USER MANUAL

RNAscope™ Multiomic LS Detection Kit

For use with BOND RX™ System, from Leica Biosystems

For Research Use Only. Not for use in diagnostic procedures.

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Citing RNAscope in Publications

When describing a procedure for publication using this product, please refer to it as the RNAscope Assay and cite: Wang F, Flanagan J, Su N, Wang L-C, Bui S, Nielson A, Wu X, Vo H-T, Ma X-J and Luo Y. RNAscope: A Novel *In Situ* RNA Analysis Platform for Formalin Fixed Paraffin Embedded Tissues. J. Mol. Diagnostics, 2012, 14:22–29.

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1 Chapter 1. Product Information

Before using this product, read and understand the information in **Appendix K. Safety** of this document.

IMPORTANT! We recommend reading the entire user manual before beginning any protocols.

About this guide

This user manual provides guidelines and protocols to use the RNAscope Multiomic LS Fluorescent Reagent Kit with the BOND RX Research Advanced Staining System.

For questions or support, contact your ACD representative at +1 (877) 576-3636.

Product description

Background

The RNAscope Multiomic LS Assay uses a novel and proprietary method of *in situ* hybridization (ISH) to simultaneously visualize up to six RNA or protein targets in samples mounted on slides. The assay is based on ACD's patented signal amplification and background suppression technology and incorporates signal amplification systems that enable users to investigate expression as well as positional relationships of multiple genes within a cellular context. The RNAscope Multiomic LS Assay allows users to automate the highly sensitive Multiomic Assay using the BOND RX System.

Overview

You can use the RNAscope Multiomic LS assay in different workflows to detect RNA targets or RNA and protein targets simultaneously. For RNA-only detection, you can use *in situ* hybridization using RNAscope probes. For multiomic detection of both RNA and proteins on the same section, two multiomic workflows are available. In sequential multiomics, the RNAscope ISH detection can be immediately followed by standard immunohistochemistry (IHC) or immunofluorescent (IF) detection of proteins. For greater protein detection capability, a full Multiomic workflow utilizing RNAscope conjugated primary and secondary antibodies is also available. In this workflow, the assay integrates RNA and protein detection in a single automated protocol. Both multiomic workflows leverage the assay's PretreatPro reagent, a protease-free pre-treatment reagent that preserves tissue morphology and maintains antibody functionality that is often lost when using traditional protease pre-treatment. To maintain compatibility with existing ISH protocols, the kit also includes protease. **Figure 1** shows an overview of the three supported workflows.

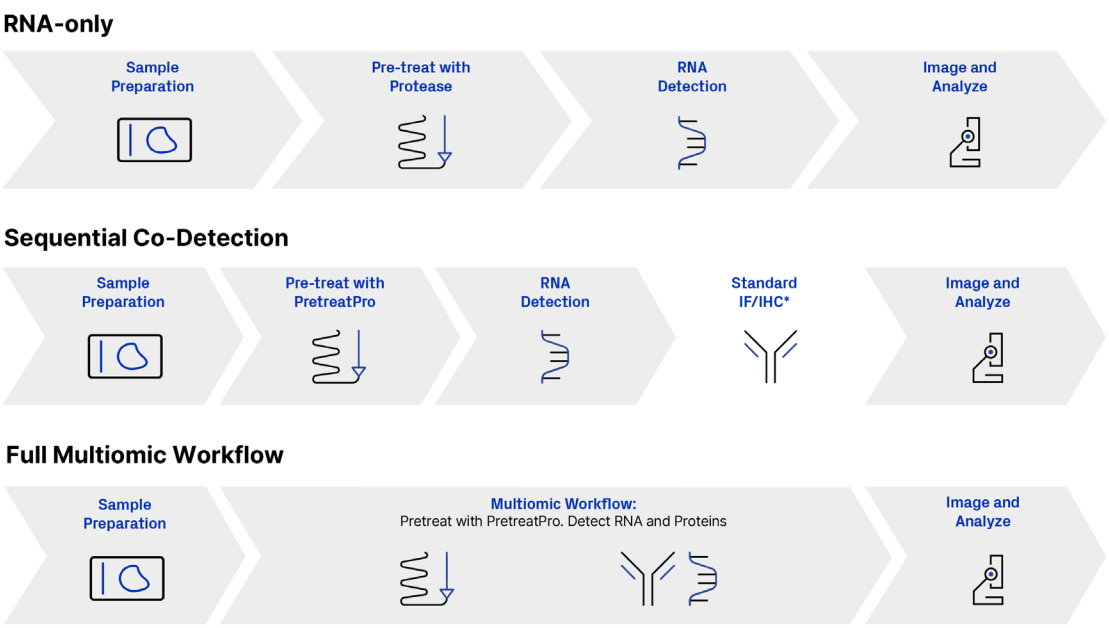
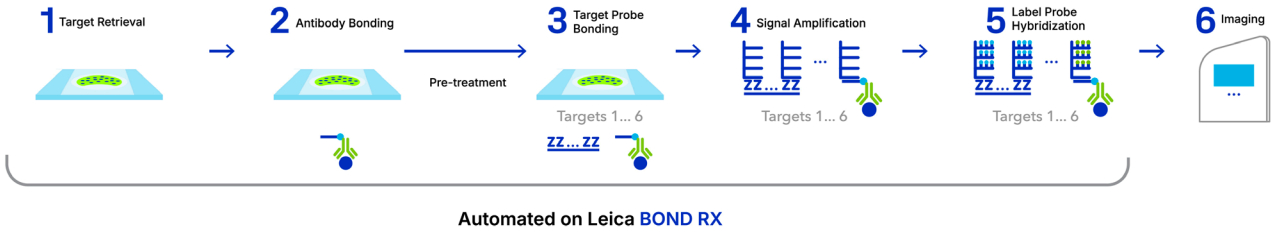


Figure 1. Workflow overviews. The RNAscope Multiomic LS assay supports three separate workflows. For RNA-only detection, u**se** RNAscope probes to detect mRNA transcripts. The Sequential Multiomic workflow is for detection of mRNA and proteins on the same section and consists of performing the RNAscope ISH followed by standard IHC or IF. We recommend starting with primary antibodies that you have already qualified when using this workflow. The RNAscope Full Multiomic workflow easily supports a greater number of protein targets and integrates the mRNA and protein detection in a single automated workflow. The Full Multiomic workflow u**ses** RNAscope primary antibodies along with user provided primaries and RNAscope secondary antibodies.



1: Target retrieval	2: Antibody binding	3: Target probe hybridization	4: Signal amplification and label probe hybridization	5: Label probe hybridization	6: Imaging
Start with appropriately prepared tissue sections and perform target retrieval to allow access to target.	Incubation with target antibodies.	Perform pretreatments and hybridize multiple sets of gene-specific probe pairs to target RNAs.	RNAscope technology sensitively and specifically amplifies signal.	Label probe hybridization and TSA amplification results in deposition of up to six spectrally distinct fluorophores.	Visualize protein and RNA targets using a fluorescent microscope or slide scanner.

Figure 2. Procedure overview. The RNAscope Multiomic LS Fluorescent Assay procedure can be completed on the instrument in about 20–26 hours. Properly prepared a**nd** sectioned samples are incubated with target retrieval agents, followed by antibodies to allow binding to their protein targets. Then the sections are incubated with pretreatment reagents and RNA-specific probes to hybridize to their target mRNAs. The RNAscope Multiomic LS Fluorescent Assay enables up to six independent signal amplification systems each using a different fluorophore to enable independent detection of the six protein or mRNA targets or a combination of both. Protein detection when using this kit is similar to standard immune-fluorescence signals. But when detecting mRNA, this assay is sensitive enough to detect single mRNA transcripts which appear as punctate dots that are visible using a fluorescent microscope or slide scanner.

Kit contents and storage

The RNAscope Multiomic LS Assay requires RNAscope LS Probes for RNA detection and RNAscope primary and/or secondary antibodies for protein detection using the RNAscope Multiomic workflow, available from Advanced Cell Diagnostics. Additional reagents available from common laboratory suppliers and Leica Biosystems are also required.

RNAscope LS Probes

The RNAscope LS Probes include the user-specified Target Probe and the Positive and Negative Control Probes. Visit **https://www.bio-techne.com/reagents/rnascope-ish-technology** to learn more about probes and find a gene specific target probe or appropriate control probe. Each target probe contains a mixture of short oligonucleotides designed to bind to a specific target mRNA, and detectable in one of six probe channels C1, C2, C3, C4, C5 or C6. Signal detection is performed using Tyramide Signal Amplification (TSA) linked fluorophores. Different colors are assigned to the C1, C2, C3, C4, C5 and C6 channel tags depending on the TSA Vivid™ or Opal™ dye fluorophore selected for that channel.

Channel C1 target probes are Ready-To-Use (RTU), while channels C2, C3, C4, C5 and C6 probes are shipped as a 50X concentrated stock. To independently detect multiple target RNAs, each target probe must be in a different channel.

Note: If you are using only the C2, C3, C4, C5 and C6 probes, you can use 2.5 LS Blank Probe Diluent (Cat. No. 300048).

Each probe is sufficient to stain ~30 standard slides. The probes have a shelf life of two years from the manufacturing date when stored as indicated in the following tables:

Target Probes					
☑	**Reagent**	**Cat. No.**	**Content**	**Quantity**	**Storage**
	RNAscope Multiomic LS Target Probe – *[species]* – *[gene]* – C1	Various	Ready-To-Use (RTU) probe for channel C1	16 mL x 1 bottle	2°C to 8°C
	2.5 LS Blank Probe Diluent	300048	Required if not using a C1 probe. C2, C3, C4, C5 and C6 probes should be diluted in this diluent.	16 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic LS Target Probe – *[species]* – *[gene]* – C2	Various	50X probe for channel C2	320 µL x 1 tube	2°C to 8°C
	RNAscope Multiomic LS Target Probe – *[species]* – *[gene]* – C3	Various	50X probe for channel C3	320 µL x 1 tube	2°C to 8°C
	RNAscope Multiomic LS Target Probe – *[species]* – *[gene]* – C4	Various	50X probe for channel C4	320 µL x 1 tube	2°C to 8°C
	RNAscope Multiomic LS Target Probe – *[species]* – *[gene]* – C5	Various	50X probe for channel C5	320 µL x 1 tube	2°C to 8°C
	RNAscope Multiomic LS Target Probe – *[species]* – *[gene]* – C6	Various	50X probe for channel C6	320 µL x 1 tube	2°C to 8°C
Control Probes					
☑	**Reagent**	**Cat. No.**	**Content**	**Quantity**	**Storage**
	RNAscope Multiomic LS 6-plex Positive Control Probe-Hs	323198	RNAscope Multiomic LS Positive Control Probe for the RNAscope Multiomic LS Fluorescent Assay – *POLR2A* (C1 channel), *PPIB* (C2 channel), *UBC* (C3 channel), *HPRT1* (C4 channel), *ACTB* (C5 channel), *TUBB* (C6 channel).	16 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic LS 6-plex Negative Control Probe	323208	RNAscope Multiomic LS Negative Control Probe for the RNAscope Multiomic LS Fluorescent Assay – *dapB* (*Bacillus subtilis* strain, C1-C6 channels).	16 mL x 1 bottle	2°C to 8°C

RNAscope antibodies

RNAscope antibodies, either primary or secondary, are conjugated to RNAscope oligonucleotides to enhance protein detection sensitivity. Pre-configured panels of RNAscope conjugated primary antibodies can work individually or together and may be combined with RNA-specific probes for simultaneous protein and RNA detection. RNAscope conjugated secondary antibodies can be paired with user-supplied primary antibodies to detect additional proteins of interest.

RNAscope Human Tumor Infiltrating Lymphocyte (TIL) Primary Antibody Panel					
☑	**Reagent**	**Dilution factor**	**Cat. No.**	**Quantity**	**Storage**
	RNAscope Ab Hs CD4-C3	75X	322949	103 µL, (20 slides)	Consult product label
	RNAscope Ab Hs CD8-C4	75X	322951	103 µL, (20 slides)	
	RNAscope Ab Hs PanCK-C5	75X	322952	103 µL, (20 slides)	
	RNAscope Ab Hs FoxP3-C6	75X	322953	103 µL, (20 slides)	
RNAscope Neural Primary Antibody Panel					
☑	**Reagent**	**Dilution factor**	**Cat. No.**	**Quantity**	**Storage**
	RNAscope Ab NeuN-C3	75X	AB0014-C3	105 µL, (20 slides)	Consult product label
	RNAscope Ab GFAP-C4	75X	AB0024-C4	105 µL, (20 slides)	
	RNAscope Ab IBA-1-C5	75X	AB0034-C5	105 µL, (20 slides)	
RNAscope Secondary Antibodies					
☑	**Reagent**	**Dilution factor**	**Cat. No.**	**Quantity**	**Storage**
	RNAscope anti-rabbit-C1	25X	322954	930 µL, (60 slides)	Consult product label
	RNAscope anti-mouse-C2	25X	322956	930 µL, (60 slides)	

RNAscope Multiomic LS Reagents

To perform the RNAscope Multiomic LS assay, the RNAscope Multiomic LS CORE Reagents and at least one of the Channel Reagents needs to be purchased. The kits provide enough reagents to stain ~ 20 or ~60 standard slides. The assay reagents are then used with RNA-specific probes (if RNA detection is desired), antibodies (if protein detection is desired), TSA linked fluorophores and mounting medium.

The assay reagents are Ready-To-Use (RTU) except for the TSA buffer and are stored as indicated in the following tables:

RNAscope Multiomic LS CORE Reagents				
☑	**Reagent**	**Quantity 60-slide kit Cat. No. 322930**	**Quantity 20-slide kit Cat. No. 323425**	**Storage**
	RNAscope 2.5 LS Protease III	21 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic LS Rinse	29 mL x 3 bottles	11 mL x 2 bottles	2°C to 8°C
	RNAscope Multiomic LS AMP 1	21 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic LS AMP 2	21 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic LS AMP 3	21 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope PretreatPro™	21 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C

RNAscope Multiomic LS CORE Reagents				
☑	**Reagent**	**Quantity 60-slide kit Cat. No. 322930**	**Quantity 20-slide kit Cat. No. 323425**	**Storage**
	RNAscope Multiomic LS Hydrogen Peroxide	21 mL x 1 bottle	8 mL 1 x bottle	2°C to 8°C
	RNAscope Multiomic LS DAPI	21 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic Antibody Diluent	29 mL x 3 bottles	14 mL x 3 bottles	2°C to 8°C
RNAscope Multiomic C1 Channel Reagents				
☑	**Reagent**	**Quantity 60-slide kit Cat. No. 322935**	**Quantity 20-slide kit Cat. No. 323430**	**Storage**
	RNAscope Multiomic TSA Buffer	29 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic HRP Blocker	29 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic LS HRP C1	21 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
RNAscope Multiomic C2 Channel Reagents				
☑	**Reagent**	**Quantity 60-slide kit Cat. No. 322940**	**Quantity 20-slide kit Cat. No. 323435**	**Storage**
	RNAscope Multiomic TSA Buffer	29 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic HRP Blocker	29 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic LS HRP C2	21 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
RNAscope Multiomic C3 Channel Reagents				
☑	**Reagent**	**Quantity 60-slide kit Cat. No. 322945**	**Quantity 20-slide kit Cat. No. 323440**	**Storage**
	RNAscope Multiomic TSA Buffer	29 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic HRP Blocker	29 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic LS HRP C3	21 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
RNAscope Multiomic C4 Channel Reagents				
☑	**Reagent**	**Quantity 60-slide kit Cat. No. 322950**	**Quantity 20-slide kit Cat. No. 323445**	**Storage**
	RNAscope Multiomic TSA Buffer	29 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic HRP Blocker	29 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic LS HRP C4	21 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
RNAscope Multiomic C5 Channel Reagents				
☑	**Reagent**	**Quantity 60-slide kit Cat. No. 322955**	**Quantity 20-slide kit Cat. No. 323450**	**Storage**
	RNAscope Multiomic TSA Buffer	29 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C

RNAscope Multiomic LS CORE Reagents				
☑	**Reagent**	**Quantity 60-slide kit Cat. No. 322930**	**Quantity 20-slide kit Cat. No. 323425**	**Storage**
	RNAscope Multiomic HRP Blocker	29 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic LS HRP C5	21 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
RNAscope Multiomic C6 Channel Reagents				
☑	**Reagent**	**Quantity 60-slide kit Cat. No. 322960**	**Quantity 20-slide kit Cat. No. 323455**	**Storage**
	RNAscope Multiomic TSA Buffer	29 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic HRP Blocker	29 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic LS HRP C6	21 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C

Required materials and equipment

Assay design

The RNAscope Multiomic LS assay provides flexibility in assay design to enable detection of up to a total of six protein or RNA targets. Proteins can be detected with user-provided primary antibodies paired with ACD's RNAscope oligo-conjugated secondary antibodies. These protein targets can be augmented with available pre-qualified RNAscope conjugated primary antibodies against common human targets. The RNAscope secondary antibodies and RNAscope primary antibodies are designed for specific RNAscope channels. Probes to RNA targets can be designed for any open channel not used by one of the conjugated antibodies.

Use this table to plan the channels needed for your assay based on your targets.

Target	**RNAscope channel assignment**					
	C1	**C2**	**C3**	**C4**	**C5**	**C6**
Protein targets	User-provided primary antibody-rabbit + RNAscope anti-rabbit-C1	User-provided primary antibody-mouse + RNAscope anti-mouse-C2	—	—	—	—
	—	—	RNAscope Primary C3	RNAscope Primary C4	RNAscope Primary C5	RNAscope Primary C6
RNA targets	RNAscope C1 probe	RNAscope C2 probe	RNAscope C3 probe	RNAscope C4 probe	RNAscope C5 probe	RNAscope C6 probe

Note: Antibodies are available only in the listed channels. Probes can be designed for any open channel.

User-supplied primary antibodies

If you wish to use the RNAscope Multiomic LS assay with your own primary antibodies, you can pair them with the RNAscope conjugated secondary antibodies (listed in the previous tables). This provides the greatest sensitivity and specificity for your selected targets. Your primary antibodies should be hosted in either rabbit (for use with the RNAscope anti-rabbit-C1 antibody) or hosted in mouse (for use with the RNAscope anti-mouse-C2 antibody).

Recommended fluorophores

The RNAscope Multiomic LS assay requires purchase of TSA Vivid from ACD or Opal from Akoya Biosciences. For a 3-plex assay, TSA Vivid dyes are recommended. For a 4 or 6-plex assay, Opal dyes are recommended.

Dilute the fluorophores to the desired working concentration in the TSA Buffer provided in the RNAscope Kit. Choose a dilution factor for each fluorophore based on recommendations from ACD and your specific experimental conditions including target expression levels, tissue quality, or microscope setting. Materials are qualified with 1:1500 dilution for all fluorophores. We cannot guarantee assay results if you use other fluorescent dyes.

Note: To reconstitute dyes, follow the manufacturer instructions available on the tube labels. Dilute the fluorophores in TSA buffer provided in the Channel Reagent kits.

See the following tables for recommended fluorophore combinations. Other combinations are also acceptable if the spectra are non-overlapping and compatible with your imaging system while considering tissue autofluorescence.

3-Plex assay using TSA Vivid Fluorophores

☑	Fluorophores	Cat. No.	Recommended dilution range
	TSA Vivid Fluorophore 520	323271	1:750–1:3000
	TSA Vivid Fluorophore 570	323272	1:750–1:3000
	TSA Vivid Fluorophore 650	323273	1:750–1:3000

Assays using Akoya Biosciences Opal Fluorophores

☑	Fluorophores	Akoya Biosciences Cat. No.	Recommended dilution range	3-plex †	4-plex	5- or 6-plex
	Opal 480 Reagent Pack	FP1500001KT	1:750–1:3000			✓
	Opal 520 Reagent Pack	FP1487001KT	1:750–1:3000	✓	✓	✓
	Opal 570 Reagent Pack	FP1488001KT	1:750–1:3000	✓	✓	✓
	Opal 620 Reagent Pack	FP1495001KT	1:750–1:3000			✓
	Opal 690 Reagent Pack	FP1497001KT	1:750–1:3000	✓	✓	✓
	Opal Polaris 780 Reagent Pack*	FP1501001KT	TSA-DIG: 1:750–1:3000 Polaris 780: 1:187.5–1:750		✓	✓

* The Opal Polaris 780 Reagent Pack contains two reagents: Opal TSA-DIG and Opal Polaris 780. We recommend diluting Polaris TSA-DIG in TSA buffer, and diluting Opal Polaris 780 in Antibody Diluent/Block from Akoya Biosciences (PN: ARD1001EA). We recommend keeping the dilution factors of Opal TSA-DIG and Opal Polaris 780 at a constant ratio. For example, when using 1:1500 dilution for Opal TSA-DIG, use 1:375 dilution for Opal Polaris 780. When using 1:750 dilution for Opal TSA-DIG, use 1:187.5 dilution for Opal Polaris 780.

† Opal 650 or Polaris 780 can be used instead of Opal 690, depending on your imager configuration.

Required slide scanner or microscope

A system with multispectral capabilities is recommended, especially for imaging tissue with high autofluorescence. For optimal fluorescence detection, we recommend using a high resolution and high sensitivity cooled CCD camera that is 64 µm pixel size or smaller with > 65% peak quantum efficiency. Common models include Orca-Flash 4.0 (Hamamatsu) and Nuance® EX (Perkin Elmer).

Slide scanner or microscope	Optics
• Akoya PhenoImager HT • Leica DM series or equivalent • Zeiss Axio Imager, Axioscan or equivalent • Inverted microscope if optics and condenser meet requirements. • Required excitation/emission filter cube for 6-plex: DAPI/ Opal480/Opal520/Opal570/Opal620/Opal690/Opal780	• 20X (N.A 0.75) air • 40X (N.A. 0.8) air (recommended) • 40X (N.A. 1.3) oil • 63X (N.A. 1.3) oil – use for low expression targets, if needed • Use 20X and 40X to visualize high expression genes and low expression genes, respectively

Required materials and equipment from Leica Biosystems

The RNAscope Multiomic LS Fluorescent Assay is designed for the BOND RX and requires specific materials and equipment available from Leica Biosystems.

☑	Component	Cat. No.	Storage
	BOND RX System — automated slide stainer	—	—
	BOND 30 mL Open containers	OP309700	Room temp (20 to 25°C)
	BOND 6 mL Titration containers and inserts*	OPT9049	Room temp (20 to 25°C)
	BOND Research Detection System	DS9455	Room temp (20 to 25°C)
	BOND Universal Covertile	S21.4611	Room temp (20 to 25°C)
	BOND Epitope Retrieval Solution 1-1L (RTU)	AR9961	2°C to 8°C
	BOND Epitope Retrieval Solution 2-1L (RTU)	AR9640	2°C to 8°C
	BOND Dewax Solution – 1L (RTU)	AR9222	2°C to 8°C
	BOND Wash Solution 10X Concentrate – 1L	AR9590	2°C to 8°C
	BOND Aspirating Probe Cleaning System	CS9100	2°C to 8°C
	BOND Mixing Stations	S21.1971	Room temp (20 to 25°C)

* BOND 7 mL Containers can be used instead but offer less flexibility.

Other user-supplied materials

IMPORTANT! Do not substitute other materials for the SuperFrost® Plus Slides listed in the following table.

☑	Description	Supplier	Cat. No.
	SuperFrost Plus Slides (required)	Fisher Scientific	12-550-15
	Salmon Sperm DNA, sheared (10mg/ml)	Thermo Fisher	AM9680
	Normal Rabbit IgG Control	R&D Systems	MAB1050
	Mouse IgG2A Isotype Control	R&D Systems	MAB003
	ProLong™ Gold Antifade Mountant	Thermo Fisher	P36930; P10144; P36934
	Opal dyes fluorophores (if not using TSA Vivid Dyes from ACD)	Akoya Biosciences	—
	Either BOND Primary Antibody Diluent or Antibody Diluent/Block (if Opal Polaris 780 is used)	Leica Biosystems Akoya Biosciences	AR9352 ARD1001EA
	Xylene	Fisher Scientific/MLS	X3P-1GAL
	100% alcohol (EtOH)	American Master Tech Scientific/MLS*	ALREACS
	10% neutral-buffered formalin (NBF)	MLS	—
	Paraffin wax	MLS	—
	1X PBS	MLS	—
	Microtome	MLS	—
	Drying oven, capable of holding temperature at 60 +/– 1°C (optional)	MLS	—
	Water bath or incubator, capable of holding temperature at 40 +/– 1°C	MLS	—
	Vertical 24-slide racks (or other slide racks or holders)	American Master Tech Scientific/MLS	LWSRA24
	Vertical staining dishes (or similar containers)	American Master Tech Scientific/MLS	LWT4457EA
	Clearing agent dishes, xylene resistant	American Master Tech Scientific/MLS	LWT4456EA
	Cover glass 24 x 50 mm	Fisher Scientific/MLS	12-545-F
	Distilled water	MLS	—
	Fume hood	MLS	—

* Major Laboratory Supplier in North America. For other regions, please check Catalog Numbers with your local laboratory supplier.

2 Chapter 2. Before You Begin

Prior to running the RNAscope Multiomic LS Fluorescent Assay on your samples for the first time, we recommend that you:

- Become familiar with BOND RX Research Advanced Staining System from Leica Biosystems. Refer to the *BOND RX System User Manual*.
- Run the assay on Control Slides (Cat. No. 310045 for Human HeLa Cell Pellet, and Cat. No. 310023 for Mouse 3T3 Cell Pellet) using the RNAscope Multiomic LS Positive and Negative RNAscope Multiomic Control Probes.

Important procedural guidelines

- Start with appropriately fixed and prepared sections. Refer to the sample preparation and pretreatment chapters in this manual.
- Regularly maintain and clean your automated staining instrument.
- Always run positive and negative control probes on your sample to assess sample RNA quality and optimal permeabilization.
- Do not substitute required materials. The assay has been validated with these materials only.
- Follow the protocol exactly for the best results.
- Do not let your sections dry out during the procedure.
- Use good laboratory practices and follow all necessary safety procedures. Refer to **Appendix K. Safety** for more information.

3 Chapter 3. Prepare Samples

The following protocols describe formalin-fixed, paraffin-embedded (FFPE), fixed frozen and fresh frozen sample preparation.

IMPORTANT! We highly recommend following these guidelines. We cannot guarantee assay results with other preparation methods.

IMPORTANT! RNA-only staining has been validated by ACD for all sample preparations described in this manual, but only FFPE samples have been fully tested for combined RNA + protein staining. Please see the following guidance for details.

Prepare FFPE sections

Materials required

- 10% neutral buffered formalin (NBF)
- 1X PBS
- Paraffin wax
- 95% Ethanol (EtOH)
- Xylene
- Microtome
- Water bath
- SuperFrost Plus slides

Fix the sample

1. Immediately following dissection cut the tissue into blocks of 3–4 mm in thickness**.**
2. Place the tissue blocks into fixative within **1 HR** of biopsy.
3. Fix the tissue in 10% NBF for **16–32 HRS** at **ROOM TEMPERATURE (RT)**. Fixation time will vary depending on tissue type and size.

⚠ CAUTION! Handle biological specimens appropriately.

IMPORTANT! Fixation for **<16 HRS** or **>32 HRS** will impair the performance of the assay.

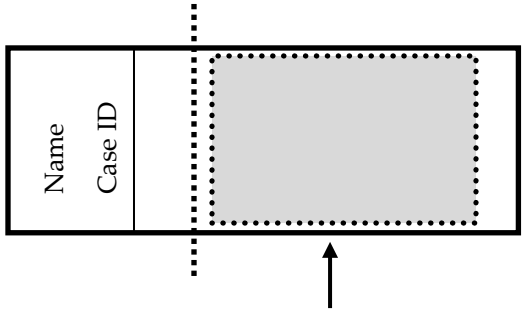
Dehydrate, embed, and cut the sample

IMPORTANT! Use fresh reagents.

1. Wash sample with 1X PBS.
2. Dehydrate sample using a standard ethanol series, followed by xylene.
3. Embed sample in paraffin using standard procedures.

Note: Embedded samples may be stored at room temperature with desiccation. To better preserve RNA quality over a long period (>1 yr), storing at 2–8°C with desiccation is recommended.

4. Trim paraffin blocks as needed and cut embedded tissue into **5 +/– 1 µm** sections using a microtome.
5. Place the paraffin ribbon in a **40–45°C** water bath and mount the sections on **SUPERFROST PLUS SLIDES.** Place tissue as shown for optimal staining:



IMPORTANT! Do not mount more than one section per slide. Place sections in the center of the slide.

6. Air dry slides **OVERNIGHT** at **RT**.

OPTIONAL STOPPING POINT. Use sectioned tissue within three months. Store sections with desiccants at room temperature.

Prepare fixed-frozen sections

Materials required

- 1X PBS
- 10% Neutral Buffered Formalin (NBF) or 4% Paraformaldehyde (PFA)
- 100% ethanol (EtOH)
- Tissue-Tek Vertical 24 Slide Rack
- Tissue-Tek Staining Dishes
- Drying oven

Fix sample

1. If needed, perfuse tissue with freshly prepared 4% Paraformaldehyde (PFA) in 1X PBS, or go directly to step 2.
2. Dissect tissue and place in freshly prepared 4% Paraformaldehyde (PFA) for **24 HRS** at **4°C**.

Freeze tissue

1. Immerse the tissue in 10% sucrose in 1X PBS at **4°C** until the tissues sink to the bottom of the containers (approximately **18 HRS** for brain tissue.)

Note: The time needed for the tissue to sink varies with the tissue type and size.

2. Immerse the tissue in 20% sucrose in 1X PBS at **4°C** until the tissue sinks to the bottom of the container.
3. Immerse the tissue in 30% sucrose in 1X PBS at **4°C** until the tissue sinks to the bottom of the container.
4. Freeze the tissue in OCT (Optimal Cutting Temperature) embedding media or TFM (Tissue Freezing Media) with crushed dry ice, iso-pentane, or liquid nitrogen.
5. Store tissue blocks in an airtight container at **-80°C**.

Prepare sections

1. Before sectioning, equilibrate the tissue blocks at **-20°C for at least 1 HR in a cryostat.**
2. Section blocks by cutting sections to a thickness of 7 – 15 µm. Mount sections on SuperFrost Plus slides ONLY (other slide types could result in tissue loss).
3. Air dry the slides for **2 HR** at **-20°C** and overnight at **-80°C.** If slides are not used immediately, store them at **-80°C** for up to **3 MONTHS.**
4. On the day of starting the assay, remove fixed-frozen tissue slides from **-80°C**.
5. Wash the slides with 200 mL 1X PBS for **5 MIN** while moving the rack to remove OCT.
6. Bake slides in drying oven for **15 - 60 MIN** at **60°C**.
7. *Immediately* post-fix slides by immersing them in prechilled 10% NBF or 4% PFA for **15–60 MIN** at **4°C**.

Note: If experiencing issues with sample detachment, the longer post-fix and baking times could be helpful.

Dehydrate and dry the sections

Reagents can be prepared ahead of time. Ensure all containers remain covered.

1. Prepare 200 mL 50% ethanol, 200 mL 70% ethanol, and 2 X 200 mL 100% ethanol in Tissue Tek Staining Dishes.
2. Remove the slides from the 10% NBF or 4% PFA, and immerse them in 50% EtOH for **5 MIN** at **RT**.
3. Place the slides in 70% ethanol for **5 MIN** at **RT**.
4. Place the slides in 100% ethanol for **5 MIN** at **RT**.
5. Place slides in fresh 100% ethanol for **5 MIN** at **RT**.
6. Remove slides from ethanol, and let them dry for **5 MIN** at **RT**.

Prepare fresh-frozen sections

Materials required

- 1X PBS
- 10% Neutral Buffered Formalin (NBF) or 4% Paraformaldehyde (PFA)
- 100% ethanol (EtOH)
- Tissue-Tek Vertical 24 Slide Rack
- Tissue-Tek Staining Dishes

Prepare fresh frozen tissue sections

Remove tissue and cut to fit into cryomolds.

⚠ CAUTION! Handle biological specimens appropriately.

1. Freeze the specimen within **5 MIN** of tissue harvest.
2. Embed the frozen tissue in cryo-embedding medium (OCT):
   a. Add two drops of OCT into a cryomold.
   b. Place the frozen tissue on the OCT in the correct orientation for cutting.
   c. Add more OCT to fill the cryomold. Do not allow any air bubbles to form.
   d. Hold the block with forceps on the surface of the liquid nitrogen or isopentane cooled by dry ice or liquid nitrogen or place the cryomold on dry ice.
3. Store the frozen block in an air-tight container at **–80°C** prior to sectioning.

Note: Embedded tissue may be stored for up to three months.

OPTIONAL STOPPING POINT (1). Section tissue within **3 MONTHS**.

4. Section the block:
   a. Equilibrate block to **–20°C** in a cryostat **~1 HR**.
   b. Cut 10–20 µm thick sections and mount onto **SUPERFROST PLUS SLIDES**.
   c. Dry the sections at **60 –120 MIN** at **–20°C** to retain tissue adherence.
5. Store the sections in slide boxes wrapped air-tight with aluminum foil or zip-lock bags at **–80°C** until use.

Note: Sections may be stored for up to three months.

IMPORTANT! Do not fix the slides prior to this step.

OPTIONAL STOPPING POINT (2). Use sectioned tissue within **3 MONTHS**.

Fix the sections

1. Pre-chill 200 ml of 10% NBF or 4% PFA in 1x PBS to **4°C.**
2. Remove fresh-frozen tissue slides from -**80°C** and place in a Tissue Tek Slide Rack.
3. *Immediately* immerse the slides in 200 mL of 10% NBF or freshly prepared 4% PFA.
4. Incubate the slides for at least **90 MIN** at **ROOM TEMPERATURE (RT)**.

Note: Formalin that has been stored for more than six months, exposed to air for more than a week, or used repeatedly may result in suboptimal tissue fixation. 4% PFA must be freshly prepared for each experiment.

Dehydrate the sections

Reagents can be prepared ahead of time. Ensure all containers remain covered.

1. Prepare 200 mL 50% ethanol, 200 mL 70% ethanol, and 2X 200 mL 100% ethanol in Tissue Tek Staining Dishes.
2. Place the slides in 50% ethanol for **5 MIN** at **RT**.
3. Place the slides in 70% ethanol for **5 MIN** at **RT**.
4. Place the slides in 100% ethanol for **5 MIN** at **RT**.
5. Place slides in fresh 100% ethanol for **5 MIN** at **RT**.

Note: If needed, slides can be stored in 100% EtOH at -**20°C** for up to **1 WEEK**. Prolonged storage may degrade sample RNA.

6. Remove slides from ethanol, and let them dry for **5 MIN** at **RT**.

4 Chapter 4. Determine Pretreatment Conditions

The following protocols describe formalin-fixed, paraffin-embedded (FFPE), fixed-frozen and fresh-frozen sample pretreatment. For other sample types and preparation methods, contact **support.acd@bio-techne.com** for the latest protocols and guidelines.

IMPORTANT! We highly recommend following these guidelines. We cannot guarantee assay results with other preparation methods.

IMPORTANT! RNA-only staining has been validated by ACD for all sample preparations described in this manual, but only FFPE samples have been fully tested for combined RNA and protein staining. Please see the following guidance for details.

Sample preparation for RNA-only staining

Pretreat FFPE sections

Target retrieval

FFPE samples must be de-crosslinked with a target retrieval step. The Multiomic LS Assay uses the BOND RX's ER2 solution exclusively for this step.

Permeabilization

Two options are available:

- Protease-based permeabilization is recommended for experiments that stain only RNA. This option uses LS Protease III.
- Protease-free permeabilization uses the LS PretreatPro reagent. This allows co-detection of RNA and proteins that were previously incompatible with protease on the same tissue section using immunohistochemistry (IHC).

To determine the optimal permeabilization option, please refer to the following flowchart:

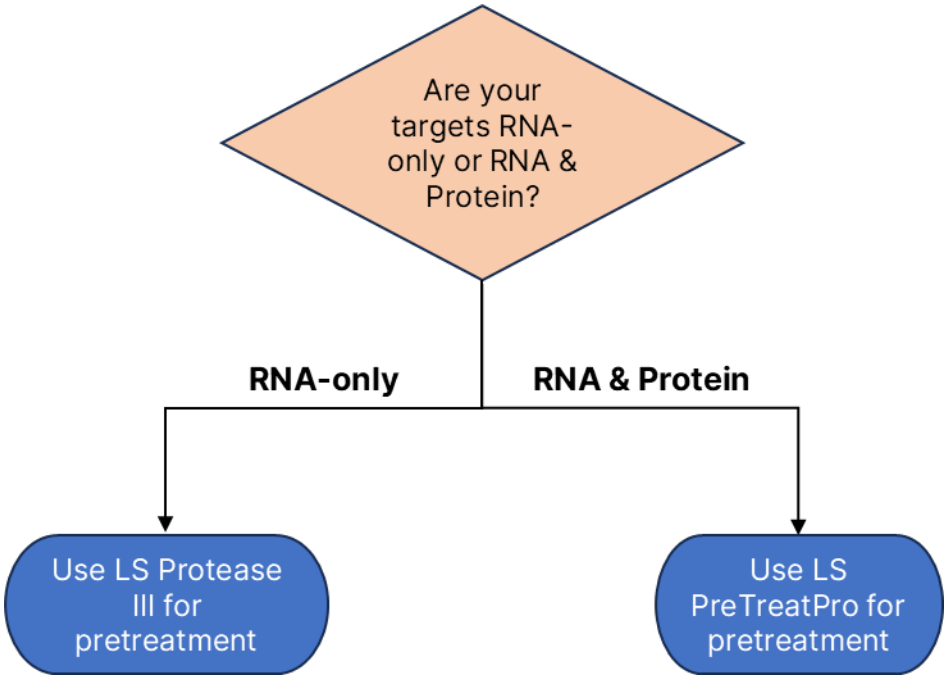


Figure 3. Permeabilization options. For experiments where only RNA is being detected, we recommend using Protease III for best results. However, if tissue morphology is adversely affected, you can also use PretreatPro in place of protease to better preserve morphology. For multiomic detection of RNA and protein, PretreatPro maintains antigen integrity for the widest antibody compatibility.

Tissue pretreatment recommendations

Use these conditions as a starting point when tissues are prepared as described in **Appendix H**.

Depending on your tissue type, vary the amount of time for ER2 until the positive RNA control signal is maximized with minimal or no signal in the negative control.

Reagent	Mild	Standard
BOND ER2*	15 MIN at 88°C	15 MIN at 95°C
LS Protease III	15 MIN at 40°C	15 MIN at 40°C
OR		
LS PretreatPro	30 MIN at 40°C	

*Sample types, such as certain xenografts and cell pellets, might require shorter incubation time. For these tissue types, reduce the BOND ER2 incubation time. You can also adjust LS Protease III incubation times but this is rarely needed. There is no need to adjust PretreatPro time or temperature. If you have a tissue type not listed, contact ACD Support at **support.acd@bio-techne.com.**

Pretreat fixed-frozen sections

Target Retrieval

Fixed-frozen samples must be gently de-crosslinked with a target retrieval step. The RNAscope 2.5 LS Assay uses the BOND RX's ER2 solution exclusively for this step.

Permeabilization

Only LS Protease III has been tested for use with fixed-frozen sections. Check with ACD Support for any updates.

Pretreat fresh-frozen sections

Target Retrieval

Fresh-frozen sections do not need target retrieval.

Permeabilization

Only LS Protease IV has been tested for use with fresh-frozen sections. Check with ACD Support for any updates.

Tissue pretreatment recommendations

Use this condition as a starting point when tissues are prepared as described in **Chapter 3. Prepare Samples.** Depending on your tissue type, vary the amount of time for Protease IV until positive RNA control signal is maximized with minimal/no negative RNA control signal (see **Appendix I. Slide Setup for Additional Tissue Types** for details).

Reagent	Standard
LS Protease IV (ACD Part Number 322140)	30 MIN at ambient temperature*

* You might need to create this enzyme treatment protocol. Please refer to **Appendix F** for further instructions.

Sample preparation for RNA plus protein staining

When performing dual staining for RNA and protein targets, detailed guidance is available for FFPE sample types. For other sample types, you can extrapolate from the RNA-only staining guidance provided in the previous section. If you need further assistance, please contact ACD Support.

Pretreat FFPE sections

Target retrieval

FFPE samples must be de-crosslinked with a target retrieval step. The Multiomic LS Assay specifically uses the BOND RX's ER2 solution for this step.

Permeabilization

Only LS PretreatPro has been tested on FFPE samples stained for both RNA and protein. Using LS Protease could negatively impact staining performance of protease sensitive antigens.

Tissue pretreatment recommendations

Use these conditions as a starting point when FFPE tissues are prepared as described in **Chapter 3. Prepare Samples**. Depending on your tissue type, vary the amount of time for ER2 until the positive RNA control signal is maximized with minimal or no negative RNA control signal (see **Appendix H** for a list of tissues). Heat retrieval recommendations are higher with PretreatPro than with Protease III to minimize nuclear trapping of the fluorescent dyes.

Reagent	Mild	Standard
BOND ER2	15 MIN at 92°C	20 MIN at 100°C
LS PretreatPro*	30 MIN at 40°C	

* Sample types, such as certain xenografts and cell pellets, might require shorter incubation time. For these tissue types, reduce the BOND ER2 incubation time If you have a tissue type not listed, contact ACD Support at **support.acd@bio-techne.com**.

5 Chapter 5. Set Up Staining Protocols

The RNAscope Multiomic LS assay can be used in various workflows to detect RNA targets or simultaneously detect RNA and protein targets. For RNA-only detection, you can perform *in situ* hybridization using RNAscope probes. For detecting both RNA and proteins on the same sample, two multiomic workflows can be employed. RNA and protein are detected sequentially, first by performing an RNAscope ISH detection followed by a standard IHC detection. For enhanced protein detection, a comprehensive RNAscope Multiomic workflow that uses RNAscope conjugated antibodies is available. This method combines RNA and protein detection in a single automated process. To support these workflows, default protocols have been integrated into BXD42. These protocols are tailored for the comprehensive Multiomic workflow previously mentioned. Following, you will find instructions on how to use these protocols for the full Multiomic assay, as well as modifications to adapt them for multiomic RNA/protein detection or RNA-only detection.

Workflow

Reagents: prefer, assign, and transfer to containers

⬇

Create and register the BOND Research Detection System (ONE TIME ONLY)

⬇

Create the RNAscope Multiomic Staining protocol

⬇

RNA Probe: create, assign, and transfer to container

⬇

Set up a study and slides for the assay

Create and register the BOND Research Detection System (one time only)

A BOND Research Detection System from Leica Biosystems is required to set up the RNAscope Multiomic LS Assays. The BOND Research Detection System can stain up to two hundred tests. For a two-part sequential stain assay, each part uses up one test from the kit.

1. Ensure that *Detection Wash reagent has been marked as **Preferred**.
2. Scan the barcode on the tray of a new BOND Research Detection System.
3. To set up a new detection system for the assay, enter **ACD LS Multiomic Detection Kit** in the Name text box.
4. Place one new BOND 30 mL Open container into position 1 of the Detection System tray.
5. Scan the container and select the registration name ***Detection Wash**. This container will be filled with 1X Bond Wash Solution
6. Select **Add.**

Notes:
When one Research Detection System is finished (up to two hundred tests), register a new detection system by scanning the barcode on the tray and select **ACD LS Multiomic Detection Kit** from the drop-down menu on the right. Creating the detection system needs to be performed only once on each BOND RX controller.

If you prefer to use a previously created Research Detection System, ensure that at least one reagent from the kit is included in each staining protocol and that the correct research kit is selected as the "Preferred Detection System."

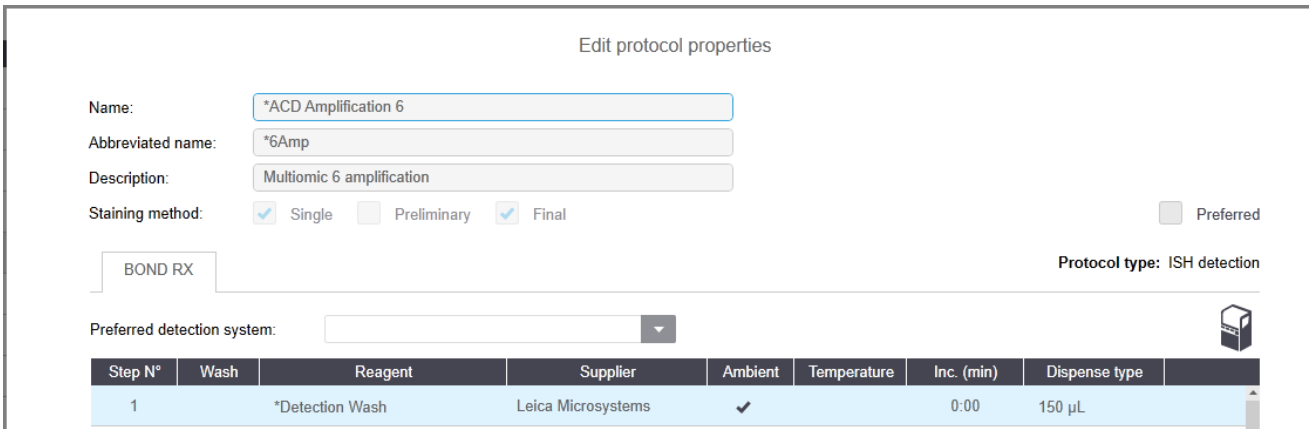
Staining protocols

IMPORTANT! Heated *Bond Wash solution steps come from the bulk reagents and are heated by the instrument. You cannot delete these steps. You may delete other wash steps.

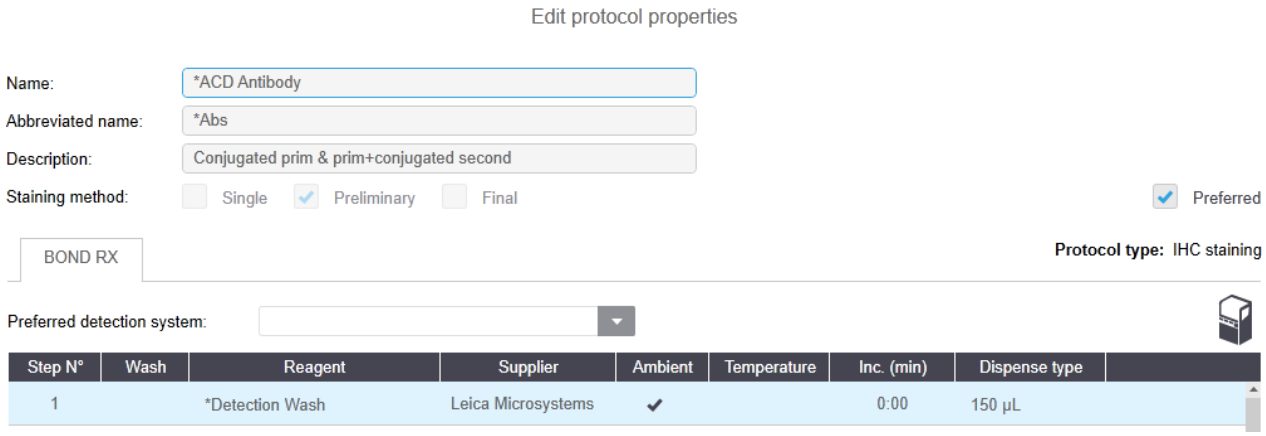
IMPORTANT! Different hardware configurations of the BOND RX can have different protocol step limits. This impacts the total number of trays that can be run, especially for 6-plex runs. Older instrument configurations can only execute 6-plex workflows on one tray due to their length. Follow the protocol steps listed in the following table. More recent instrument configurations can support a larger number of protocol steps, allowing more trays to be run. See **Appendix J. Protocol Step Limits** for more details.

Two staining protocol templates are pre-built into the BOND RX software to support each Multiomic Detection workflow:

- ***ACD Amplification 6 –** This protocol includes amplification and fluorescent detection steps for both hybridized RNA probes and bound ACD-provided primary and secondary antibodies. The protocol can be truncated depending on the number of markers and the channels used in your panel.



- ***ACD Antibody** – This protocol includes steps for ACD-provided antibody incubation, blocking, and post-fixation. If only RNAscope conjugated primaries or RNAscope conjugated secondaries are used in your panel, the protocol can be truncated to exclude the relevant steps.



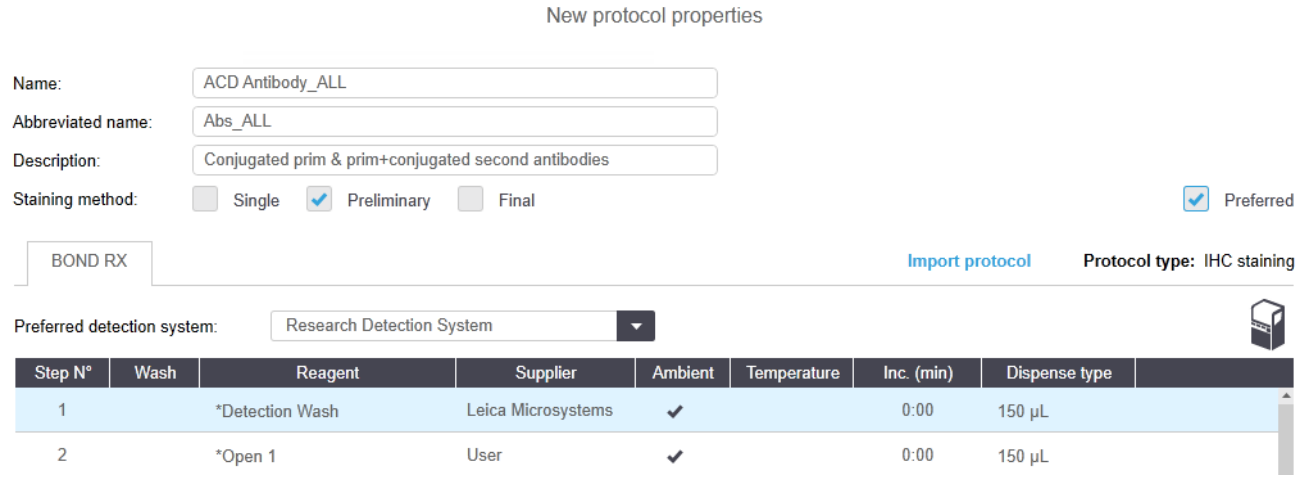
The following are examples of multiomic panel selections. The full protocols for these assays are provided in the appendices. Since both primary and secondary antibodies are used as cocktails, the protocol steps remain the same regardless of the number of antibodies you choose to include in your panel. Your ACD Field Application Specialist (FAS) can help implement procedures.

Assay 1. Full Multiomic protocol

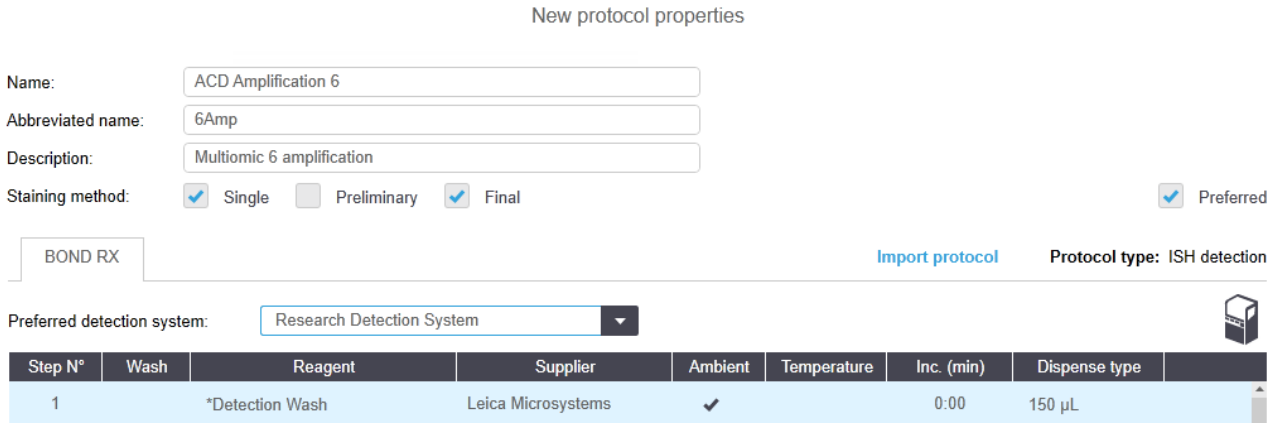
The full Multiomic assay includes mRNA, as well as RNAscope primary antibody and user provided primary along with RNAscope secondary antibodies. It is a 2-plex sequential stain using the *ACD Antibody and *ACD Amplification 6 Protocol Templates. To be used on a slide, both protocols require a BOND Research Detection System to be assigned to them as the "Preferred Detection System."

Copy, rename, and prefer the protocol:

1. Go to the **Protocol** setup screen.
2. Set the **Protocol Group** filter at the bottom left to **Staining** and the Protocol Type to **IHC staining**.
3. Set the **Preferred** filter at the bottom right of the screen to **All**.
4. Find the ***ACD Antibody** protocol, highlight it, and click on **Copy**.
5. Rename the protocol by deleting the * from both the name and abbreviated name.
6. Select your previously created BOND Research Detection System from the **Preferred Detection System** drop-down.
7. Ensure the **Preferred** box is selected.
8. Save the protocol (see **Appendix B** for all the steps in this protocol).



9. Repeat the previous steps for the ***ACD Amplification 6** protocol (see **Appendix A** for all the steps in this protocol).



Now both the protocols are ready for selection when adding your slides.

*If you have used an existing detection system, you will need to replace *Detection Wash with a reagent from your Research Detection System.

Assay 2. Protocol with mRNA and RNAscope primary antibodies

This assay is a 2-plex sequential stain using a modified *ACD Antibody Protocol Template and *ACD Amplification 6 Protocol template. Refer to the previous instructions to prepare the ACD Amplification 6 protocol.

1. Follow steps 1–4 from the "Full Multiomic" protocol procedure to create the modified *ACD Antibody protocol.
2. Rename the protocol with a name and abbreviated name of your choice.
3. Delete steps 8–26 from your copied and renamed protocol (see **Appendix C** for all the steps in this protocol).
4. Follow steps 6–9 from the full Multiomic protocol procedure.

New protocol properties

Name: ACD AB-Conjugated Antibody

Abbreviated name: Abs_CgAb

Description: Conjugated Primary Antibody

Staining method: ☐ Single ☑ Preliminary ☐ Final ☑ Preferred

BOND RX | Import protocol | **Protocol type:** IHC staining

Preferred detection system: Research Detection System

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
1		*Detection Wash	Leica Microsystems	✔		0:00	150 µL
2		*Open 1	User	✔		0:00	150 µL
3		*Open 1	User	✔		60:00	150 µL
8		*Co-Detection Antibody 3	ACD	✔		0:00	150 µL
9		*Co-Detection Antibody 3	ACD	✔		60:00	150 µL
15		*LS Rinse	Advanced Cell Diagnostics	✔		5:00	150 µL
16		*LS Rinse	Advanced Cell Diagnostics	✔		5:00	150 µL
21		*10% Neutral Buffered Formalin	Cell Signalling Technology	✔		30:00	150 µL

Assay 3. Protocol with mRNA and user primary with RNAscope secondary

This assay is a 2-plex sequential stain using a modified *ACD Antibody protocol template and *ACD Amplification 6 protocol template. Refer to the instructions in the full Multiomic protocol procedure to prepare the ACD Amplification 6 protocol.

1. Follow steps 1–4 from the "Full Multiomic" protocol procedure to create the modified *ACD Antibody protocol.
2. Rename the protocol with a name and abbreviated name of your choice.
3. On the instrument, delete steps 27–33 from your copied and renamed protocol (see **Appendix D** for all the steps in this protocol).

4. Follow steps 6–9 from the full Multiomic protocol procedure.

Assay 4. RNAscope primary + user primary w/ RNAscope secondary

This assay is for detecting proteins only and does not include mRNA detection. It uses RNAscope primary and user primary with RNAscope secondary antibody staining using the *ACD Antibody Protocol template followed by *ACD Amplification 6 protocol template. It does not require the use of an RNA probe. Refer to the instructions in the "Full Multiomic" assay procedure to prepare the ACD Antibody and ACD Amplification 6 protocols.

Assay 5. mRNA only

This assay is a single stain using *ACD Amplification 6 as the protocol template. Refer to the instructions in the "Full Multiomic" protocol procedure to prepare the ACD Amplification 6 protocol.

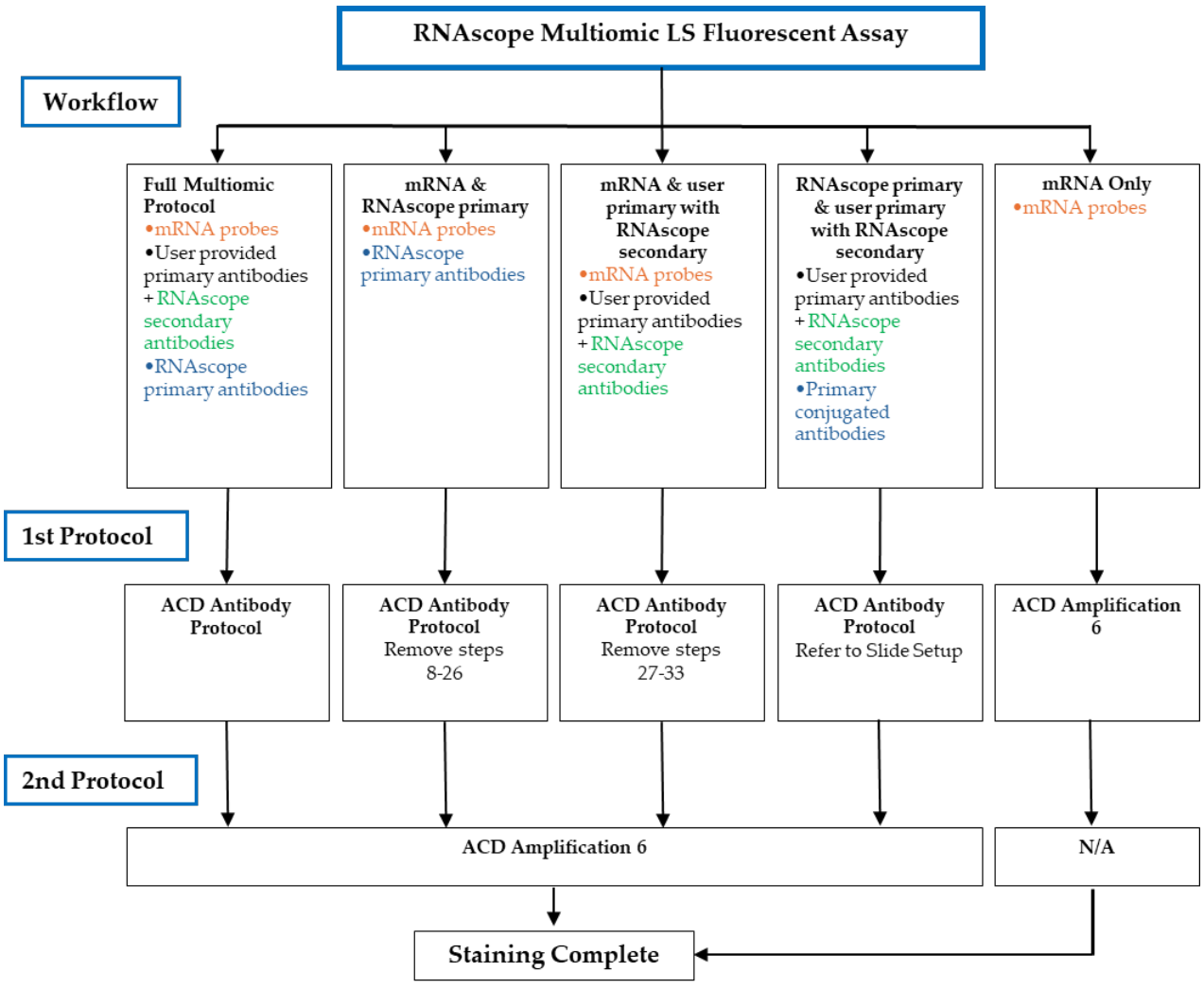


Figure 3. Diagram showing an overview of the supported protocols.

Prepare reagents

Prefer the reagents

1. Select the **Reagent Setup** icon at the top of the screen.
2. Select **All** for the filter at the bottom right of the screen.
3. Refer to the following table. Find these reagents in the Reagent Setup screen, open them, and mark them as **Preferred.**

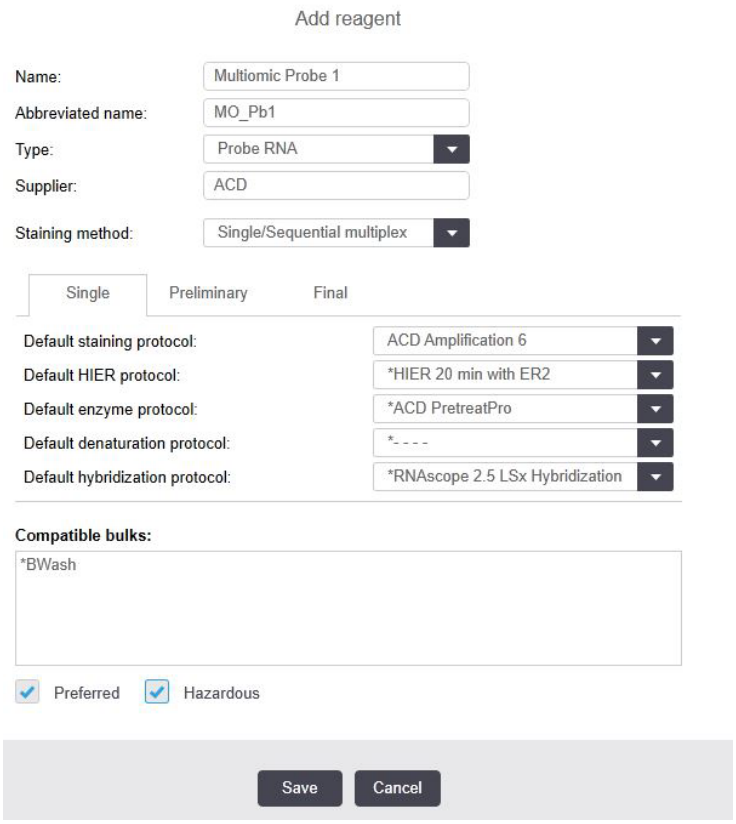
Reagent Name	Abbreviated Name
*RNAscope Multiomic LS AMP 1	*MO-Amp1
*RNAscope Multiomic LS AMP 2	*MO-Amp2
*RNAscope Multiomic LS AMP 3	*MO-Amp3
*RNAscope Multiomic LS HRP C1	*MO-HRPC1
*RNAscope Multiomic LS HRP C2	*MO-HRPC2
*RNAscope Multiomic LS HRP C3	*MO-HRPC3
*RNAscope Multiomic LS HRP C4	*MO-HRPC4
*RNAscope Multiomic LS HRP C5	*MO-HRPC5
*RNAscope Multiomic LS HRP C6	*MO-HRPC6
*RNAscope Multiomic LS HRP Blocker	*HRPBK
*Multiomic TSA-F1	*MO-TSAF1
*Multiomic TSA-F2	*MO-TSAF2
*Multiomic TSA-F3	*MO-TSAF3
*Multiomic TSA-F4	*MO-TSAF4
*Multiomic TSA-F5	*MO-TSAF5
*Antibody Blocker	*AbBlock
*Opal 780 Reagent	*Opal 780
*Co-Detection Antibody 1	*Cd-D Ab1
*Co-Detection Antibody 2	*Cd-D Ab2
*Co-Detection Antibody 3	*Cd-D Ab3
*RNAscope LS PretreatPro	*PretPro
*Opal TSA-DIG	*TSA-DIG
*DAPI	*DAPI
*LS Rinse	*LS Rinse
*Open 1	*Open1
*10% Neutral Buffered Formalin	*10% NBF
*Open 0 Haz	*Open0H

4. Select **Save**.

Create an RNA probe for assays with mRNA

1. Go to the Reagent setup screen (refer to the screenshots following this procedure).
2. Click on **Add**.
3. Enter in a name and an abbreviated name for your RNA Probe
4. Select **Probe RNA** from the Type drop-down menu.
5. Enter **ACD** into the Supplier text box.
6. Ensure **Single/Sequential MS** is selected.
7. For the **Single** tab, select the following:
   a. Default staining protocol = ACD Amplification 6
   b. Default HIER protocol = *HIER 20 min with ER2
   c. Default enzyme protocol = *ACD PretreatPro
   d. Default denaturation protocol = *----
   e. Default hybridization protocol = *RNAscope 2.5 LSx Hybridization
8. For the **Final** tab, select the following:
   a. Default staining protocol = ACD Amplification 6
   b. Default HIER protocol = *----
   c. Default enzyme protocol = *ACD PretreatPro
   d. Default denaturation protocol = *----
   e. Default hybridization protocol = *RNAscope 2.5 LSx Hybridization
9. Ensure that the **Hazardous** box is checked.
10. Click on **Save**.

Note: To run multiple probes on the BOND RX at the same time, create each probe separately with unique names and abbreviated names.



Reagent volumes required

Refer to the following table to calculate the volume of reagent required to run your assay. In addition to the slide volume, you need to add extra reagent to account for container dead volumes:

- 600 µL dead volume when using a BOND Titration container (6 mL)
- 1 mL dead volume when using a BOND 7 mL Open container.
- 2.5 mL dead volume when using a BOND 30 mL Open container.

Table 1. Reagent volumes

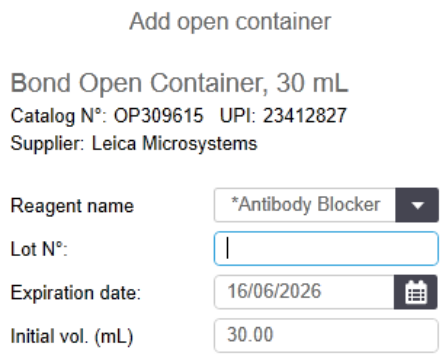
Reagent Name	# Dispenses/volume required per slide for each assay				
	Full assay	mRNA, RNAscope primary	mRNA, user primary w/ RNAscope secondary	RNAscope primary, user primary w/RNAscope secondary	mRNA assay 6-plex
*RNAscope LS PretreatPro/*LS Protease III	2/300 µL	2/300 µL	2/300 µL	2/300 µL	2/300 µL
*Open 0 Haz/ RNAscope Multiomic LS Hydrogen Peroxide	1/150 µL	1/150 µL	1/150 µL	1/150 µL	1/150 µL

Reagent Name	# Dispenses/volume required per slide for each assay				
	Full assay	mRNA, RNAscope primary	mRNA, user primary w/ RNAscope secondary	RNAscope primary, user primary w/RNAscope secondary	mRNA assay 6-plex
*Open 1	2/300 µL	2/300 µL	2/300 µL	2/300 µL	—
*10% Neutral Buffered Formalin	1/150 µL	1/150 µL	1/150 µL	1/150 µL	—
*Antibody Blocker	2/300 µL	—	—	2/300 µL	—
*Co-Detection Antibody 1	2/300 µL	—	2/300 µL	2/300 µL	—
*Co-Detection Antibody 2	2/300 µL	—	2/300 µL	2/300 µL	—
*Co-Detection Antibody 3	2/300 µL	2/300 µL	—	2/300 µL	—
*LS Rinse	6/900 µL	6/900 µL	6/900 µL	6/900 µL	4/600 µL
ACD RNA Probe (user-defined)	3/370 µL	3/370 µL	3/370 µL	—	3/370 µL
*RNAscope Multiomic LS AMP 1	2/300 µL	2/300 µL	2/300 µL	2/300 µL	2/300 µL
*RNAscope Multiomic LS AMP 2	2/300 µL	2/300 µL	2/300 µL	2/300 µL	2/300 µL
*RNAscope Multiomic LS AMP 3	2/300 µL	2/300 µL	2/300 µL	2/300 µL	2/300 µL
*RNAscope Multiomic LS HRP C1	2/300 µL	2/300 µL	2/300 µL	2/300 µL	2/300 µL
*RNAscope Multiomic LS HRP C2	2/300 µL	2/300 µL	2/300 µL	2/300 µL	2/300 µL
*RNAscope Multiomic LS HRP C3	2/300 µL	2/300 µL	2/300 µL	2/300 µL	2/300 µL
*RNAscope Multiomic LS HRP C4	2/300 µL	2/300 µL	2/300 µL	2/300 µL	2/300 µL
*RNAscope Multiomic LS HRP C5	2/300 µL	2/300 µL	2/300 µL	2/300 µL	2/300 µL
*RNAscope Multiomic LS HRP C6	2/300 µL	2/300 µL	2/300 µL	2/300 µL	2/300 µL
*RNAscope Multiomic LS HRP Blocker	12/1800 µL	12/1800 µL	12/1800 µL	12/1800 µL	12/1800 µL
*Multiomic TSA-F1	2/300 µL	2/300 µL	2/300 µL	2/300 µL	2/300 µL
*Multiomic TSA-F2	2/300 µL	2/300 µL	2/300 µL	2/300 µL	2/300 µL
*Multiomic TSA-F3	2/300 µL	2/300 µL	2/300 µL	2/300 µL	2/300 µL
*Multiomic TSA-F4	2/300 µL	2/300 µL	2/300 µL	2/300 µL	2/300 µL
*Multiomic TSA-F5	2/300 µL	2/300 µL	2/300 µL	2/300 µL	2/300 µL
*Opal 780 Reagent	2/300 µL	2/300 µL	2/300 µL	2/300 µL	2/300 µL
*Opal TSA-DIG	2/300 µL	2/300 µL	2/300 µL	2/300 µL	2/300 µL

Reagent Name	# Dispenses/volume required per slide for each assay				
	Full assay	mRNA, RNAscope primary	mRNA, user primary w/ RNAscope secondary	RNAscope primary, user primary w/RNAscope secondary	mRNA assay 6-plex
*DAPI	1/150 µL	1/150 µL	1/150 µL	1/150 µL	1/150 µL

Assign the reagents to Open Containers

1. Assay reagents need to be assigned to BOND Open Containers. Refer to your protocol to identify which reagents are required. Select an appropriately sized container, considering the volume required and whether reagents need to be freshly made for each run.
2. Label each BOND Open Container with the relevant reagent name.
3. Scan the front barcode of the BOND Open Container and select the reagent name from the drop-down menu.
4. Enter in a Lot Number (if required) and the Expiry Date.
5. Click on **Save**.



6. Repeat for each BOND Open Container.

Preparing the reagents

1. Fill the *Detection Wash open container with 1X BOND Wash Solution. The kit requires 150 µL reagent per slide, per stain (for example, sequential = 300 µL).
2. Carefully transfer all other RNAscope LS kit reagents ***except for the TSA buffer*** into their labelled, empty BOND Open containers. Transfer RNAscope Multiomic LS Hydrogen Peroxide into *Open 0 Haz container.

Note: Before each run, make sure you have enough of each reagent. See the table above for the reagent volume required per slide.

IMPORTANT! Do not introduce bubbles into the solutions by shaking the containers. To mix reagents, gently invert the containers several times. If bubbles are present, leave the containers out at room temperature until the bubbles dissipate.

Note: You may use your own DAPI or other counterstain in place of the DAPI provided in the kit.

3. Prepare the RNAscope LS target probe mix:
   a. Refer to **Table 1** to determine the volume of probe required. Make sure you add an extra amount for dead volume.
   b. Dilute the 50X C2, C3, C4, C5 and C6 probe stocks 1:50 into the Ready-To-Use C1 probe. For example, add 320 µL of each of 50X C2, 50X C3, 50X C4, 50X C5 and 50X C6 probe to a tube, then add enough C1 probe to bring the final volume to 16 mL.

Note: If not using C1 probe then dilute 50X C2, 50X C3, 50X C4, 50X C5 and 50X C6 probe to a 2.5 LS blank Probe Diluent (PN: 300048).

   c. Transfer the RNAscope Multiomic LS probe mix into the labeled BOND open container.

Note: The RNAscope probe mix is stable for one year at **2–8°C**.

4. If performing an RNA and protein staining, prepare RNAscope antibodies and ancillaries:
   a. Refer to **Table 1** to determine the volume of antibody mix required. Refer to the following table for suggested antibody concentrations. Make sure you add an extra amount for dead volume.

Reagent	BOND Reagent Container Name	Details (concentration, dilution)
Primary raw antibody mix	*Co-Detection Antibody 1	Multiomic Antibody Diluent (see **Appendix G**. RNAscope Antibody Concentration for concentration)
Secondary conjugated antibody mix	*Co-Detection Antibody 2	Multiomic Antibody Diluent (see **Appendix G** for concentration)
Primary conjugated antibody mix	*Co-Detection Antibody 3	Multiomic Antibody Diluent (see **Appendix G** for concentration)
Salmon sperm DNA	*Open 1	500 µg/mL, in Multiomic Antibody Diluent
Antibody blocker (Mouse and/or Rabbit IgG)	*Antibody Blocker	5 µg/mL, in Multiomic Antibody Diluent

   b. Dilute primary antibody conjugates together into one tube.
   c. Dilute the antibody conjugates using the Multiomic Antibody Diluent provided with the antibody.
   d. If using a secondary conjugate, use a separate container to dilute it.
   e. Add diluted antibody conjugates and ancillaries to the labeled BOND open containers.

IMPORTANT! Do not pool secondaries with primary antibody conjugates.

5. Prepare the Opal fluorophore dilutions:
   a. Refer to **Table 1** to determine the volume of Opal fluorophore required. Make sure you add an extra amount for dead volume.
   b. Dilute the Opal fluorophore stock using the TSA buffer provided in the reagent kit.
   c. Add the diluted fluorophores to the appropriate BOND open containers.

Reagents	Recommended dilution range	Dye Intensity
Opal 480*	1:750–1:3000 (in TSA buffer)	Highest

Reagents	Recommended dilution range	Dye Intensity
Opal 520*	1:750–1:3000 (in TSA buffer)	Highest
Opal 570*	1:750–1:3000 (in TSA buffer)	Medium
Opal 620*	1:750 -1:3000 (in TSA buffer)	Medium
Opal 690*	1:750–1:3000 (in TSA buffer)	Low
Opal TSA-DIG* (if using Opal 780)	1:750–1:3000 (in TSA buffer)	Lowest
Opal Polaris 780* (if using Opal 780)	1:187.5–1:750† (in Akoya or Bond diluent)	Lowest

* Reconstitute all Opals (except Opal Polaris 780) with 75 µL Dimethylsulfoxide (DMSO). Reconstitute Opal Polaris 780 with 300 µL double distilled water (ddH2O).

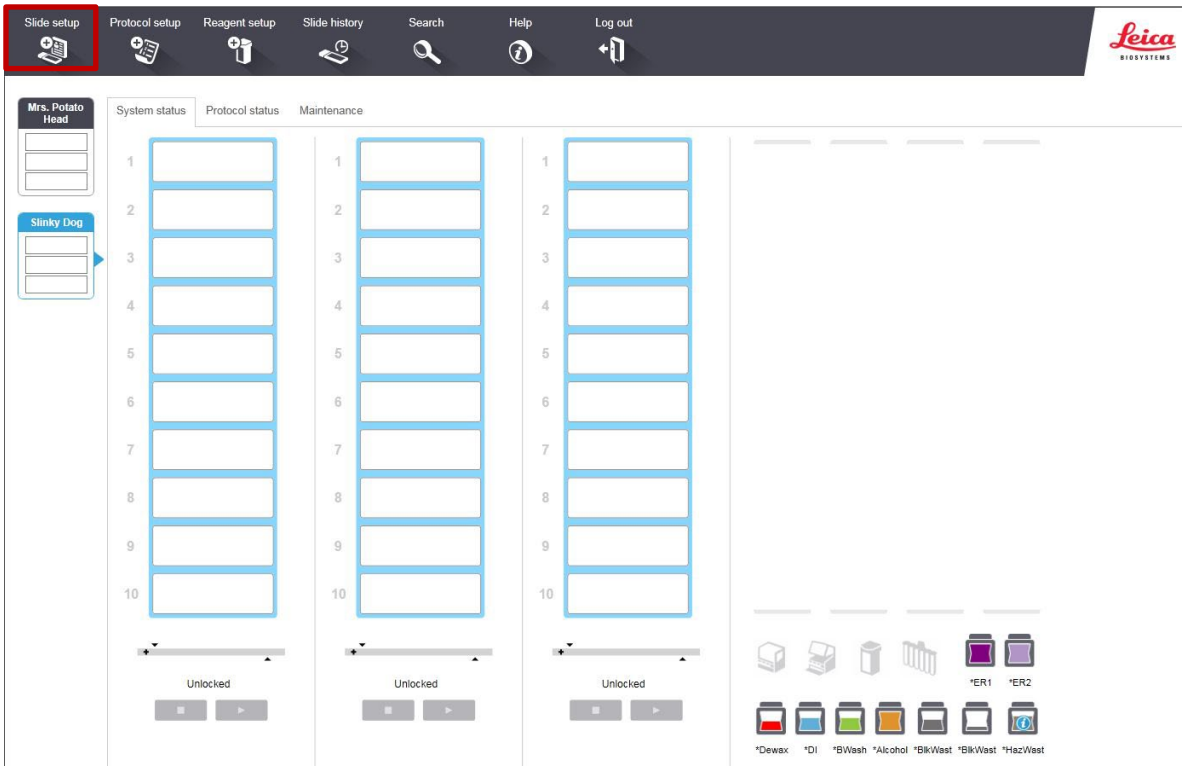
† We recommend keeping the dilution factors of Opal TSA-DIG and Opal Polaris 780 at a constant ratio. For example, when using 1:1500 dilution for Opal TSA-DIG, use 1:375 dilution for Opal Polaris 780. When using 1:750 dilution for Opal TSA-DIG, use 1:187.5 dilution for Opal Polaris 780.

For best results, assign brighter fluorophores to low expressors, develop high expressors last and low expressors first, and assign co-expressing markers to spectrally distinct fluorophores.

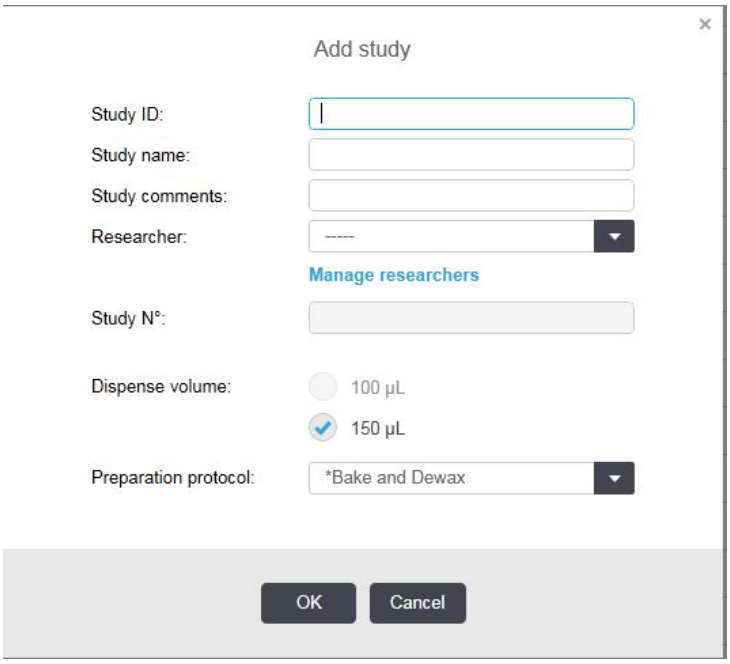
Setting up the study and slides

Creating the study

1. Select the **Slide setup** icon at the top of the screen.



2. Select **Add study** and enter a name in the Study ID field (keep the Dispense volume at 150 µL as shown).



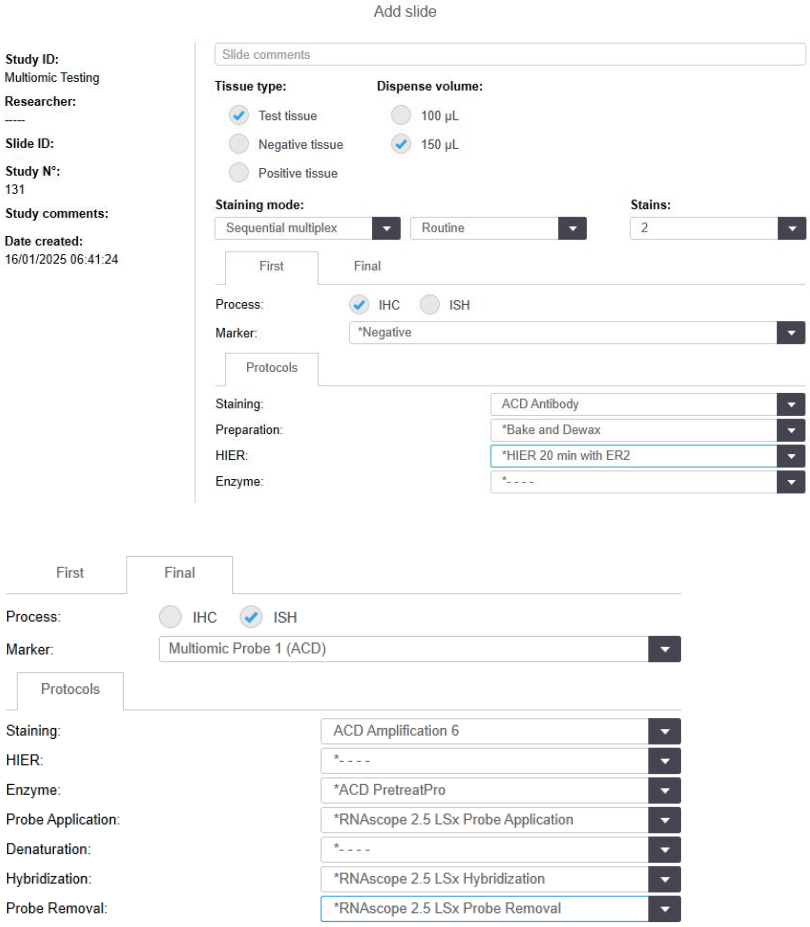
3. For FFPE tissues, select ***Bake and Dewax** as the Preparation protocol. For frozen tissues, select ***----** instead.
4. Select **OK**.

Creating the slides

1. Set up the slides for the "Full Multiomic" assay (Assay 1) with mRNA, primary and RNAscope secondary antibodies.
   a. Select **Add slide** to assign a protocol to each slide.
   b. Enter the tissue type and probe name under the Comments field.
   c. Select **Sequential Multiplex** as the default from the Staining mode drop-down menu.
   d. Select **2** from the Stains drop-down menu.
   e. On the **First** tab, select the following:
      i. Process = IHC
      ii. Marker = *Negative
      iii. Staining protocol = ACD Antibody
      iv. Preparation = *Bake and Dewax
      v. HIER = *HIER 20 min with ER2
      vi. Enzyme = *----
   f. On the **Final** tab, select the following:
      i. Process = ISH
      ii. Marker = Probe created in **Create an RNA probe for assays with mRNA**
      iii. Staining protocol = ACD Amplification 6
      iv. HIER = *----

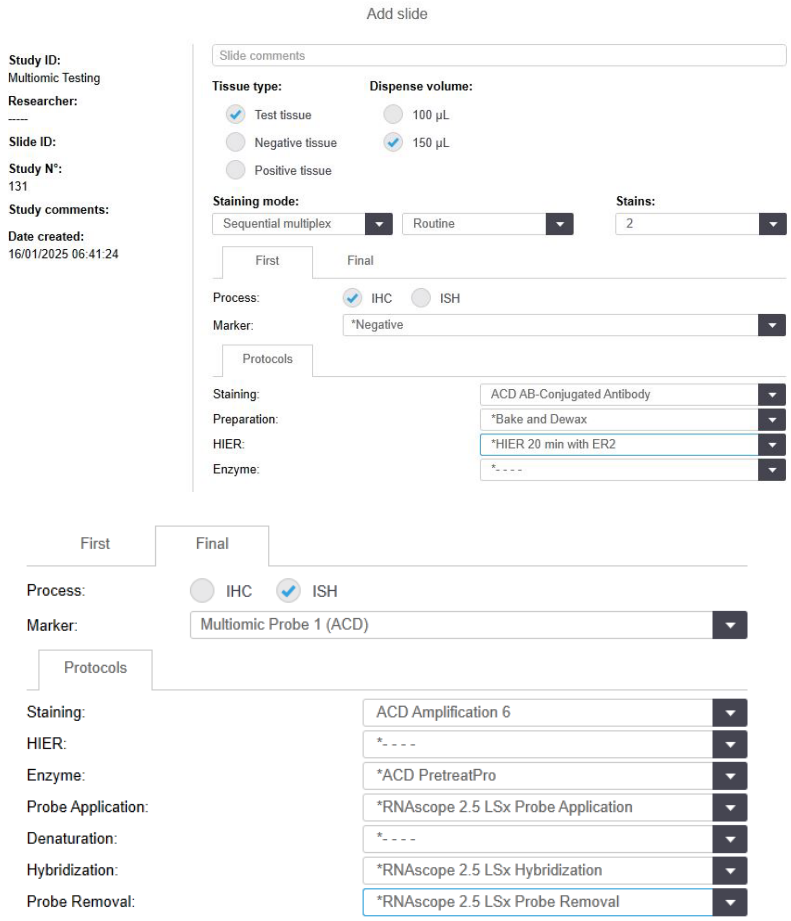
v. Enzyme = *ACD PretreatPro
vi. Probe Application = *RNAscope 2.5 LSx Probe Application
vii. Denaturation = *----
viii. Hybridization = *RNAscope 2.5 LSx Hybridization
ix. Probe Removal = *RNAscope 2.5 LSx Probe Removal

g. Select **Add slide** for each target probe and for each of the slides used in the run.
h. After adding all the slides to the study, select **Close** to return to the Slide setup screen.
i. Select **Print labels** to print barcodes to attach to the slides.



2. Set up the slides for Assay 2 with mRNA and RNAscope primaries:
   a. Select **Add slide** to assign a protocol to each slide.
   b. Enter the tissue type and probe name under the Comments field.
   c. Select **Sequential Multiplex** as default from the Staining mode drop-down menu.
   d. Select **2** from the Stains drop-down menu.
   e. On the **First** tab, select the following:
      i. Process = IHC
      ii. Marker = *Negative
      iii. Staining protocol = your 'First' protocol created above (modified from ACD Antibody)
      iv. Preparation = *Bake and Dewax
      v. HIER = *HIER 20 min with ER2
      vi. Enzyme = *----

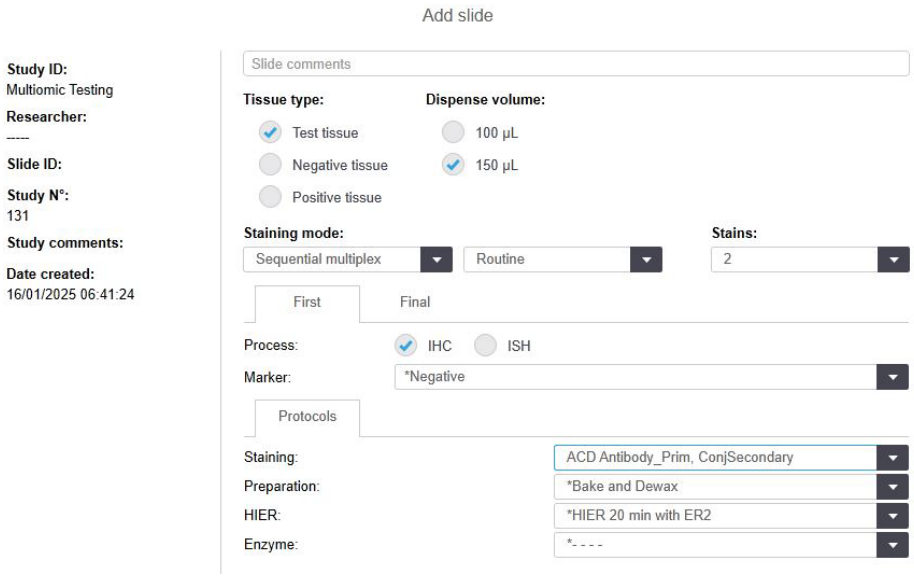
f. On the **Final** tab, select the following:
   vii. Process = ISH
   viii. Marker = Probe created in **Create an RNA probe for assays with mRNA**
   ix. Staining protocol = ACD Amplification 6
   x. HIER = *----
   xi. Enzyme = *ACD PretreatPro
   xii. Probe Application = *RNAscope 2.5 LSx Probe Application
   xiii. Denaturation = *----
   xiv. Hybridization = *RNAscope 2.5 LSx Hybridization
   xv. Probe Removal = *RNAscope 2.5 LSx Probe Removal
g. Select **Add slide** for each target probe and for each of the slides used in the run.
h. After adding all the slides to the study, select **Close** to return to the Slide setup screen.
i. Select **Print labels** to print barcodes to attach to the slides.

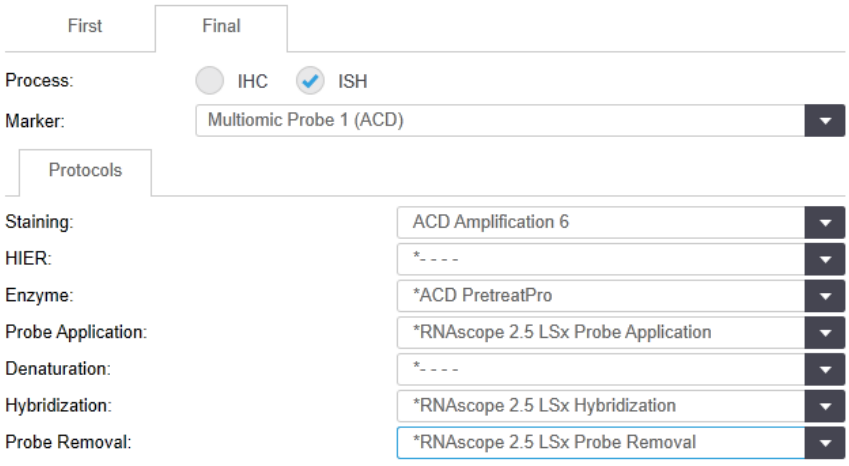


3. Set up the slides for Assay 3 with mRNA and user primaries and RNAscope secondaries:
   a. Select **Add slide** to assign a protocol to each slide.
   b. Enter the tissue type and probe name under the Comments field.
   c. Select **Sequential Multiplex** as default from the Staining mode drop-down menu.
   d. Select **2** from the Stains drop-down menu.
   e. On the **First** tab, select the following:
      i. Process = IHC
      ii. Marker = *Negative

iii. Staining protocol = your 'First' protocol created above (modified from ACD Antibody)
iv. Preparation = *Bake and Dewax
v. HIER = *HIER 20 min with ER2
vi. Enzyme = *----

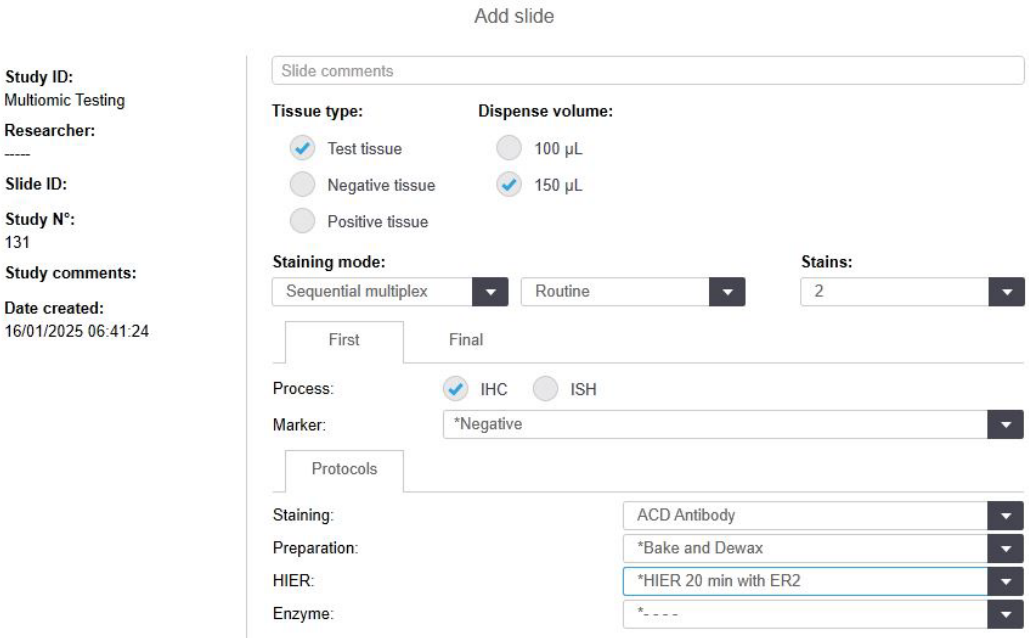
f. On the **Final** tab, select the following:
i. Process = ISH
ii. Marker = Probe created in **Create an RNA probe for assays with mRNA**
iii. Staining protocol = ACD Amplification 6
iv. HIER = *----
v. Enzyme = *ACD PretreatPro
vi. Probe Application = *RNAscope 2.5 LSx Probe Application
vii. Denaturation = *----
viii. Hybridization = *RNAscope 2.5 LSx Hybridization
ix. Probe Removal = *RNAscope 2.5 LSx Probe Removal

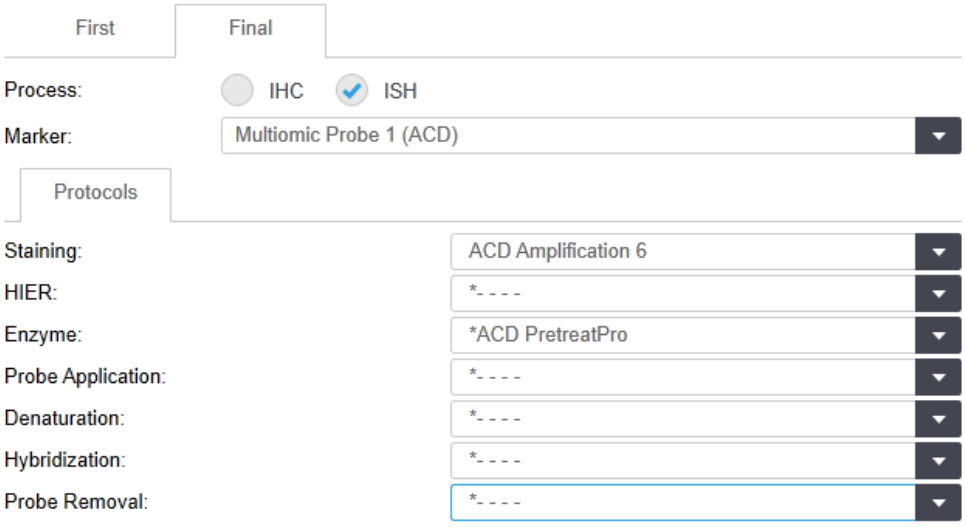




4. Set up the slides for assay using user primaries with RNAscope secondaries:
a. Select **Add slide** to assign a protocol to each slide.

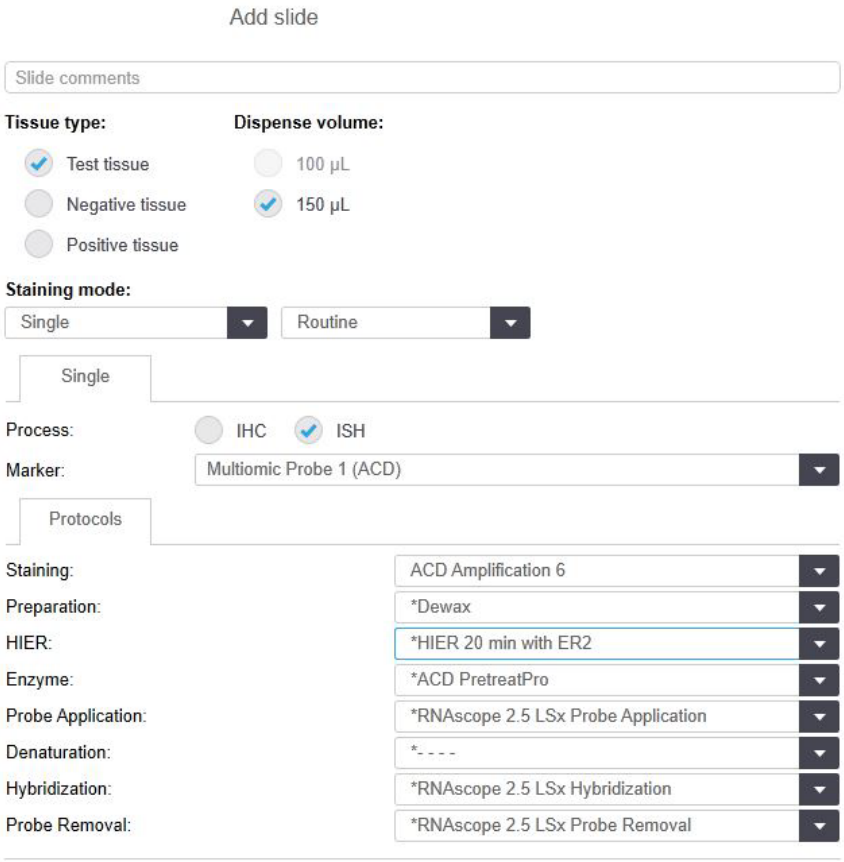
b. Enter the tissue type and probe name under the Comments field.
c. Keep **Sequential Multiplex** as default from the Staining mode drop-down menu.
d. Select **2** from the Stains drop-down menu.
e. On the **First** tab, select the following:
    i. Process = IHC
    ii. Marker = *Negative
    iii. Staining protocol = ACD Antibody
    iv. Preparation = *Bake and Dewax
    v. HIER = *HIER 20 min with ER2
    vi. Enzyme - *----
f. On the **Final** tab, select the following:
    i. Process = ISH
    ii. Marker = Because this selection is only used for slide generation, you can choose any probe.
    iii. Staining protocol = ACD Amplification 6
    iv. HIER = *----
    v. Enzyme = *ACD PretreatPro
    vi. Probe Application = *----
    vii. Denaturation = *----
    viii. Hybridization = *----
    ix. Probe Removal = *----
g. Select **Add slide** for each target probe and for each of the slides used in the run.
h. After adding all the slides to the study, select **Close** to return to the Slide setup screen.
i. Select **Print labels** to print barcodes to attach to the slides.





5. Set up the slides for the mRNA only assay.
   a. Select **Add slide** to assign a protocol to each slide.
   b. Enter the tissue type and probe name under the Comments field.
   c. Keep **Single** as default from the Staining mode drop-down menu.
      i. Process = ISH
      ii. Marker = Probe created in **Create an RNA probe for assays with mRNA**
      iii. Staining protocol = ACD Amplification 6
      iv. HIER = *HIER 20 min with ER2
      v. Enzyme – *ACD PretreatPro
      vi. Probe Application = *RNAscope 2.5 LSx Probe Application
      vii. Denaturation = *----
      viii. Hybridization = *RNAscope 2.5 LSx Hybridization
      ix. Probe Removal = *RNAscope 2.5 LSx Probe Removal
   d. Select **Add slide** for each target probe and for each of the slides used in the run.
   e. After adding all the slides to the study, select **Close** to return to the Slide setup screen.

f. Select **Print labels** to print barcodes to attach to the slides.



6. Set up slides for a mRNA only assay followed by sequential IF.
It is possible to also detect protein via traditional immunofluorescent (IF) methods following the RNAscope portion of the assay. In many cases, protocols and antibodies validated previously via IF can be applied as the second protocol in a sequential stain. Following are basic instructions, but further optimization may be required.

   a. Select **Add slide** to assign a protocol to each slide.
   b. Enter the tissue type and probe name under the Comments field.
   c. Keep **Sequential Multiplex** as default from the Staining mode drop-down menu.
   d. Select **2** from the Stains drop-down menu.
   e. On the **First** tab:
      i. Process = ISH
      ii. Marker = Probe created in **Create an RNA probe for assays with mRNA**
      iii. Staining protocol = Modified ACD Amplification 6. Delete the DAPI step and any channel detection steps that are not used from the protocol.
      iv. HIER = *HIER 20 min with ER2
      v. Enzyme – *ACD PretreatPro
      vi. Probe Application = *RNAscope 2.5 LSx Probe Application
      vii. Denaturation = *----
      viii. Hybridization = *RNAscope 2.5 LSx Hybridization
      ix. Probe Removal = *RNAscope 2.5 LSx Probe Removal

f. On the **Final** tab:
   i. Process = IHC
   ii. Marker = Select your antibody of choice
   iii. Staining protocol = Select your protocol of choice

6 Chapter 6. Run the Multiomic LS Assay

Prepare the instrument

1. Fill the large containers located in the bottom of the instrument with the BOND RX bulk reagents.
2. Dilute BOND Wash Solution 1:10.

Note: Insufficient bulk reagent volumes may lead to run failure.

IMPORTANT! Do not introduce bubbles into the solutions by shaking the containers. To mix reagents, gently invert the containers several times. If bubbles are present, leave the containers out at room temperature until the bubbles dissipate.

3. Use clean, dry covertiles for every run. Follow Leica Biosystems instructions to clean used covertiles with water, bleach, and ethanol. Air dry before reuse.
4. Before starting a run, empty bulk waste containers. Discard waste according to all local, state/provincial, and/or national regulations.

Start the run

1. Attach the barcoded labels to the slides and add the slides to the slide tray with the label sides facing up.
2. Add a covertile on top of each slide and verify placement and seating of each covertile.

Note: The rectangular-shaped neck of the covertile should fit into the groove of the slide tray.

3. Place the tray in the BOND RX and press the button to lock the slide tray onto the machine.
4. Once the slides have been scanned, select the **PLAY** (triangular) button on the software screen, located under the slide tray, to start the run. Alternatively, right-click on scanned label images, and select **Delayed Start** to start the run at a future time. Do not use Delayed Start with fixed or fresh-frozen tissue.
    - Full Multiomic assay with mRNA, RNAscope primary and user primary with RNAscope secondary antibodies = 26 hours for 10 slides
    - Multiomic assay with mRNA and RNAscope primary antibodies = 24 hours for 10 slides
    - Multiomic assay with mRNA, user primary with RNAscope secondary antibodies = 25 hours for 10 slides
    - Protein assay with RNAscope primary and user primary with RNAscope secondary antibodies = 22 hours for 10 slides
    - mRNA-only 6-plex assay = 20 hours for 10 slides

IMPORTANT! Before leaving the instrument unattended, ensure that the instrument is running successfully.

Complete the run and mount the samples

1. After the run is complete, press the button on the front of the instrument to unlock the slide trays.
2. Remove the slide trays, followed by the covertile and slides.
3. Add a drop of ProLong Gold Antifade Mountant to each slide. Avoid introducing bubbles.
4. Carefully place a glass coverslip on the slides, and dry overnight in the dark.
5. Store the slides at **4˚C** in the dark for up to two weeks.

7 Chapter 7. Evaluate the Results

Evaluate the samples

Examine tissue sections under a standard fluorescent microscope at 20–40X magnification. You may also use a confocal microscope.

- Assess tissue and cell morphology.
- Assess the negative control background first. One dot to every 10 cells displaying background staining per 20X microscope field is acceptable. Set the light source and exposure time of image acquisition to acceptable background levels.
- Assess positive control signal strength. Positive control signal should be visible as punctate dots within the cell at 20–40X magnification.

Scoring Guidelines

When used for RNA detection, the assay enables a semiquantitative scoring guideline utilizing the estimated number of punctate dots present within each cell boundary. An example of how to develop such a guideline for semi-quantitative assessment of mRNA staining intensity is shown for a gene with expression level varying between 1 to > 10 dots per cell.

Note: If your gene expression level is higher or lower than this range, you may need to scale the criteria accordingly.

mRNA scoring is divided into five categories:

Staining Score	Microscope Objective Scoring
0	No staining or less than 1 dot per 10 cells
1	1–3 dots per cell
2	4–9 dots per cell, no or very few dot clusters
3	10–15 dots per cell and/or <10% dots are in clusters
4	>15 dots per cell and/or >10% dots are in clusters

Example assay images

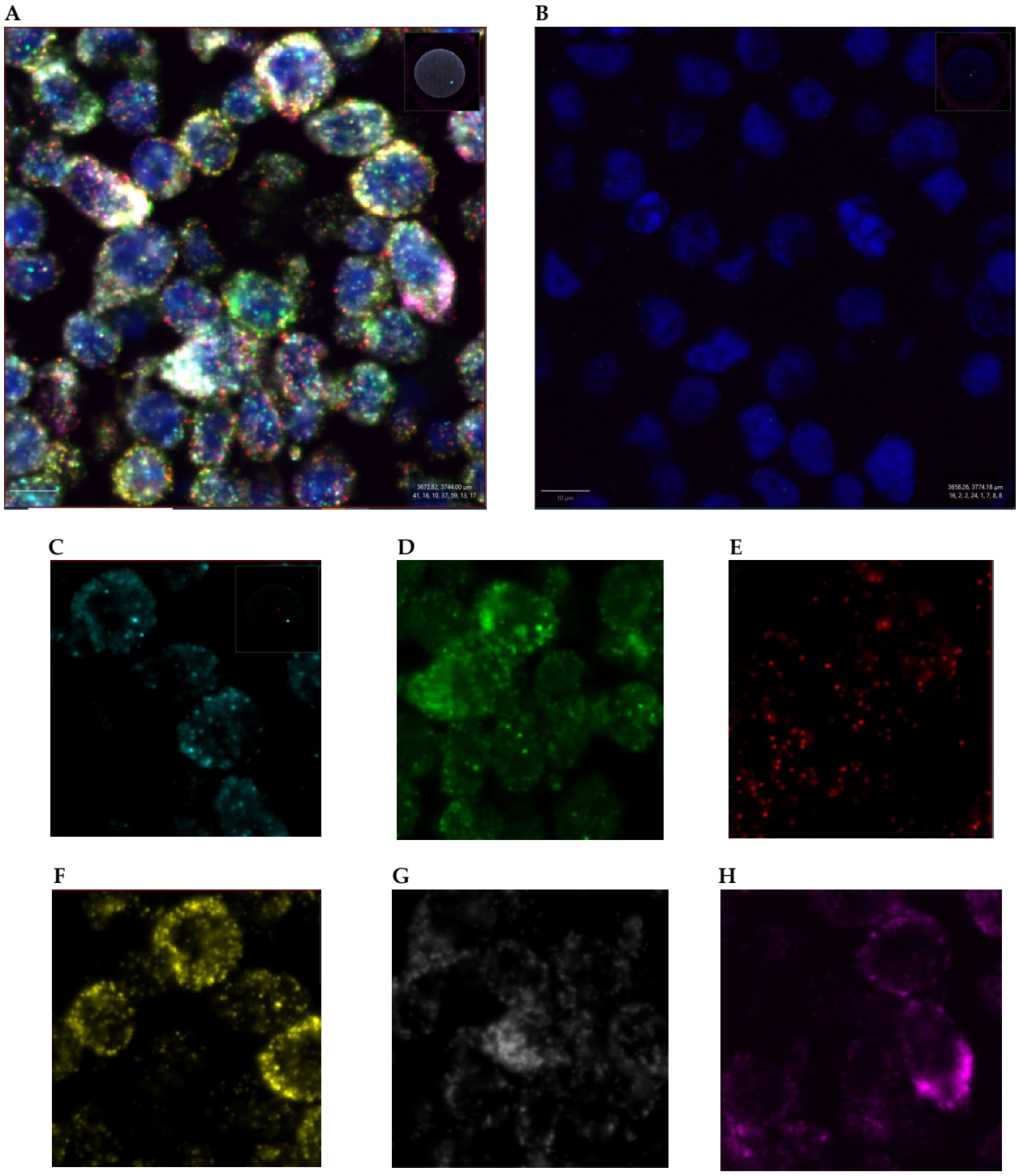


Figure 4. mRNA staining examples from FFPE HeLa cell pellet using RNAscope Multiomic LS Fluorescent Assay at 40X magnification. A) Positive control slide showing 6 RNA targets: *POLR2A* (teal), *ACTB* (green), *HPRT1* (red), *UBC* (yellow), *PPIB* (white), *TUBB* (pink). B) Negative control slide with *dapB*. C-H) Individual channels at higher magnification.

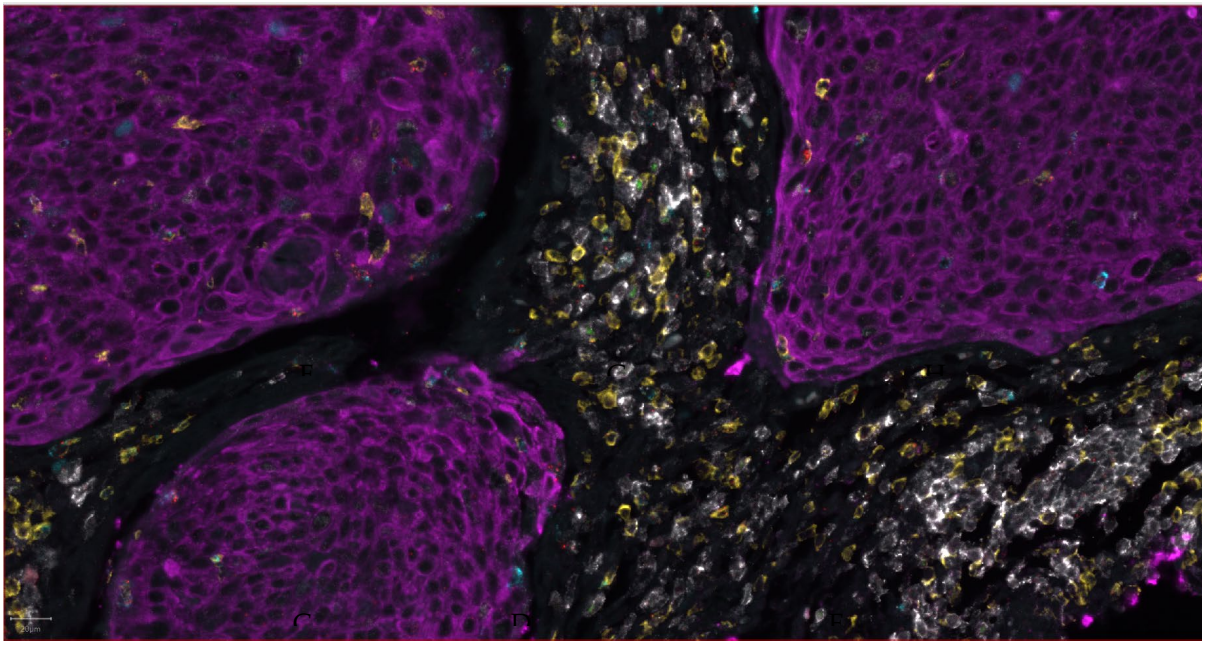


Figure 5. RNAscope Multiomic LS Fluorescent Assay detection of 2 RNAs and 3 proteins. *IFNG* (red) *GZMB* (blue) CD4 (yellow) CD8a (white) PanCK (pink) in Cervical cancer FFPE tissue at 20X magnification.

Troubleshooting

If you obtain less than satisfactory results, troubleshoot your assay by following these simple guidelines:

- Always use optimal fluorescent filter settings and imaging tools.
- If signal intensity is too low for your imaging tools, increase the fluorophore concentration.
- Use optimized fluorescence filter sets to reduce signal bleed-through. If you observe fluorescence bleed-through, reduce the fluorophore concentration of the affected channel and/or reduce the exposure time during image acquisition to avoid over-exposure.
- If your RNA ISH signal cannot be distinguished from autofluorescence in tissues with high autofluorescence, increase the fluorophore concentration.
- If you observe the presence of background staining, limit the sensitivity of image acquisition, or reduce the corresponding Opal fluorophore concentration. Always acquire images in the setting where background is minimally detected. If the signal-to-noise ratio is low due to high background, optimize pretreatment conditions. Contact ACD support for recommendations.
- The RNAscope Multiomic LS Fluorescence Assay uses only the Leica Biosystem BOND Research Detection System. Do not use BOND Polymer Refine DAB/Red Detection kits or any other chromogen kits.
- Do not shake the contents in the dispensers as this will form bubbles and may lead to weak or no staining. If bubbles are present, leave the containers out at room temperature until the bubbles dissipate.
- For troubleshooting information, please contact technical support at **support.acd@bio-techne.com**.

A Appendix A. Standard 6-plex Multiomic Detection Protocol

The ***ACD Amplification 6** template protocol is pre-programmed into Bond 6.0+ software versions on BXD 42 and above. Use as the basis for detection of RNA probes, ACD conjugated primaries, ACD conjugated secondaries. Detection steps can be removed for lower-plexed assays.

IMPORTANT! Heated *Bond Wash solution steps come from the bulk reagents and are heated by the instrument. You cannot delete these steps. You may delete other wash steps.

IMPORTANT! Different hardware configurations of the BOND RX can have different protocol-step limits. This impacts the total number of trays that can be run, especially for 6-plex runs. Older instrument configurations can only execute 6-plex workflows on one tray due to their length. Follow the protocol steps listed in the following table. More recent instrument configurations can support a larger number of protocol steps, allowing more trays to be run. See **Appendix J. Protocol Step Limits** for more details.

Step No.	Reagent	Step Type	Incubation Time	Temperature†
1	*Detection Wash (from Research Detection System)	Reagent	0 MIN	Ambient
2	*RNAscope Multiomic LS Amp 1	Reagent	1 MIN	42°C
3	*RNAscope Multiomic LS Amp 1	Reagent	30 MIN	42°C
4	*Bond Wash Solution	Wash	0 MIN	Ambient
5	*Bond Wash Solution	Wash	0 MIN	Ambient
6	*Bond Wash Solution	Wash	0 MIN	Ambient
7	*Bond Wash Solution	Wash	3 MIN	Ambient
8	*Bond Wash Solution	Wash	3 MIN	Ambient
9	*Bond Wash Solution	Wash	0 MIN	Ambient
10	*Bond Wash Solution	Wash	0 MIN	Ambient
11	*Bond Wash Solution	Wash	0 MIN	Ambient
12	*RNAscope Multiomic LS Rinse	Reagent	5 MIN	Ambient
13	*RNAscope Multiomic LS Rinse	Reagent	5 MIN	Ambient
14	*Bond Wash Solution	Wash	0 MIN	Ambient
15	*Bond Wash Solution	Wash	0 MIN	Ambient
16	*Bond Wash Solution	Open Wash	0 MIN	Ambient
17	*Bond Wash Solution	Wash	0 MIN	Ambient
18	*RNAscope Multiomic LS Amp 2	Reagent	1 MIN	42°C
19	*RNAscope Multiomic LS Amp 2	Reagent	30 MIN	42°C

Step No.	Reagent	Step Type	Incubation Time	Temperature†
20	*Bond Wash Solution	Wash	0 MIN	Ambient
21	*Bond Wash Solution	Wash	0 MIN	Ambient
22	*Bond Wash Solution	Wash	0 MIN	Ambient
23	*Bond Wash Solution	Wash	3 MIN	Ambient
24	*Bond Wash Solution	Wash	3 MIN	Ambient
25	*Bond Wash Solution	Wash	0 MIN	Ambient
26	*Bond Wash Solution	Wash	0 MIN	Ambient
27	*Bond Wash Solution	Wash	0 MIN	Ambient
28	*RNAscope Multiomic LS Rinse	Reagent	5 MIN	Ambient
29	*RNAscope Multiomic LS Rinse	Reagent	5 MIN	Ambient
30	*Bond Wash Solution	Wash	0 MIN	Ambient
31	*Bond Wash Solution	Wash	1 MIN	Ambient
32	*Bond Wash Solution	Open Wash	1 MIN	Ambient
33	*Bond Wash Solution	Wash	1 MIN	Ambient
34	*RNAscope Multiomic LS Amp 3	Reagent	1 MIN	42°C
35	*RNAscope Multiomic LS Amp 3	Reagent	15 MIN	42°C
36	*Bond Wash Solution	Wash	0 MIN	Ambient
37	*Bond Wash Solution	Wash	0 MIN	Ambient
38	*Bond Wash Solution	Wash	0 MIN	Ambient
39	*Bond Wash Solution	Wash	1 MIN	Ambient
40	*Bond Wash Solution	Wash	1 MIN	Ambient
41	*Bond Wash Solution	Wash	1 MIN	Ambient
42	*Bond Wash Solution	Open Wash	1 MIN	Ambient
43	*Bond Wash Solution	Wash	1 MIN	Ambient
44	*RNAscope Multiomic LS HRP C1	Reagent	1 MIN	42°C
45	*RNAscope Multiomic LS HRP C1	Reagent	15 MIN	42°C
46	*Bond Wash Solution	Wash	0 MIN	Ambient
47	*Bond Wash Solution	Wash	0 MIN	Ambient
48	*Bond Wash Solution	Wash	0 MIN	Ambient
49	*Bond Wash Solution	Wash	1 MIN	Ambient
50	*Bond Wash Solution	Wash	1 MIN	Ambient
51	*Bond Wash Solution	Wash	1 MIN	Ambient
52	*Bond Wash Solution	Open Wash	1 MIN	Ambient
53	*Bond Wash Solution	Wash	1 MIN	Ambient
54	*RNAscope Multiomic TSA-F1	Reagent	1 MIN	Ambient
55	*RNAscope Multiomic TSA-F1	Reagent	30 MIN	Ambient
56	*Bond Wash Solution	Wash	0 MIN	Ambient
57	*Bond Wash Solution	Wash	0 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature†
58	*Bond Wash Solution	Wash	0 MIN	Ambient
59	*Bond Wash Solution	Wash	1 MIN	Ambient
60	*Bond Wash Solution	Wash	1 MIN	Ambient
61	*Bond Wash Solution	Wash	1 MIN	Ambient
62	*Bond Wash Solution	Wash	1 MIN	Ambient
63	*RNAscope Multiomic LS HRP Blocker	Reagent	1 MIN	42°C
64	*RNAscope Multiomic LS HRP Blocker	Reagent	15 MIN	42°C
65	*Bond Wash Solution	Wash	0 MIN	Ambient
66	*Bond Wash Solution	Wash	0 MIN	Ambient
67	*Bond Wash Solution	Wash	0 MIN	Ambient
68	*Bond Wash Solution	Wash	1 MIN	Ambient
69	*Bond Wash Solution	Wash	1 MIN	Ambient
70	*Bond Wash Solution	Wash	1 MIN	Ambient
71	*Bond Wash Solution	Wash	1 MIN	Ambient
72	*RNAscope Multiomic HRP C2	Reagent	1 MIN	42°C
73	*RNAscope Multiomic HRP C2	Reagent	15 MIN	42°C
74	*Bond Wash Solution	Wash	0 MIN	Ambient
75	*Bond Wash Solution	Wash	0 MIN	Ambient
76	*Bond Wash Solution	Wash	0 MIN	Ambient
77	*Bond Wash Solution	Wash	1 MIN	Ambient
78	*Bond Wash Solution	Wash	1 MIN	Ambient
79	*Bond Wash Solution	Open Wash	1 MIN	Ambient
80	*Bond Wash Solution	Wash	1 MIN	Ambient
81	*RNAscope Multiomic TSA-F2	Reagent	1 MIN	Ambient
82	*RNAscope Multiomic TSA-F2	Reagent	30 MIN	Ambient
83	*Bond Wash Solution	Wash	0 MIN	Ambient
84	*Bond Wash Solution	Wash	0 MIN	Ambient
85	*Bond Wash Solution	Wash	0 MIN	Ambient
86	*Bond Wash Solution	Wash	1 MIN	Ambient
87	*Bond Wash Solution	Wash	1 MIN	Ambient
88	*Bond Wash Solution	Wash	1 MIN	Ambient
89	*Bond Wash Solution	Wash	1 MIN	Ambient
90	*RNAscope Multiomic LS HRP Blocker	Reagent	1 MIN	42°C
91	*RNAscope Multiomic LS HRP Blocker	Reagent	15 MIN	42°C
92	*Bond Wash Solution	Wash	0 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature†
93	*Bond Wash Solution	Wash	0 MIN	Ambient
94	*Bond Wash Solution	Wash	0 MIN	Ambient
95	*Bond Wash Solution	Wash	1 MIN	Ambient
96	*Bond Wash Solution	Wash	1 MIN	Ambient
97	*Bond Wash Solution	Wash	1 MIN	Ambient
98	*Bond Wash Solution	Wash	1 MIN	Ambient
99	*RNAscope Multiomic LS HRP C3	Reagent	1 MIN	42°C
100	*RNAscope Multiomic LS HRP C3	Reagent	15 MIN	42°C
101	*Bond Wash Solution	Wash	0 MIN	Ambient
102	*Bond Wash Solution	Wash	0 MIN	Ambient
103	*Bond Wash Solution	Wash	0 MIN	Ambient
104	*Bond Wash Solution	Wash	1 MIN	Ambient
105	*Bond Wash Solution	Wash	1 MIN	Ambient
106	*Bond Wash Solution	Open Wash	1 MIN	Ambient
107	*Bond Wash Solution	Wash	1 MIN	Ambient
108	*RNAscope Multiomic TSA-F3	Reagent	1 MIN	Ambient
109	*RNAscope Multiomic TSA-F3	Reagent	30 MIN	Ambient
110	*Bond Wash Solution	Wash	0 MIN	Ambient
111	*Bond Wash Solution	Wash	0 MIN	Ambient
112	*Bond Wash Solution	Wash	0 MIN	Ambient
113	*Bond Wash Solution	Wash	1 MIN	Ambient
114	*Bond Wash Solution	Wash	1 MIN	Ambient
115	*Bond Wash Solution	Wash	1 MIN	Ambient
116	*Bond Wash Solution	Wash	1 MIN	Ambient
117	*RNAscope Multiomic HRP Blocker	Reagent	1 MIN	42°C
118	*RNAscope Multiomic HRP Blocker	Reagent	15 MIN	42°C
119	*Bond Wash Solution	Wash	0 MIN	Ambient
120	*Bond Wash Solution	Wash	0 MIN	Ambient
121	*Bond Wash Solution	Wash	0 MIN	Ambient
122	*Bond Wash Solution	Wash	1 MIN	Ambient
123	*Bond Wash Solution	Wash	1 MIN	Ambient
124	*Bond Wash Solution	Wash	1 MIN	Ambient
125	*RNAscope Multiomic LS HRP C4	Reagent	1 MIN	42°C
126	*RNAscope Multiomic LS HRP C4	Reagent	15 MIN	42°C
127	*Bond Wash Solution	Wash	0 MIN	Ambient
128	*Bond Wash Solution	Wash	0 MIN	Ambient
129	*Bond Wash Solution	Wash	0 MIN	Ambient
130	*Bond Wash Solution	Wash	1 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature†
131	*Bond Wash Solution	Open Wash	1 MIN	Ambient
132	*Bond Wash Solution	Wash	1 MIN	Ambient
133	*RNAscope Multiomic TSA-F4	Reagent	1 MIN	Ambient
134	*RNAscope Multiomic TSA-F4	Reagent	30 MIN	Ambient
135	*Bond Wash Solution	Wash	0 MIN	Ambient
136	*Bond Wash Solution	Wash	0 MIN	Ambient
137	*Bond Wash Solution	Wash	0 MIN	Ambient
138	*Bond Wash Solution	Wash	1 MIN	Ambient
139	*Bond Wash Solution	Wash	1 MIN	Ambient
140	*Bond Wash Solution	Wash	1 MIN	Ambient
141	*Bond Wash Solution	Wash	1 MIN	Ambient
142	*RNAscope Multiomic LS HRP Blocker	Reagent	1 MIN	42°C
143	*RNAscope Multiomic LS HRP Blocker	Reagent	15 MIN	42°C
144	*Bond Wash Solution	Wash	0 MIN	Ambient
145	*Bond Wash Solution	Wash	0 MIN	Ambient
146	*Bond Wash Solution	Wash	0 MIN	Ambient
147	*Bond Wash Solution	Wash	1 MIN	Ambient
148	*Bond Wash Solution	Wash	1 MIN	Ambient
149	*Bond Wash Solution	Wash	1 MIN	Ambient
150	*RNAscope Multiomic LS HRP C5	Reagent	1 MIN	42°C
151	*RNAscope Multiomic LS HRP C5	Reagent	15 MIN	42°C
152	*Bond Wash Solution	Wash	0 MIN	Ambient
153	*Bond Wash Solution	Wash	0 MIN	Ambient
154	*Bond Wash Solution	Wash	0 MIN	Ambient
155	*Bond Wash Solution	Wash	1 MIN	Ambient
156	*Bond Wash Solution	Open Wash	1 MIN	Ambient
157	*Bond Wash Solution	Wash	1 MIN	Ambient
158	*RNAscope Multiomic TSA-F5	Reagent	1 MIN	Ambient
159	*RNAscope Multiomic TSA-F5	Reagent	30 MIN	Ambient
160	*Bond Wash Solution	Wash	0 MIN	Ambient
161	*Bond Wash Solution	Wash	0 MIN	Ambient
162	*Bond Wash Solution	Wash	0 MIN	Ambient
163	*Bond Wash Solution	Wash	1 MIN	Ambient
164	*Bond Wash Solution	Wash	1 MIN	Ambient
165	*Bond Wash Solution	Wash	1 MIN	Ambient
166	*Bond Wash Solution	Wash	1 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature†
167	*RNAscope Multiomic LS HRP Blocker	Reagent	1 MIN	42°C
168	*RNAscope Multiomic LS HRP Blocker	Reagent	15 MIN	42°C
169	*Bond Wash Solution	Wash	0 MIN	Ambient
170	*Bond Wash Solution	Wash	0 MIN	Ambient
171	*Bond Wash Solution	Wash	0 MIN	Ambient
172	*Bond Wash Solution	Wash	1 MIN	Ambient
173	*Bond Wash Solution	Wash	1 MIN	Ambient
174	*Bond Wash Solution	Wash	1 MIN	Ambient
175	*RNAscope Multiomic LS HRP C6	Reagent	1 MIN	42°C
176	*RNAscope Multiomic LS HRP C6	Reagent	15 MIN	42°C
177	*Bond Wash Solution	Wash	0 MIN	Ambient
178	*Bond Wash Solution	Wash	0 MIN	Ambient
179	*Bond Wash Solution	Wash	0 MIN	Ambient
180	*Bond Wash Solution	Wash	1 MIN	Ambient
181	*Bond Wash Solution	Open Wash	1 MIN	Ambient
182	*Bond Wash Solution	Wash	1 MIN	Ambient
183	*Opal TSA-DIG†	Reagent	1 MIN	Ambient
184	*Opal TSA-DIG†	Reagent	30 MIN	Ambient
185	*Bond Wash Solution	Wash	0 MIN	Ambient
186	*Bond Wash Solution	Wash	0 MIN	Ambient
187	*Bond Wash Solution	Wash	0 MIN	Ambient
188	*Bond Wash Solution	Wash	1 MIN	Ambient
189	*Bond Wash Solution	Wash	1 MIN	Ambient
190	*RNAscope Multiomic LS HRP Blocker	Reagent	1 MIN	42°C
191	*RNAscope Multiomic LS HRP Blocker	Reagent	15 MIN	42°C
192	*Bond Wash Solution	Wash	0 MIN	Ambient
193	*Bond Wash Solution	Wash	0 MIN	Ambient
194	*Bond Wash Solution	Wash	0 MIN	Ambient
195	*Bond Wash Solution	Wash	1 MIN	Ambient
196	*Bond Wash Solution	Wash	1 MIN	Ambient
197	*Polaris 780†	Reagent	1 MIN	Ambient
198	*Polaris 780†	Reagent	30 MIN	Ambient
199	*Bond Wash Solution	Wash	0 MIN	Ambient
200	*Bond Wash Solution	Wash	0 MIN	Ambient
201	*Bond Wash Solution	Wash	0 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature†
202	*Bond Wash Solution	Wash	1 MIN	Ambient
203	*Bond Wash Solution	Wash	1 MIN	Ambient
204	*RNAscope Multiomic LS DAPI‡	Reagent	10 MIN	Ambient
205	*Bond Wash Solution	Wash	0 MIN	Ambient
206	*Bond Wash Solution	Wash	0 MIN	Ambient
207	*Bond Wash Solution	Wash	0 MIN	Ambient

* Indicates reagent is hard coded in the software by Leica Biosystems.

† If Opal 780 is used, the fluorophore step must be last. TSA-DIG can still be performed earlier in the protocol, with the necessary HRP Blocker steps. Please consult your local FAS to adjust the amplification protocol properly.

‡ The standard protocol uses DAPI. Use BOND Wash instead of DAPI, if you are using DAPI offline or performing IHC steps afterwards on your samples.

B Appendix B. Antibody Protocol: Secondary + RNAscope Primary

The ***ACD Antibody** template protocol is pre-programmed into Bond 6.0+ software versions on BXD 42 and above. The full protocol (below) includes steps for both conjugated primary incubation as well as unconjugated primaries with conjugated secondaries. Because primary and secondary antibodies are combined into the same container, this protocol can be used for up to four conjugated primaries and up to two unconjugated primaries (rabbit and mouse).

Additional reagents to register:

Step No.	Reagent	Container name	Details (concentration, dilution)
1	Salmon sperm DNA	Open1	500 µg/ml, in multiomic antibody diluent
2	10% NBF	10% NBF	None
3	RNA probe	User Defined	1:50 in RNAscope probe diluent
4	Primary raw antibody mix	CoDetection Antibody 1	Multiomic antibody diluent (user defined)
5	Secondary conjugated antibody mix	CoDetection Antibody 2	Multiomic antibody diluent (see **Appendix G**. RNAscope Antibody Concentration for concentration)
6	Primary conjugated antibody mix	CoDetection Antibody 3	Multiomic antibody diluent (see **Appendix G**. RNAscope Antibody Concentration for concentration)
7	Antibody blocker (mouse and/or rabbit IgG)	Antibody Blocker	5 µg/ml, in multiomic antibody diluent

IMPORTANT! When using RNAscope conjugated secondary antibodies, they can bind to RNAscope primary conjugated antibodies, causing cross-detection. To prevent this, use an antibody blocker. Add 5 µg/ml of both mouse IgG (Mouse IgG2A Isotype Control, R&D Systems MAB003) and rabbit IgG (Normal Rabbit IgG Control, R&D Systems MAB1050) in multiomic antibody diluent for 30 minutes at room temperature between the incubations of secondary and primary conjugated antibodies. This step will help eliminate cross-reactivity. Ensure the IgG blocker is placed in an open container registered as *Antibody Blocker for the pre-programmed protocol.

Step No.	Reagent	Step Type	Incubation Time	Temperature†
1	*Detection Wash (from Research Detection System)	Reagent	0 MIN	Ambient
2	*Open 1 (will contain the Salmon Sperm DNA)	Reagent	0 MIN	Ambient
3	*Open 1 (will contain the Salmon Sperm DNA)	Reagent	60 MIN	Ambient
4	*Bond Wash Solution	Wash	0 MIN	Ambient
5	*Bond Wash Solution	Wash	0 MIN	Ambient
6	*Bond Wash Solution	Wash	0 MIN	Ambient
7	*Bond Wash Solution	Wash	0 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature†
8	*Co-Detection Antibody 1	Reagent	0 MIN	Ambient
9	*Co-Detection Antibody 1	Reagent	60 MIN	Ambient
10	*Bond Wash Solution	Wash	0 MIN	Ambient
11	*Bond Wash Solution	Wash	0 MIN	Ambient
12	*Bond Wash Solution	Wash	1 MIN	Ambient
13	*Bond Wash Solution	Wash	3 MIN	Ambient
14	*Co-Detection Antibody 2	Reagent	0 MIN	Ambient
15	*Co-Detection Antibody 2	Reagent	30 MIN	Ambient
16	*Bond Wash Solution	Wash	0 MIN	Ambient
17	*Bond Wash Solution	Wash	0 MIN	Ambient
18	*Bond Wash Solution	Wash	1 MIN	Ambient
19	*Bond Wash Solution	Wash	1 MIN	Ambient
20	*Antibody Blocker	Reagent	0 MIN	Ambient
21	*Antibody Blocker	Reagent	30 MIN	Ambient
22	*Bond Wash Solution	Wash	0 MIN	Ambient
23	*Bond Wash Solution	Wash	0 MIN	Ambient
24	*Bond Wash Solution	Reagent	1 MIN	Ambient
25	*Bond Wash Solution	Reagent	3 MIN	Ambient
26	*Bond Wash Solution	Wash	3 MIN	Ambient
27	*Co-Detection Antibody 3	Reagent	0 MIN	Ambient
28	*Co-Detection Antibody 3	Reagent	60 MIN	Ambient
29	*Bond Wash Solution	Wash	0 MIN	Ambient
30	*Bond Wash Solution	Reagent	0 MIN	Ambient
31	*Bond Wash Solution	Reagent	1 MIN	Ambient
32	*Bond Wash Solution	Wash	3 MIN	Ambient
33	*Bond Wash Solution	Wash	3 MIN	Ambient
34	*LS Rinse	Reagent	5 MIN	Ambient
35	*LS Rinse	Reagent	5 MIN	Ambient
36	*Bond Wash Solution	Wash	0 MIN	Ambient
37	*Bond Wash Solution	Wash	0 MIN	Ambient
38	*Bond Wash Solution	Open Wash	0 MIN	Ambient
39	*Bond Wash Solution	Wash	0 MIN	Ambient
40	*10% Neutral Buffered Formalin	Reagent	30 MIN	Ambient
41	*Bond Wash Solution	Wash	0 MIN	Ambient
42	*Bond Wash Solution	Wash	0 MIN	Ambient
43	*Bond Wash Solution	Wash	0 MIN	Ambient
44	*Bond Wash Solution	Wash	3 MIN	Ambient
45	*Bond Wash Solution	Wash	3 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature†
46	*Bond Wash Solution	Wash	0 MIN	Ambient
47	*Bond Wash Solution	Reagent	0 MIN	Ambient
48	*Bond Wash Solution	Reagent	0 MIN	Ambient

* Indicates reagent is hard coded in the software by Leica Biosystems.

C Appendix C. RNAscope Primary Antibody

The ***ACD Antibody** template protocol is pre-programmed into Bond software versions 6.0 and higher on BXD 42and above. The template should be truncated to include just the steps in the following protocol. Multiple conjugated primaries can be added into the same open container.

Reagent	Container name	Details (concentration, dilution)
Salmon sperm DNA	Open1	500 µg/mL in multiomic antibody diluent
10% NBF	10% NBF	None
Antibody mix	CoDetection Antibody 3	Multiomic antibody diluent (see **Appendix G**. RNAscope Antibody Concentration for concentration)

Step No.	Reagent	Step Type	Incubation Time	Temperature†
1	*Detection Wash (from Research Detection System)	Reagent	0 MIN	Ambient
2	*Open 1 (will contain the Salmon Sperm DNA)	Reagent	0 MIN	Ambient
3	*Open 1 (will contain the Salmon Sperm DNA)	Reagent	60 MIN	Ambient
4	*Bond Wash Solution	Wash	0 MIN	Ambient
5	*Bond Wash Solution	Wash	0 MIN	Ambient
6	*Bond Wash Solution	Wash	0 MIN	Ambient
7	*Bond Wash Solution	Wash	0 MIN	Ambient
8	*Co-Detection Antibody 3	Reagent	0 MIN	Ambient
9	*Co-Detection Antibody 3	Reagent	60 MIN	Ambient
10	*Bond Wash Solution	Wash	0 MIN	Ambient
11	*Bond Wash Solution	Reagent	0 MIN	Ambient
12	*Bond Wash Solution	Reagent	1 MIN	Ambient
13	*Bond Wash Solution	Wash	3 MIN	Ambient
14	*Bond Wash Solution	Wash	3 MIN	Ambient
15	*LS Rinse	Reagent	5 MIN	Ambient
16	*LS Rinse	Reagent	5 MIN	Ambient
17	*Bond Wash Solution	Wash	0 MIN	Ambient
18	*Bond Wash Solution	Wash	0 MIN	Ambient
19	*Bond Wash Solution	Open Wash	0 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature†
20	*Bond Wash Solution	Wash	0 MIN	Ambient
21	*10% Neutral Buffered Formalin	Reagent	30 MIN	Ambient
22	*Bond Wash Solution	Wash	0 MIN	Ambient
23	*Bond Wash Solution	Wash	0 MIN	Ambient
24	*Bond Wash Solution	Wash	0 MIN	Ambient
25	*Bond Wash Solution	Wash	3 MIN	Ambient
26	*Bond Wash Solution	Wash	3 MIN	Ambient
27	*Bond Wash Solution	Wash	0 MIN	Ambient
28	*Bond Wash Solution	Reagent	0 MIN	Ambient
29	*Bond Wash Solution	Reagent	0 MIN	Ambient

Appendix D. Secondary Antibody

The ***ACD Antibody** template protocol is pre-programmed into Bond software versions 6.0 and higher on BXD 42and above. The template should be truncated to include just the steps in the following protocol. The same protocol can be used for rabbit, mouse, or a cocktail of primary antibodies from both species and their respective conjugated secondaries.

Additional reagents to register:

Step No.	Reagent	Container name	Details (concentration, dilution)
1	Salmon sperm DNA	Open1	500 µg/ml, in multiomic antibody diluent
2	10% NBF	10% NBF	None
4	Primary raw antibody mix	CoDetection Antibody 1	Multiomic antibody diluent (user defined)
5	Secondary conjugated antibody mix	CoDetection Antibody 2	Multiomic antibody diluent (see **Appendix G**. RNAscope Antibody Concentration for concentration)

When using RNAscope conjugated secondary antibodies, they can bind to RNAscope primary conjugated antibodies, causing cross-detection. To prevent this, use an antibody blocker. Add 5 µg/ml of both mouse IgG (Mouse IgG2A Isotype Control, R&D Systems MAB003) and rabbit IgG (Normal Rabbit IgG Control, R&D Systems MAB1050) in multiomic antibody diluent for 30 minutes at room temperature between the incubations of secondary and primary conjugated antibodies. This step will help eliminate cross-reactivity. Ensure the IgG blocker is placed in an open container registered as *Antibody Blocker for the pre-programmed protocol.

Step No.	Reagent	Step Type	Incubation Time	Temperature†
1	*Detection Wash (from Research Detection System)	Reagent	0 MIN	Ambient
2	*Open 1 (will contain the Salmon Sperm DNA)	Reagent	0 MIN	Ambient
3	*Open 1 (will contain the Salmon Sperm DNA)	Reagent	60 MIN	Ambient
4	*Bond Wash Solution	Wash	0 MIN	Ambient
5	*Bond Wash Solution	Wash	0 MIN	Ambient
6	*Bond Wash Solution	Wash	0 MIN	Ambient
7	*Bond Wash Solution	Wash	0 MIN	Ambient
8	*Co-Detection Antibody 1	Reagent	0 MIN	Ambient
9	*Co-Detection Antibody 1	Reagent	60 MIN	Ambient
10	*Bond Wash Solution	Wash	0 MIN	Ambient
11	*Bond Wash Solution	Wash	0 MIN	Ambient
12	*Bond Wash Solution	Wash	1 MIN	Ambient

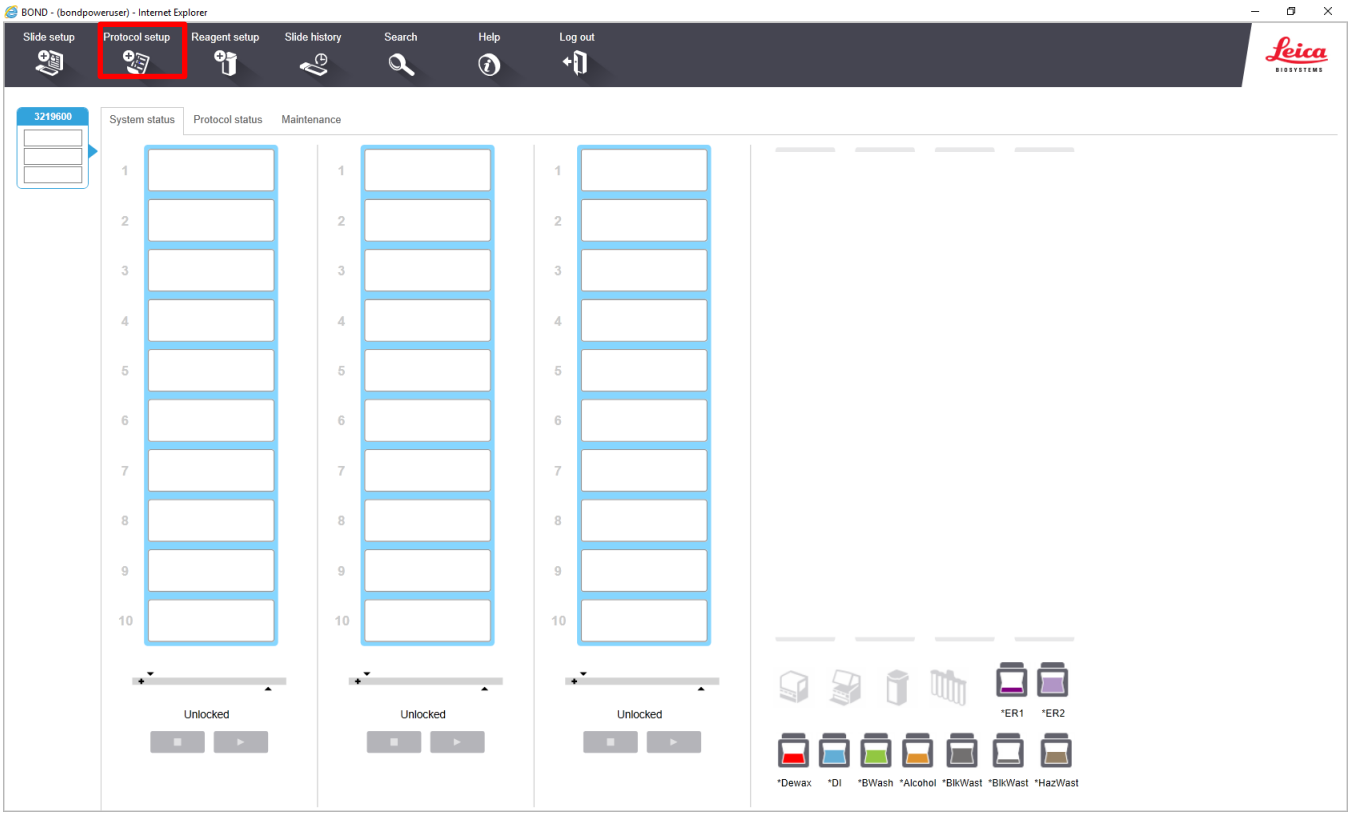
Step No.	Reagent	Step Type	Incubation Time	Temperature†
13	*Bond Wash Solution	Wash	3 MIN	Ambient
14	*Co-Detection Antibody 2	Reagent	0 MIN	Ambient
15	*Co-Detection Antibody 2	Reagent	30 MIN	Ambient
16	*Bond Wash Solution	Wash	0 MIN	Ambient
17	*Bond Wash Solution	Wash	0 MIN	Ambient
18	*Bond Wash Solution	Wash	1 MIN	Ambient
19	*Bond Wash Solution	Wash	1 MIN	Ambient
27	*LS Rinse	Reagent	5 MIN	Ambient
28	*LS Rinse	Reagent	5 MIN	Ambient
29	*Bond Wash Solution	Wash	0 MIN	Ambient
30	*Bond Wash Solution	Wash	0 MIN	Ambient
31	*Bond Wash Solution	Open Wash	0 MIN	Ambient
32	*Bond Wash Solution	Wash	0 MIN	Ambient
33	*10% Neutral Buffered Formalin	Reagent	30 MIN	Ambient
34	*Bond Wash Solution	Wash	0 MIN	Ambient
35	*Bond Wash Solution	Wash	0 MIN	Ambient
36	*Bond Wash Solution	Wash	0 MIN	Ambient
37	*Bond Wash Solution	Wash	3 MIN	Ambient
38	*Bond Wash Solution	Wash	3 MIN	Ambient
39	*Bond Wash Solution	Wash	0 MIN	Ambient
40	*Bond Wash Solution	Reagent	0 MIN	Ambient
41	*Bond Wash Solution	Reagent	0 MIN	Ambient

Appendix E. Edit the Epitope Retrieval Protocol

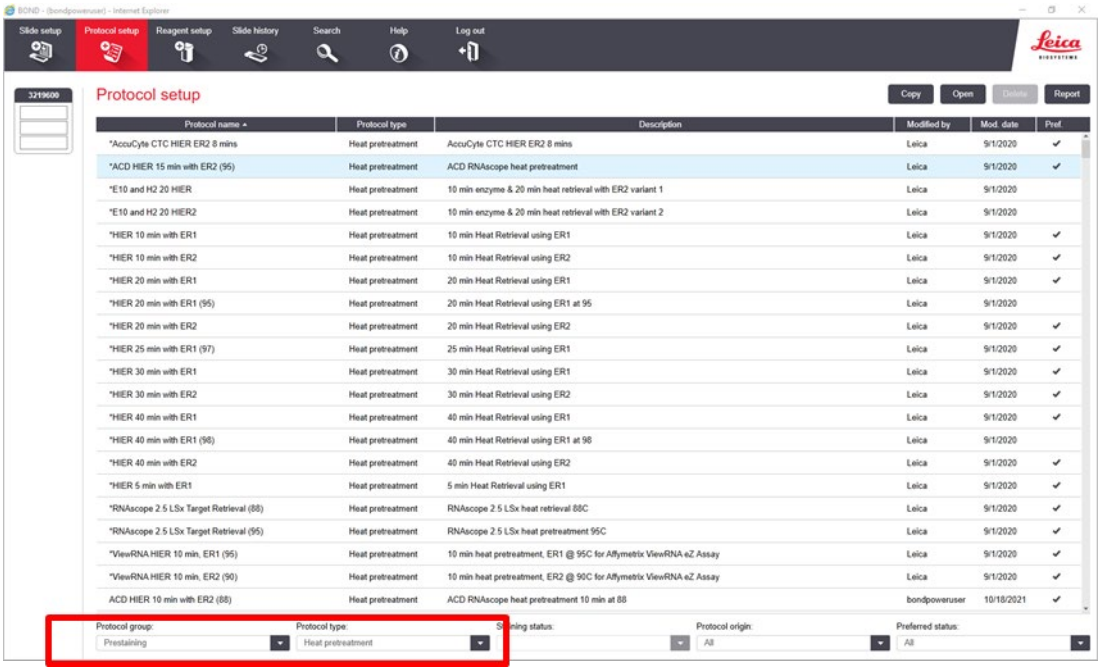
The following example shows how to edit the Epitope Retrieval procedure from within the software. For mild pretreatment with PretreatPro, 92°C heat retrieval is recommended.

Create a prestaining protocol

1. For RNAscope, ER 2 temperature varies between **100°C** and **92°C** depending on the tissue type used.
2. Open the BOND RX software and click on the **Protocol setup icon** as shown**.**



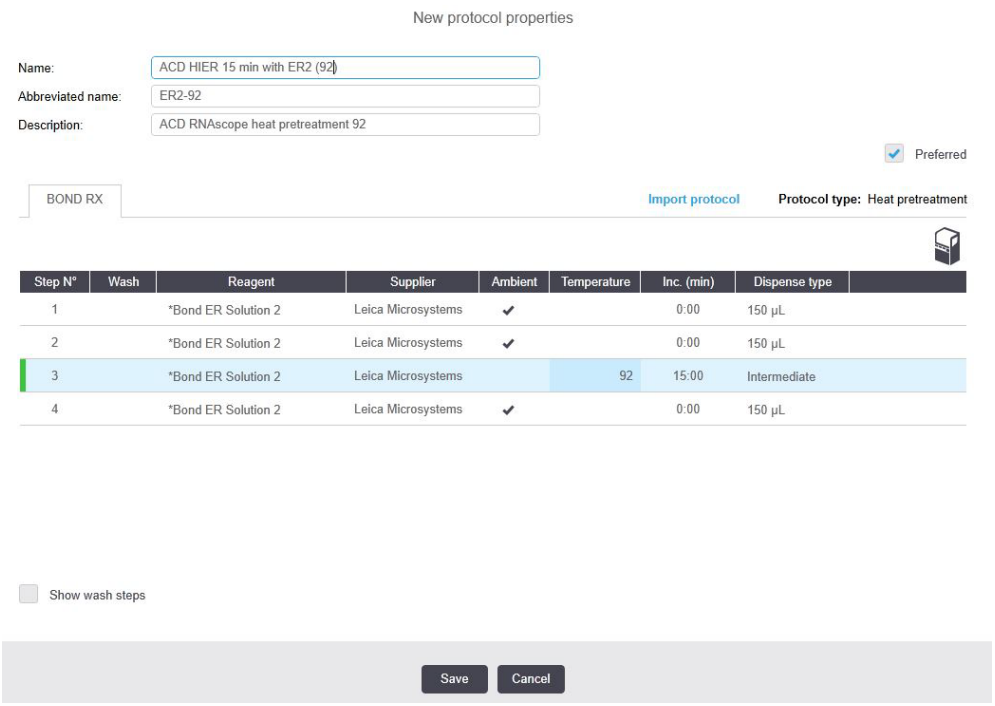
2. Select **Prestaining** under the Protocol group menu and **Heat pretreatment** under the Protocol type menu to access the heat pretreatment protocols.



3. Highlight the ***HIER 20 min with ER2** protocol**.** Select **Copy.**

Note: ER2 = Epitope Retrieval 2.

4. Rename the protocol as **ACD HIER 15 min with ER2 (92)**.
5. Rename the Abbreviated name as **ER2-92**.
6. Rename the Description to **ACD RNAscope heat pretreatment 92**.
7. Highlight the third ***BOND ER Solution 2** step (see above) and change temperature to **92°C**.
8. Change the time to **15 min**.

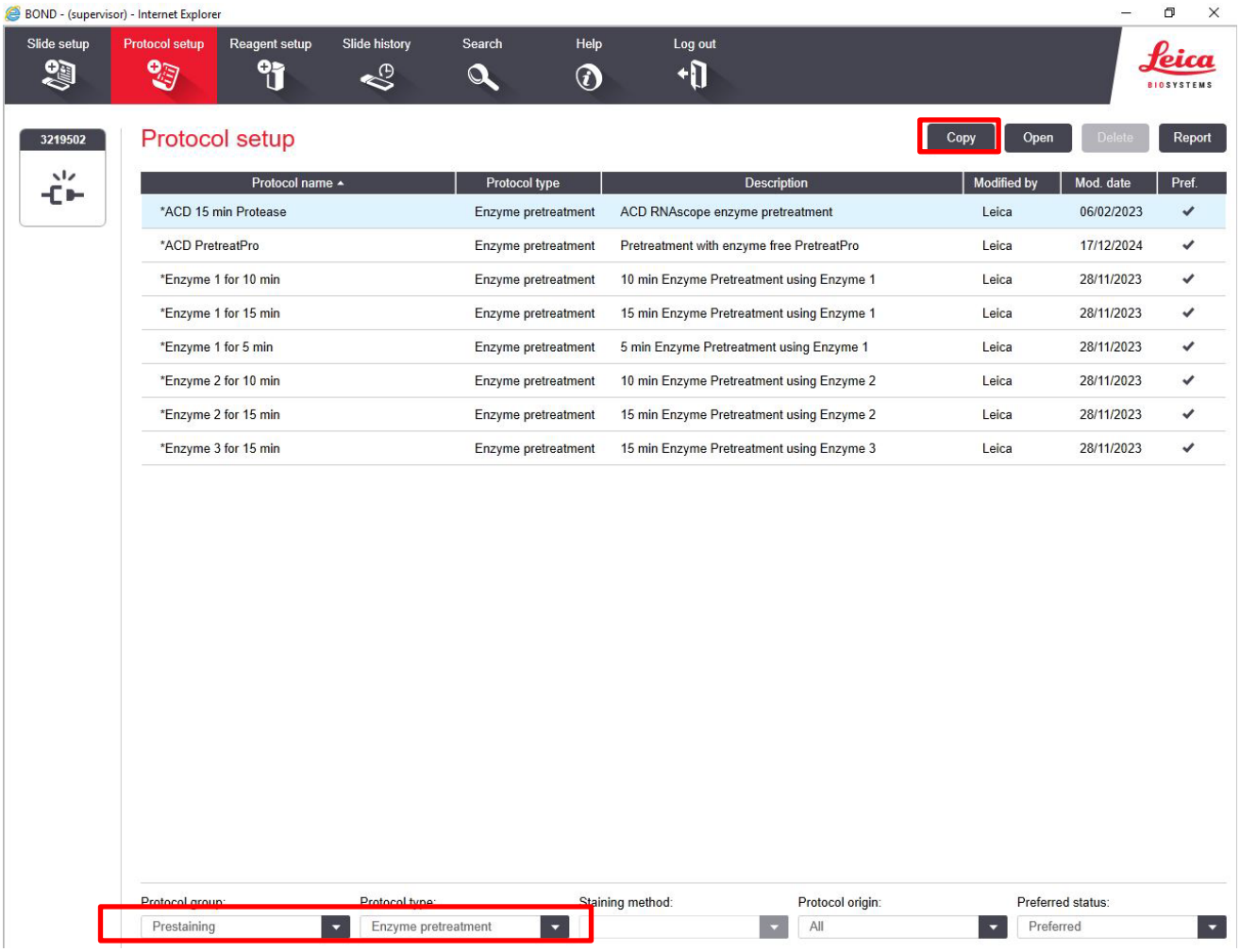


9. Select **Save**.
10. If needed, repeat Steps 1–8 to create new heating protocols for different incubation times and temperatures (for example, ACD 25minER2).

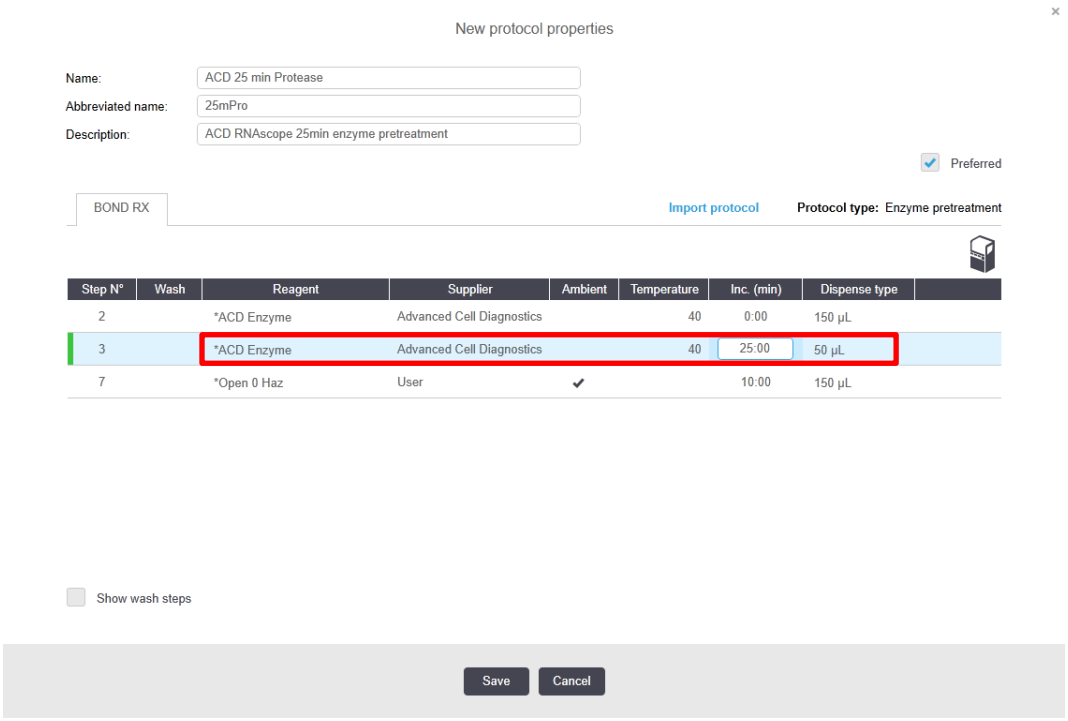
F Appendix F. Edit the Protease Protocol

PreteatPro is recommended for assays that include antibody detection. For RNA-only assays, you can use either Protease III (FFPE) or PretreatPro. 15 minutes at 95°C is the standard protease condition, but the time can be adjusted if needed. The following example shows how to edit the protease procedure from within the software.

1. Select **Enzyme Pretreatment** under the Protocol type menu (bottom left).
2. Highlight **the *ACD 15min Protease** protocol. Select **Copy**.



3. Rename the protocol to **ACD 25min Protease**.
4. Rename the Abbreviated name to **25minPro**.
5. Rename the Description to **ACD RNAscope 25min enzyme**.
6. Highlight the second ***ACD Enzyme** step. Keep the temperature at **40°C** and set the enzyme incubation time to desired time (for example, 25min).



7. Select **Save**.
8. If needed, repeat steps 1–7 to create a new protease protocol for different sample types (for example, ACD 10min Protease or ACD 15min Protease at ambient temperature).

Appendix G. RNAscope Antibody Concentrations

The following guide shows how to dilute the RNAscope primary and secondary antibodies with the multiomic antibody diluent provided.

1. Determine the number of slides needed for the run.
2. Calculate the total volume for RNAscope primary and secondary antibodies. Make sure to add enough dead volume to your calculation depending on the container type used.
3. For up to 10 slides, use 6 mL BOND Titration containers with 600 µL dead volume.
4. Use the following table for suggested antibody concentrations.
5. Dilute RNAscope secondary antibody in a separate tube.
6. Pool all four RNAscope primary antibodies together in the same tube.
7. Add the diluted antibodies to the appropriate containers.
8. Assign fluorophores using the following recommendations for best results.

RNAscope Antibody	Cat No.	Channel	Dilution factor	Opal Dye	Dye Dilution
RNAscope Ab Hs CD4-C3	322949	C3	75x	480	1:3000
RNAscope Ab Hs CD8-C4	322951	C4	75x	690	1:5000
RNAscope Ab Hs PanCK-C5	322952	C5	75x	780	1:500 (TSA-DIG) + 1:125 (Opal 780 reagent†)
RNAscope Ab Hs FoxP3-C6	322953	C6	75x	520	1:5000
RNAscope Ab NeuN-C3	AB0014-C3	C3	75x	480	1:3000
RNAscope Ab GFAP-C4	AB0024-C4	C4	75x	520	1:3000
RNAscope Ab IBA-1-C5	AB0034-C5	C5	75x	690	1:5000
RNAscope anti-rabbit-C1	322954	C1	25x	620	1:10000
RNAscope anti-mouse-C2	322956	C2	25x	570	1:10000

† We recommend keeping the dilution factors of Opal TSA-DIG and Opal Polaris 780 at a constant ratio. For example, when using 1:1500 dilution for Opal TSA-DIG, use 1:375 dilution for Opal Polaris 780. When using 1:750 dilution for Opal TSA-DIG, use 1:187.5 dilution for Opal Polaris 780.

H Appendix H. Pretreatment Guidance for FFPE Samples – RNA targets only

Follow the recommended pretreatment conditions based on your tissue type for:

- Any new or previously untested FFPE tissue types
- Samples prepared differently than the sample preparation protocol found in **Chapter 3**.
- For specific guidance on other sample preparations contact ACD Support at **support.acd@bio-techne.com**

Tissue-specific pretreatment conditions

Refer to the following table for tissue specific FFPE pretreatment conditions. For information about species or tissue type not listed here, contact support at **support.acd@bio-techne.com.**

Species	Tissue Type	Pathology	Pretreatment Condition
Mouse/ Rat	Intestine	Normal	Standard
	Intestine	Tumor	Standard
	Embryo	Normal	Standard
	Brain	Normal	Standard
	Spleen	Normal	Standard
	Eye/Retina	Normal	Extended
	Liver	Normal	Standard
	Kidney	Normal	Standard
Human	Breast	Tumor	Standard
	Colon	Tumor	Standard
	Colon	Normal	Standard
	Lung	Tumor	Standard
	Lung	Normal	Standard
	Prostate	Tumor	Standard
	Prostate	Normal	Standard
	Lymph node	Tumor	Standard
	Lymph node	Normal	Mild
	Tonsil	Normal	Mild/Standard
	Pancreas	Normal	Standard
	Cervical	Cancer	Standard
	Cervical	Normal	Standard
	Cervical dysplasia	Abnormal	Standard
	Brain	Tumor	Standard
	Brain	Normal	Standard
	Cancer	Standard	Head

Species	Tissue Type	Pathology	Pretreatment Condition
Human	Neck	Cancer	Standard
	Liver	Cancer	Standard
	Liver	Normal	Standard
	Heart	Normal	Standard
	GI tract	Normal	Standard
	Kidney	Normal	Standard
	Skin	Normal	Standard
	Lymphoma	Cancer	Standard
	Thymus	Normal	Mild/Standard
	Melanoma	Tumor	Standard
	Nevus	Benign	Standard
	Placenta	Normal	Standard
	Skin (TMA*)	Normal	Standard
	Breast (TMA*)	Normal	Standard
	Melanoma (TMA*)	Normal	Standard
	Nevus (TMA)	Benign	Standard
	Stomach (TMA)	Normal	Standard
	Stomach (TMA)	Tumor	Standard
	Cell pellets, fixed with 10% NBF	—	Mild
	HeLa or 3T3 cells, fixed with 10% Formaldehyde /PBS/ACD Control	—	Mild
	Xenograft tissue	—	Mild

Species	Tissue Type	Pathology	Pretreatment Condition
Cyno monkey	Spleen	Normal	Mild
	Lymph Node	Normal	Mild
	Tonsil	Normal	Mild
	Thymus	Normal	Mild
	Retina	Normal	Mild
	Prostate Gland	Normal	Standard/Mild
	Epididymis	Normal	Mild/Standard
	Testis	Normal	Mild/Standard
	Ovary	Normal	Mild/Standard
	Duodenum	Normal	Mild/Standard
	Jejunum	Normal	Mild/Standard
	Colon	Normal	Standard
	Adrenal Gland	Normal	Mild/Standard
Rat	Spleen	Normal	Mild
	Lymph Node	Normal	Mild
	Tonsil	Normal	N.A.
	Thymus	Normal	Mild
	Retina	Normal	Mild
	Prostate Gland	Normal	Standard/Mild
	Epididymis	Normal	Standard
	Testis	Normal	Standard
	Ovary	Normal	Standard
	Duodenum	Normal	Standard/Mild
	Jejunum	Normal	Standard
	Colon	Normal	Standard
	Adrenal Gland	Normal	N. A
Dog	Spleen	Normal	Mild
	Lymph Node	Mild	Mild
	Tonsil	N.A.	N.A.
	Thymus	Mild	Mild
	Retina	Mild	Mild
	Prostate Gland	Mild	Mild
	Epididymis	Mild	Mild
	Testis	Mild/Standard	Mild/Standard
	Ovary	Mild/Standard	Mild/Standard
	Duodenum	Normal	Mild
	Jejunum	Normal	Mild
	Colon	Normal	Mild
	Adrenal Gland	Normal	Standard/Mild

I Appendix I. Slide Setup for Additional Tissue Types –RNA targets only

Alternatively prepared samples can be stained on the BOND RX using the following slide setup parameters.

Fixed-frozen tissues

Fixed-frozen tissues need a gentle target retrieval step (see **Chapter 4**).

1. In Slide setup, select the following:
   a. Staining: Select the appropriate protocol for the chemistry and workflow you are using.
   b. Preparation: Select ***----**.
   c. HIER: Select ***ACD HIER 5 min with ER2 (95)**. See **Appendix B** to create this protocol.
   d. Enzyme: Select the appropriate protocol for the chemistry and workflow you are using; ***ACD 15min Protease**.
   e. Probe Application: Select ***RNAscope 2.5 LSx Probe Application**.
   f. Denaturation: Select ***….**
   g. Hybridization: Select ***RNAscope 2.5 LSx Hybridization**.
   h. Probe Removal: Select ***RNAscope 2.5 LSx Probe Removal**.
2. Protease incubation time may need to be adjusted but start with **15 MINS** as that works for most tissues.

Fresh-frozen tissues

Fresh-frozen tissues do not need a target retrieval (see **Chapter 4**). Instead, permeabilize the tissue at ambient temperature with a stronger protease such as RNAscope LS Protease IV (Cat. No. 322140).

1. In Slide setup, skip the following steps: 1) Bake or Bake and Dewax 2) Heat retrieval. Choose the following instead:
   a. Staining: Select the appropriate protocol for the chemistry and workflow you are using.
   b. Preparation: Select ***----**.
   c. HIER: Choose ***----**.
   d. Enzyme: Select **ACD 30min RT with LS Protease IV**†.
   e. Probe Application: Select ***RNAscope 2.5 LSx Probe Application**.
   f. Denaturation: Select ***….**
   g. Hybridization: Select ***RNAscope 2.5 LSx Hybridization**.
   h. Probe Removal: Select ***RNAscope 2.5 LSx Probe Removal**.

   †See **Appendix F** to edit the protease protocol.

Note: Start your run immediately after setting it up. Do not use a delayed start. Otherwise, your protease will not distribute equally on the slide and can result in poor permeabilization. When the run is complete, the BOND RX rinses the slides every 10 minutes which can impact the counterstain. Set up the instrument as late in the day as possible. Rinsing does not affect the RNAscope signal and counterstaining can be repeated offline in the morning if needed.

J Appendix J. Protocol Step Maximum Limit Per Instrument Run

BOND RX, from Leica Biosystems, contains a mainboard which dictates the protocol step limits.

BOND RX instruments with a serial number **higher** than 3498490 include the most advanced BOND Mainboard. This allows the BOND RX to perform staining runs of up to approximately 1000 steps per instrument run. This configuration allows the **ACD Multiomic assay to be run on up to three slide staining assemblies (SSAs) at a time (up to 30 slides).**

On BOND RX instruments with a serial number **lower** than 3498490, there was a 500-protocol step maximum limit per instrument run. This configuration supports **a single run (up to 10 slides on one SSA) for the ACD Multiomic assay** to be conducted at a time.

However, if your instrument has an earlier serial number, the About BOND screen (please see the following) can be used to determine which version of the mainboard is installed and therefore what run step capability your instrument supports. Alternatively, contact your local Leica Biosystems representative for more information.

LATEST MAINBOARD CONFIGURATION

Processing Module

Serial number:	3219502
Type:	BOND RX
PCB:	M000 **Starts with "M"**
BOM:	000
EPLD:	01.01
Firmware version:	07.01.00
XYZ firmware:	RSP9000-V4.20-07/96
Syringe firmware:	XL-3000 Hi Res 8 port April 1996 P/N 726950_B
Imager firmware:	REV_WA: 31205480-035

PREVIOUS MAINBOARD CONFIGURATION

Processing Module

Serial number:	3491000
Type:	BOND-III
PCB:	0110 **Starts with "0"**
BOM:	00000000
EPLD:	12289
Firmware version:	06.01.01
XYZ firmware:	RSP9000-V4.20-07/96
Syringe firmware:	XL-3000 Hi Res 8 port April 1996 P/N 726950_B
Imager firmware:	REV_WA: 31205480-035

Appendix K. Safety

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, visit **https://www.bio-techne.com/** and search for the product name.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- IMPORTANT! Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at **www.cdc.gov/biosafety**
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030)
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials
- Additional information about biohazard guidelines is available at **www.cdc.gov/**

In the EU:

- Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition
- Registration, Evaluation, Authorization and Restriction of Chemicals (REACH)

Documentation and Support

Obtaining SDSs

Safety Data Sheets (SDSs) are available at: **https://www.bio-techne.com/** in the documents download section of individual product pages. For the SDSs of chemicals not distributed by Advanced Cell Diagnostics, contact the chemical manufacturer.

Obtaining support

For support information, go to: **https://www.bio-techne.com/support/contact-us.**

Or for the latest resources and troubleshooting guides, go to**: https://www.bio-techne.com/resources.**

At the web pages notes above, you can:

- Search through frequently asked questions (FAQs).
- Submit a question directly to Technical Support.
- Search for user documents, SDSs, application notes, citations, training videos, and other product support documents.

Contact information

Advanced Cell Diagnostics, Inc.
7707 Gateway Blvd Suite 200
Newark, CA 94560

Toll Free: 1-877-576-3636
Direct: 1-510-576-8800
Fax: 1-510-576-8801

Information: **info.acd@bio-techne.com**

Orders: **orders.acd@bio-techne.com**

Support Email: **support.acd@bio-techne.com**

Limited product warranty

Advanced Cell Diagnostics, Inc. and/or its affiliate(s) warrant their products as set forth in the ACD General Terms and Conditions of Sale found on the ACD website. If you have any questions, please contact Advanced Cell Diagnostics at: **https://www.bio-techne.com/support/contact-us**.

Headquarters

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Phone 1-510-576-8800 Toll Free 1-877-576-3636
For support, email **support.acd@bio-techne.com**
www.bio-techne.com