		1:00	sel volume
38		Tube 4 (Cyanine dye AR)		✓		30:00	sel volume
39	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	sel volume
40	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	sel volume
41	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	sel volume
42	✓	*BOND Wash Solution	Leica Microsystems	✓		1:00	sel volume
43	✓	*BOND Wash Solution	Leica Microsystems	✓		1:00	sel volume
44	✓	*BOND Wash Solution	Leica Microsystems	✓		1:00	open
45	✓	*BOND Wash Solution	Leica Microsystems	✓		1:00	sel volume

Step No.	Wash	Reagent	Supplier	Ambient Temperature	Temp.	Inc. (min)	Dispense type
46		Tube 5 (0.3% H_2O_2)		✓		1:00	sel volume
47		Tube 5 (0.3% H_2O_2)		✓		15:00	sel volume
48	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	sel volume
49	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	sel volume
50	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	sel volume
51	✓	*BOND Wash Solution	Leica Microsystems	✓		1:00	sel volume
52	✓	*BOND Wash Solution	Leica Microsystems	✓		1:00	sel volume
53	✓	*BOND Wash Solution	Leica Microsystems	✓		1:00	open
54	✓	*BOND Wash Solution	Leica Microsystems	✓		1:00	sel volume
55		Tube 6 (Proteinase K)			37	0:00	sel volume
56		Tube 6 (Proteinase K)			37	15:00	sel volume
57	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	sel volume
58	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	sel volume
59	✓	*BOND Wash Solution	Leica Microsystems	✓		0:00	sel volume
60	✓	*BOND Wash Solution	Leica Microsystems	✓		1:00	sel volume
61	✓	*BOND Wash Solution	Leica Microsystems	✓		1:00	sel volume
62	✓	*BOND Wash Solution	Leica Microsystems	✓		1:00	sel volume
63	✓	*BOND Wash Solution	Leica Microsystems	✓		1:00	open
64	✓	*BOND Wash Solution	Leica Microsystems	✓		5:00	sel volume

Fill reagent containers

1. Fill the Home Buffer container in position 1 with 30 mL of 1X BOND Wash solution the first time you run it and as needed. Ensure you have at least 150 µL per slide plus 5 mL of dead volume. Leave the lid open.
2. Calculate the total amount of each reagent needed ((required reagent volume per slide, from Table 38 x number of slides) + dead volume of 300 µL).
3. Prepare each reagent in sufficient volume and to the specified concentration and place in its respective BOND 6 mL Titration Container.

Table 38: Reagents for the BOND containers

Reagent	Concentration	Volume per slide (µL)
Buffer W	Undiluted	450
Primary Ab	1–5 µg/ml in Buffer W	450
Secondary Ab	10 µg/ml in Buffer W	450
Cyanine dye Amplification Reagent (AR)	1:250 in 1X Plus Amplification Diluent	300
Proteinase K	1 µg/ml in 1X PBS**	300
0.3% H_2O_2	Dilute from 3% stock in DEPC water	300
3% H_2O_2	Dilute from 30% stock in water	150

**Adjust the Proteinase K concentration according to tissue type as detailed in Table 14 on page 54.

4. **Load the BOND Research Detection System onto the BOND RX/RX^m^.** The instrument will recognize containers and, depending on your Dip Test settings, may check container volumes. Once finished, you may click on the **Processing Module** icon to view the status of any of the containers in the **System Status** window. Right-click on the desired container and select **Inventory** to view the volume measured in the container. Refill if required.

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nanoString

Add your study and slides

1. Click the **Slide setup** icon (see Figure 88).

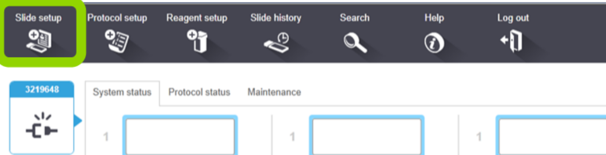

Figure 88: Slide setup icon

2. Click **Add Study** (see Figure 89).

3. In the **Add Study** window (see Figure 90), enter your study information:

Figure 89: Add study button

- **Study ID:** (required).
- **Study name:** (required).
- **Study comments:** (optional).
- **Researcher:** (optional, refer to the BOND RX user manual to add new researchers).
- **Dispense volume:** 150 µL
- **Preparation protocol:** Select ***Bake and Dewax** from the drop-down list.
- Click **OK**.

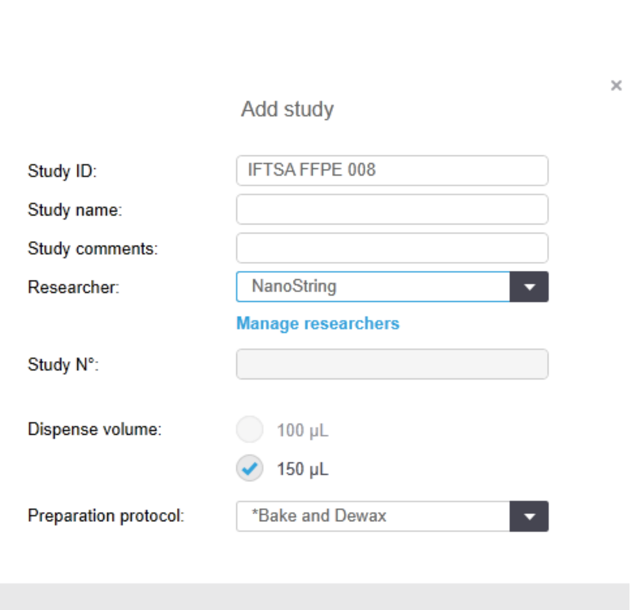

Figure 90. Add the IF/TSA study

4. Highlight your study and click Add slide.

   a. In the Add slide window (see Figure 91), enter your slide information:

      - Enter **Slide Comments**.
      - **Tissue type:** Check **Test tissue**
      - **Dispense volume:** Check **150 µL**
      - **Staining mode:** Select **Single** and **Routine** from the drop-down lists
      - **Process:** Check **IHC**
      - **Marker:** Select ***Negative** from the drop down list

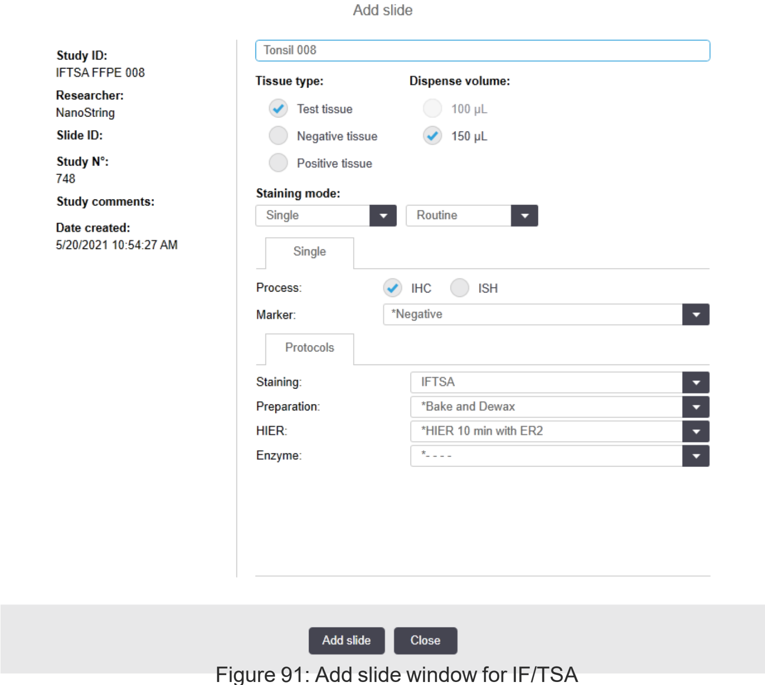

Figure 91: Add slide window for IF/TSA

   b. On the Protocols tab:

      - **Staining:** Select **IFTSA**
      - **Preparation:** Select ***Bake and Dewax**
      - **HIER**: Select a pre-defined HIER protocol from the drop-down list or select the appropriate incubation time based on Table 39. Incubation times and temperatures may differ by tissue and may need to be empirically determined. To create a new protocol, see NOTE below Table 39.

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Table 39: Target retrieval times and temps - IF/TSA

Tissue Type	Target Retrieval
Brain	*HIER 15 min with ER2 @ 95°C
Cell pellets	*HIER 10 min with ER2 @ 85°C
Heart	*HIER 10 min with ER2 @ 100°C
Kidney	*HIER 10 min with ER2 @ 100°C
Liver	*HIER 10 min with ER2 @ 100°C
Lung	*HIER 10 min with ER2 @ 100°C
Spleen	*HIER 10 min with ER2 @ 100°C

NOTE: To create a new protocol with different HIER conditions, click the Protocol Setup icon (see Figure 86). Find an existing *HIER protocol, copy it, and modify the conditions as needed. Save with a unique name and abbreviated name. Check the checkbox for "Preferred" protocol.

- **Enzyme:** Select *---- from the drop-down list. (The enzyme step is programmed into Step 55 and 56 of the TSA Protocol defined above).

c. Click **Add Slide**. Continue to click Add slide until you have sufficient labels for all of your slides. Change **Slide Comments** as needed.

d. Click **Close**.

e. Click **Print Labels**.

f. In the **Print slide labels** window, select **All slide labels not yet printed for current study** and click **Print**.

g. Place the labels on the same side of the slides as the samples, aligning them at the top of the slide.

Load and run your slides

1. Check that all bulk containers are at least half-full and that the bulk waste container is empty.
2. Referencing the embossed image on the slide tray, **load the slides onto the slide tray** in the correct orientation (see Figure 92). Ensure the sample and label are facing upwards, with the label at the top of the slide tray.
3. **Place covertiles** on top of the slides with the tab at the bottom and *Leica* facing upwards.

Figure 92: Slides in slide rack

4. Gently load the slide tray(s) with slides and covertiles onto the BOND RX/RX^{m}; ensure that none of the slides or covertiles are dislodged.

Complete the run

1. Once the run has finished, the LED button below the slide tray will blink green. Press the LED button to raise the slide tray.
2. Gently **slide the slide tray out** of the instrument and place on a flat, stable surface.
3. Handle slides one at a time so that they do not dry out. Remove each covertile by holding down the label of the slide, then carefully putting pressure downwards on the neck of the covertile to lift the end of the covertile off the slide. Do not slide the covertile across the surface of the slide, as you may damage the tissue. **Place slide in 1X PBS** then proceed to **In situ hybridization (overnight) on page 57**.
4. Clean covertiles following the BOND RX/RX^{m} User Manual.

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Troubleshooting

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

Issue	Possible Cause	Suggested Actions
Equipment, materials, or reagents are not available	Supply chain issues or regional variability	Contact GeoMxSupport@nanostring.com or your Applications Scientist.
Tissue on slide extends beyond boundaries of scan area (see diagram **on page 19**)	Tissue slides were prepared outside of GeoMx guidance	Perform slide preparation steps as usual. Just before loading on instrument, scrape excess tissue away using a sharp, clean razor blade.
Coverslip is difficult to remove	Mounting medium evaporated or was insufficient in volume	Soak slides in 1X TBS-T with gentle agitation. Gently lift a corner of the coverslip with forceps.
Probe mix evaporated or did not stay within the barrier during overnight incubation	Improper conditions in humidity chamber or broken hydrophobic barrier (in protein workflow)	Check whether the entire tissue section still has liquid in contact with it. Areas that dried may lose signal. Check conditions of humidity chamber with a test slide and mock probe mix to prevent evaporation from reoccurring.
Tissue falls off slide	Tissue did not adhere sufficiently during baking	Confirm you are using Leica BOND Plus Slides. Extending slide baking time, up to overnight at 37°C.
Some sections of tissue are not in focus	Tissue may be separating from the slide in places or have folds	See above “Tissue falls off slide”. Ensure tissue does not have folds.

Issue	Possible Cause	Suggested Actions
Fluorescent signal is not consistent across the tissue sample	Inconsistent tissue thickness	Ensure sections are cut with even thickness and don't have folds.
Streaks in scanned image	Smudges on bottom of glass slide	Clean bottom of slide with 70% ethanol and a Kimwipe thoroughly before and/or after loading in slide tray.
Morphology marker signal is weak	Morphology marker is simply a weak marker	Try adjusting exposure time on the GeoMx DSP (up to 300 ms per channel; not more) or increasing the intensity using Render Settings in the Scan Workspace.
	Conditions have not been experimentally optimized	Optimize staining conditions by testing different antibody concentrations and/or incubation times following guidance in literature or previous experience with immunohistochemistry. For the RNA workflow, in certain cases, Proteinase K concentration may need to be adjusted.
Bright speckled spots in all channels across tissue	Autofluorescence from red blood cells (RBC) or dying cells	During segmentation, try adding a positive requirement for the nuclear stain signal to exclude RBC, or a negative requirement on a different channel to remove cells fluorescing in all channels. In certain cases, ImageJ can be used to define segmentation boundaries on a mask and import them to the GeoMx. See GeoMx DSP Instrument User Manual (MAN-10152).
	Dust or debris on slide	Follow washing and blocking steps to specifications in this user manual.
Can't find reagents or protocols in BOND RX drop-down menus	Reagents and protocols are hidden per BOND RX settings	Set all reagents and protocols to Preferred status. See instructions **on page 71**.
Problems with or questions about BOND RX		Contact your Leica Biosystems representative.

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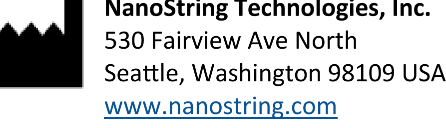

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