USER MANUAL

ProximityScope™ Protein Assay

For use with BOND RX™ System, from Leica Biosystems

This user manual is for detection of the proximity signal alone. For detection of the proximity signal together with RNA and/or other protein targets, please refer to the RNAscope Multiomic LS User Manual UM 323175.

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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.

Disclaimers

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Contents

1 Chapter 1. Product Information

Before using this product, read and understand the information in **Appendix D. 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 for using ProximityScope Secondary antibodies or ProximityScope Human PD-1/PD-L1 antibodies with detection via RNAscope Multiomic LS Reagents. This assay workflow is supported on the BOND RX Research Advanced Staining System from Leica Biosystems.

IMPORTANT! This user manual covers detection of **only** the proximity signal. Because the ProximityScope assay uses the RNAscope Multiomic LS kit for signal development, probes for RNA transcripts and other RNAscope antibodies can also be included. Integrating proximity signals with RNA and protein marker visualization enables a multiomics assay that reveals protein interactions, cell activation states, and cell phenotypes. For detection of the proximity signal together with RNA and/or other protein targets, please refer to the UM 323175 RNAscope Multiomic LS Detection Kit User Manual.

For questions or support, contact your ACD representative at +1 (877) 576-3636 or **support.acd@bio-techne.com**.

Product description

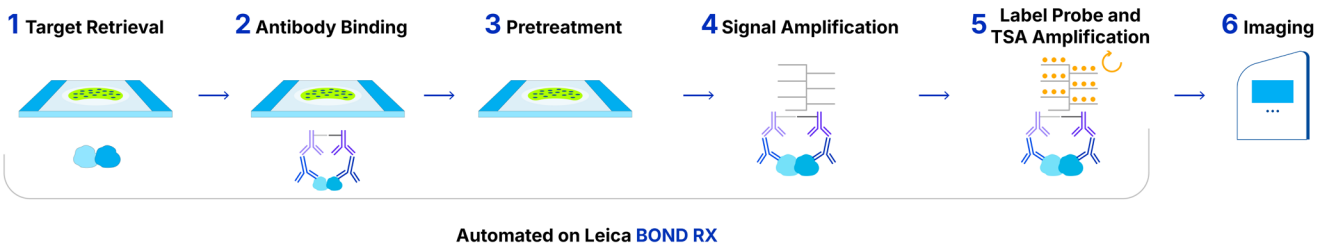
The ProximityScope Protein assay consists of pairs of antibodies designed for the C6 channel, with each antibody designated as either C6a or C6b. Signal detection is performed using the RNAscope Multiomic LS reagents. This assay generates a signal only when the two target proteins are in close physical proximity, allowing spatial visualization and analysis of potential protein interactions. This provides valuable insights into protein dynamics and function.

Both ProximityScope primary and secondary antibodies are available. ProximityScope anti-mouse and anti-rabbit secondary antibodies (also labeled as “RNAscope Proximity secondary antibodies”) are intended for use with user-supplied primary antibodies raised in mouse or rabbit. This combination offers flexibility to detect any two protein targets for which suitable primary antibodies exist.

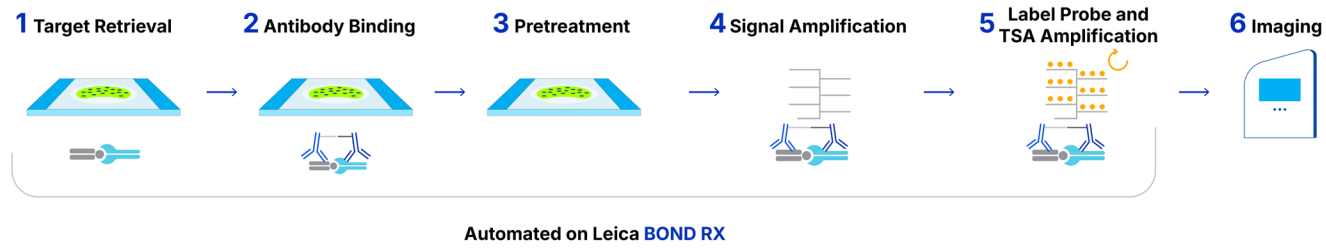
ProximityScope primary antibodies are target-specific and designed to detect specific protein pairs. The ProximityScope Human PD-1 and PD-L1 primary antibodies are specific to those proteins and are used to visualize when the two proteins are close together in tissue. These primary antibodies are directly detectable, eliminating the need for secondary antibodies. **Note:** The ProximityScope antibodies were previously named “RNAscope Proximity antibodies”. While tube labels may

display either name during this transition, the formulation, usage, function, and part numbers remain unchanged. Both versions perform identically.

A. ProximityScope Secondary Workflow



B. ProximityScope PD-1/PD-L1 Workflow



1: Target retrieval	2: Antibody binding	3: Tissue pretreatment	4: Signal amplification	5: Label probe hybridization	6: Imaging
Start with appropriately prepared tissue sections and perform target retrieval to allow access to target.	Incubation with appropriate antibodies.	Perform pretreatments.	RNAscope technology sensitively and specifically amplifies signal.	Label probe hybridization and TSA amplification results in deposition of fluorophore.	Visualize Proximity signal using a fluorescent microscope or slide scanner.

Figure 1. Procedure overview for A. ProximityScope secondaries and B. PD-1/PD-L1 assay are shown. The assay can be completed on the instrument in about 12–13 hours. Properly prepared and 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 amplification reagents to develop and detect the proximity signal.

Kit contents and storage

For detection of the Proximity signal only (without RNAs or other proteins), the ProximityScope Protein assay requires ProximityScope antibodies and the RNAscope Multiomic LS CORE and C6 channel reagents, available from Advanced Cell Diagnostics. Additional reagents available from common laboratory suppliers and Leica Biosystems are also required.

ProximityScope antibodies

ProximityScope antibodies are primary or secondary antibodies used in pairs that generate a signal only when two target proteins are in close physical proximity. This enables spatial visualization and analysis of putative protein interactions and provides insight into protein dynamics and function.

ProximityScope secondary antibodies-rabbit and mouse (PN 322415) (formerly RNAscope Protein Proximity secondary antibodies-rabbit and mouse)					
☑	**Reagent**	**Dilution**	**Component No.**	**Quantity**	**Storage**
	ProximityScope anti-mouse-C6a (formerly RNAscope Px anti-mouse-C6a)	25X	P0034-C6A	310 µL, (20 slides)	Consult product label
	ProximityScope anti-rab-C6b (formerly RNAscope Px anti-rab-C6b)	25X	P0044-C6B	310 µL, (20 slides)	
ProximityScope primary antibodies Human PD-1/PD-L1 (PN 322405) (formerly RNAscope Protein Proximity antibodies- Human PD1/PD-L1)					
☑	**Reagent**	**Dilution**	**Component No.**	**Quantity**	**Storage**
	ProximityScope Ab Hs PD1-C6a (formerly RNAscope Px Ab Hs PD1-C6a)	50X	P0014-C6A	155 µL, (20 slides)	Consult product label
	ProximityScope Ab Hs PDL1-C6b (formerly RNAscope Px Ab Hs PDL1-C6b)	50X	P0024-C6B	155 µL, (20 slides)	

Note: The ProximityScope antibodies were previously named "RNAscope Proximity antibodies". The newly named ProximityScope antibodies maintain their formulation, usage, function, and part numbers. Some users may have tube labels with either branding, but their usage and performance is the same regardless of label name.

RNAscope Multiomic LS Reagents

To perform the ProximityScope Protein assay, the RNAscope Multiomic LS CORE Reagents and the C6 Channel Reagents need to be purchased. These kits provide enough reagents to stain ~ 20 or ~60 standard slides. The assay reagents are then used with the ProximityScope antibodies, TSA linked fluorophores and mounting medium.

All assay reagents are Ready-To-Use (RTU), except for the TSA buffer and the Multiomic Antibody Diluent, which require preparation. Storage conditions for each reagent are listed in the tables below.

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 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 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

Guidelines for choosing user-supplied primary antibodies

When using ProximityScope rabbit and mouse secondary antibodies, please consider the following when selecting primary antibodies:

- Users must provide one mouse IgG primary antibody and one rabbit IgG primary antibody. The primary antibodies should generate specific immunohistochemistry staining on FFPE tissue using BOND Epitope Retrieval Solution 2 (ER2).
- Users can test the compatibility of their primary antibodies with the RNAscope Multiomic LS assay using RNAscope anti-rabbit-C1 (PN 322954) and anti-mouse-C2 (PN 322956). We recommend titrating primary antibody concentrations to minimize non-specific signals.

Recommended fluorophores

The ProximityScope Protein assay requires purchase of Opal fluorophore from Akoya Biosciences.

Dilute the fluorophores to the desired working concentration in the TSA Buffer provided in the RNAscope Multiomic C6 Channel Reagents. 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.

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 table for recommended fluorophores and dilution range.

Assays using Akoya Biosciences Opal Fluorophores

☑	Fluorophores	Akoya Biosciences Cat. No.	Recommended dilution range	Proximity secondary antibodies	Proximity primary antibodies
	Opal 520 Reagent Pack	FP1487001KT	1:500–1:3000	✓	✓
	Opal 570 Reagent Pack	FP1488001KT	1:500–1:3000	✓	✓

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 compatible with fluorophore used: DAPI/Opal 520/Opal 570	• 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 ProximityScope Protein 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)

☑	Component	Cat. No.	Storage
	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
	ProLong™ Gold Antifade Mountant	Thermo Fisher	P36930; P10144; P36934
	Opal dyes fluorophores	Akoya Biosciences	—
	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
	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 ProximityScope Protein 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*.
- If using the ProximityScope secondaries, ensure that the primary antibodies are producing a sensitive and specific signal when used on their own. Consult the guidelines in Chapter 1.

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.
- 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 D. Safety** for more information.

3 Chapter 3. Prepare Samples

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

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

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** after 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.

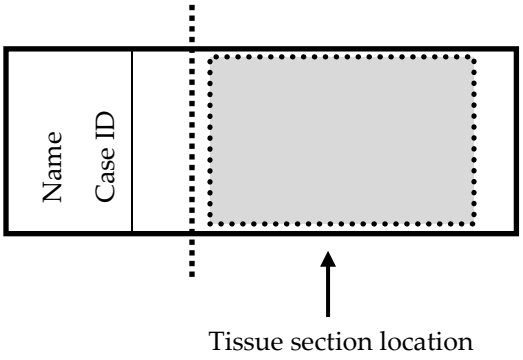
Dehydrate, embed, and cut the sample

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.

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.

4 Chapter 4. Determine Pretreatment Conditions

The following protocols describe formalin-fixed, paraffin-embedded (FFPE) sample pretreatment.

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

Pretreat FFPE sections

Target retrieval

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

Permeabilization

Only LS Pretreat Pro has been tested on FFPE samples for protein detection, including the ProximityScope Protein assay. Although LS Protease is included in the reagent kit, its use is not recommended for the ProximityScope assay, as it may negatively affect 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 proximity signal is maximized with minimal or no negative control signal.

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

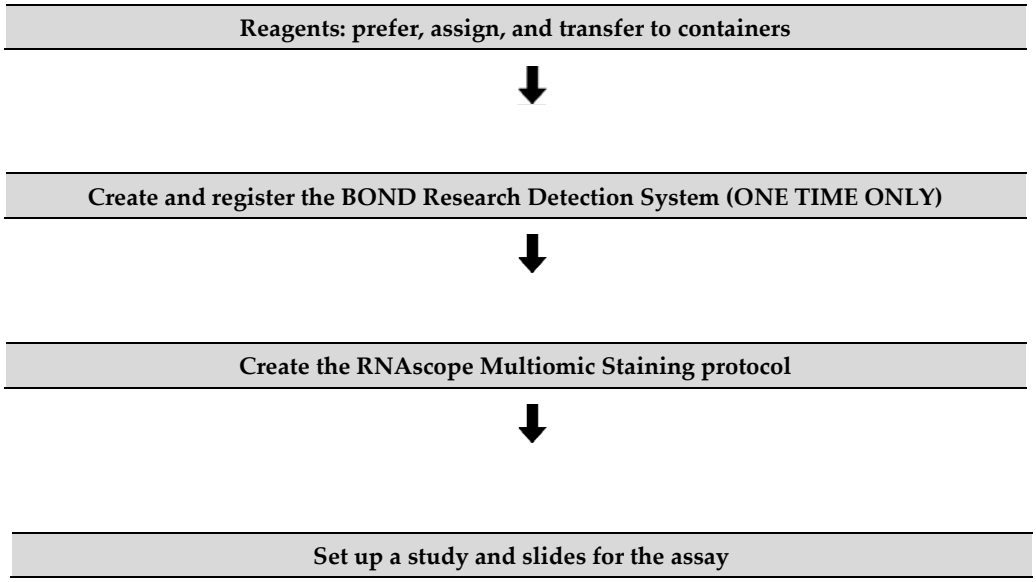
* Sample types, such as certain xenografts and cell pellets, might require shorter incubation time. For these tissue types, reduce the BOND ER2 incubation time.

5 Chapter 5. Set Up Staining Protocols

In this section, you will find instructions on how to use protocols integrated into the Leica BXD software for ProximityScope detection on the BOND RX.

IMPORTANT! The RNAscope Multiomic LS assay also supports same-section detection of ProximityScope signals alongside RNA and protein expression. If you wish to combine ProximityScope detection with RNA and/or other protein targets, please consult the RNAscope Multiomic LS assay user manual (UM 323175 rev F or later).

Workflow



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.

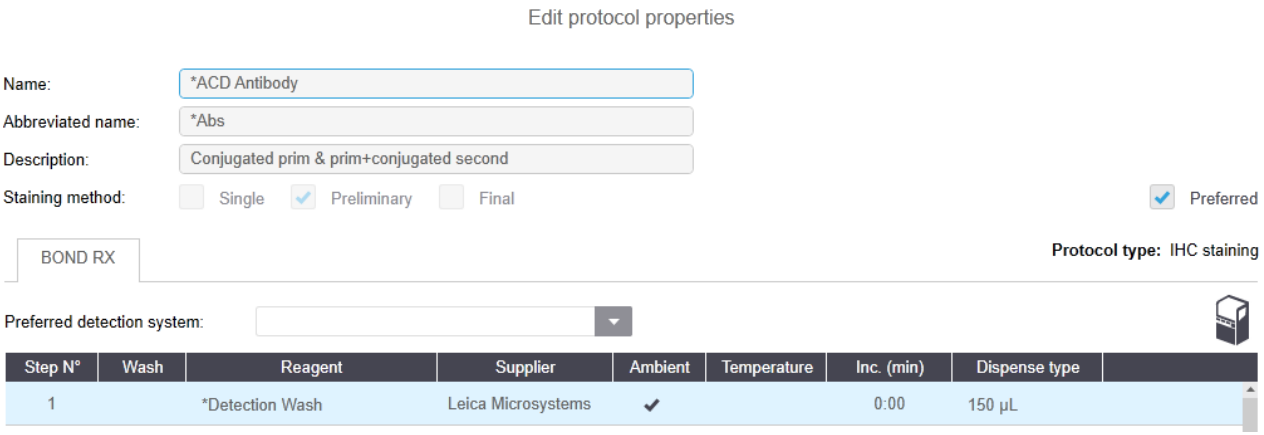
If you prefer to use a previously created Research Detection System, ensure that at least one reagent from the kit is included in each staining protocol and that the correct research kit is selected as the "Preferred Detection System."

Staining protocols

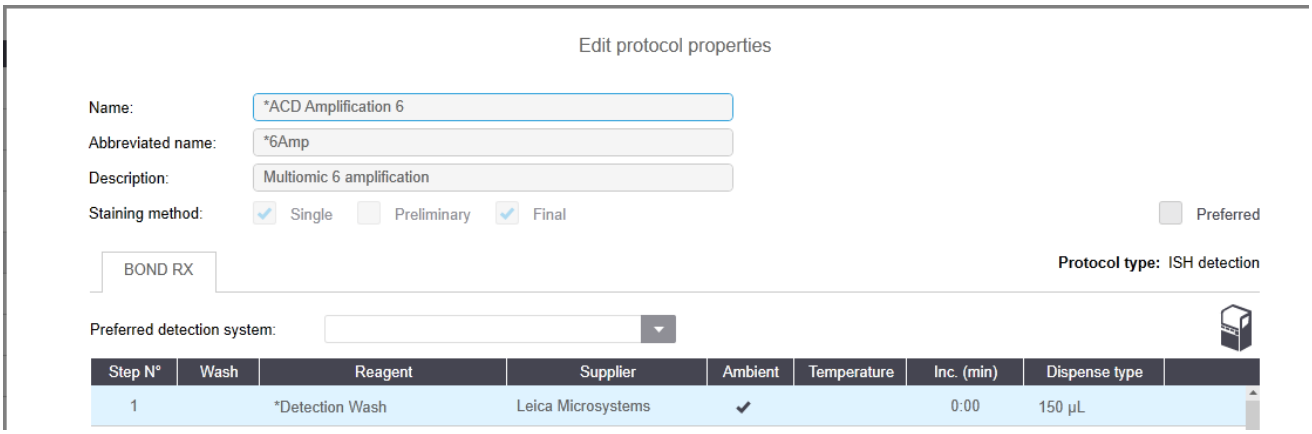
IMPORTANT! Heated *Bond Wash solution steps come from the bulk reagents and are heated by the instrument. You cannot delete these steps. You may delete other wash steps.

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

- ***ACD Antibody** – This protocol template includes steps for RNAscope antibody incubation and post-fixation. To run a ProximityScope only assay, the protocol can be truncated to exclude irrelevant steps. Refer to the following sections for instruction on deleting steps.



- ***ACD Amplification 6 –** This protocol template includes amplification and fluorescent detection steps for up to 6 target detection. For ProximityScope only assay, delete protocol steps from 72 to 203 and change reagent in step 44 and 45 to *Multiomic LS HRP C6.



The following are detailed instructions on editing the two protocols for ProximityScope Protein assays. All steps for the two protocols are provided in the appendices. Your ACD Field Application Specialist (FAS) can help implement procedures.

The diagram below outlines which protocol to follow on the Leica BOND RX and any required modifications to those protocols.

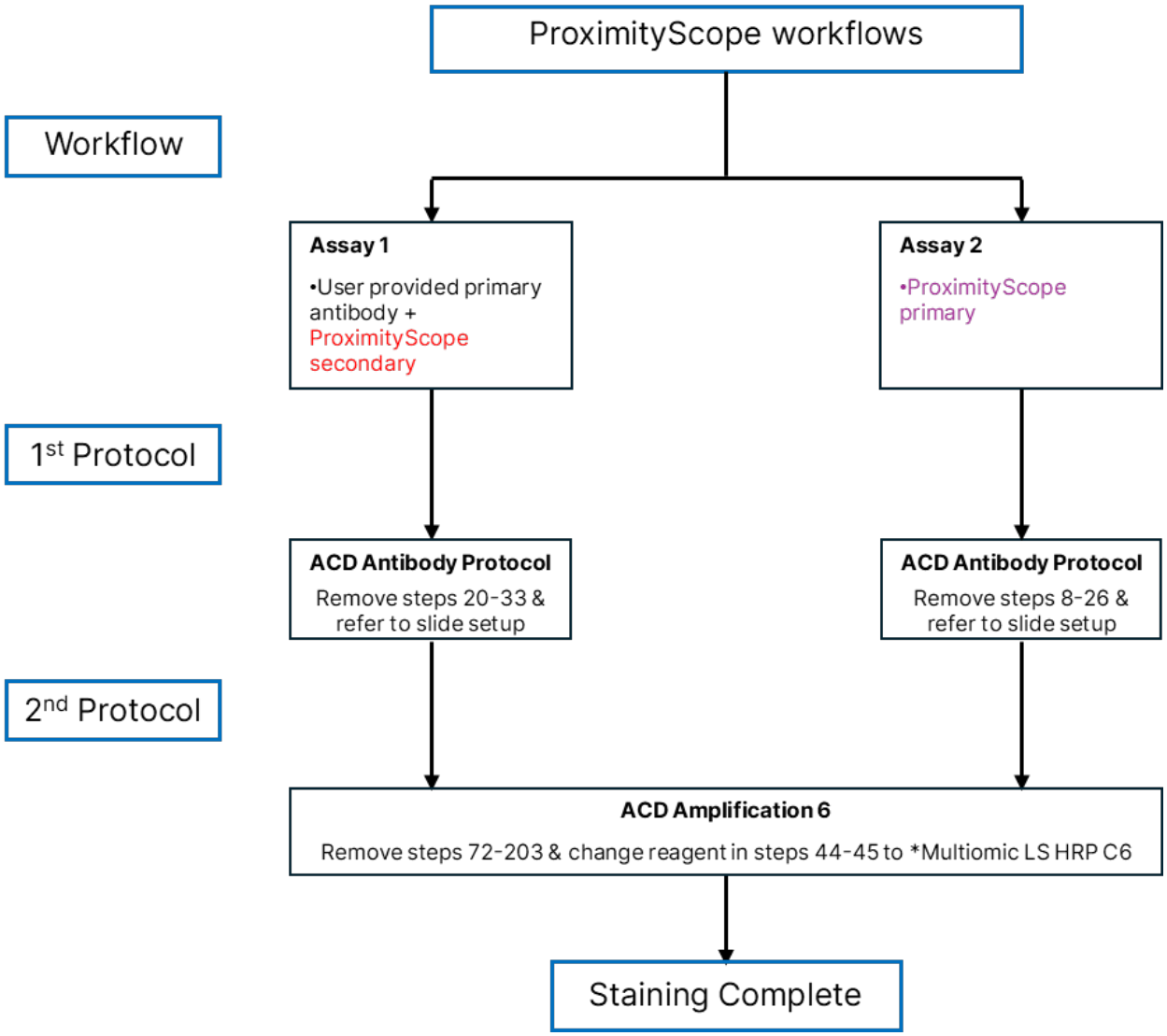


Figure 2. Overview of ProximityScope workflows.

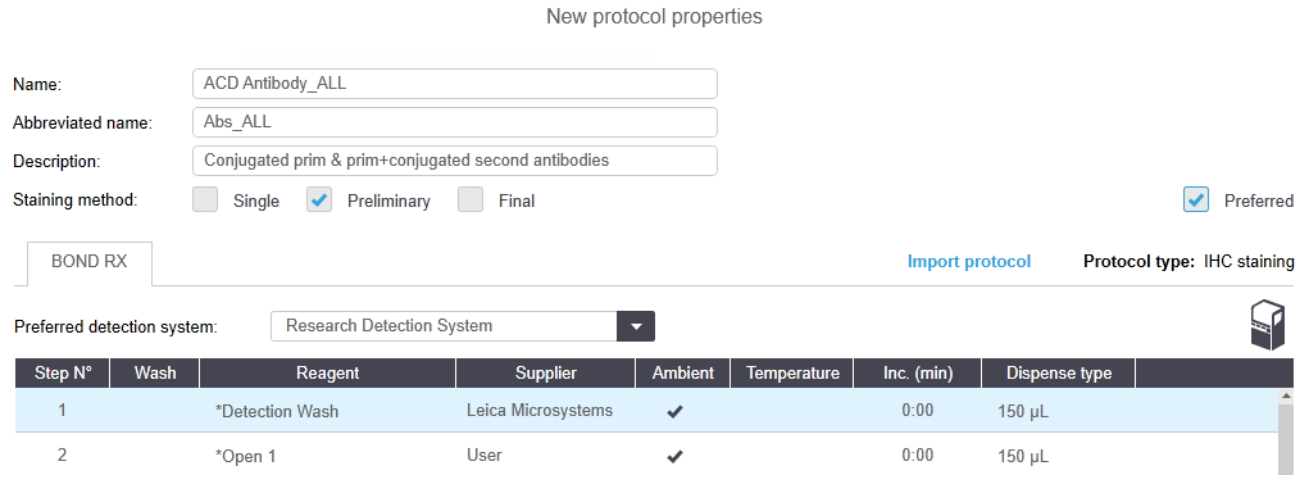
Assay 1. Assay with ProximityScope Secondary Antibodies

The ProximityScope Protein assay with secondary antibodies requires user provided rabbit- and mouse-hosted primary antibodies and ProximityScope secondary antibodies. It is a two-protocol 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."

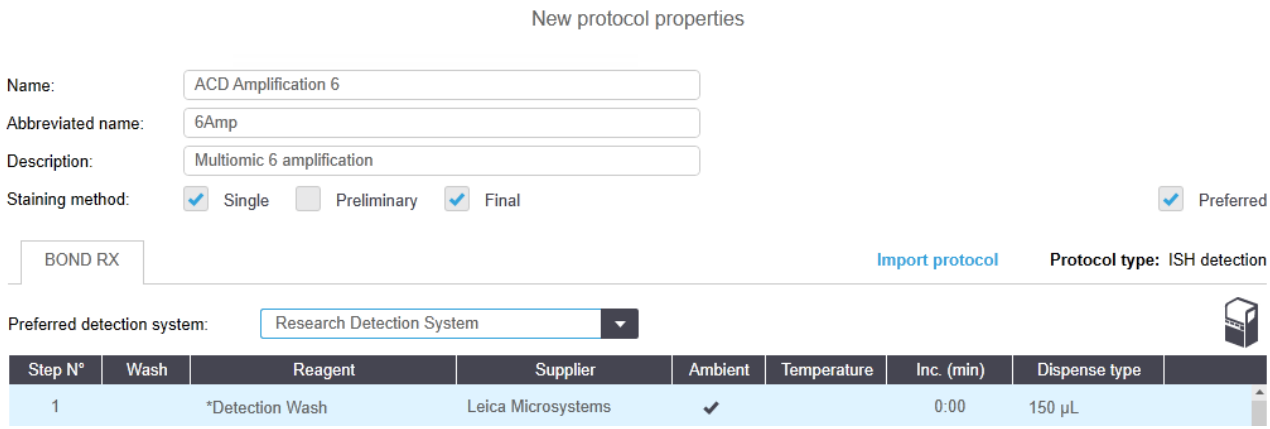
Copy, rename, and prefer the protocol:

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 with a name and abbreviated name of your choice.
6. Select your previously created BOND Research Detection System from the **Preferred Detection System** drop-down.
7. Ensure the **Preferred** box is selected.
8. On the instrument, delete steps 20–33 from your copied and renamed protocol (see **Appendix A** for all the steps in this protocol).
9. Save the protocol.



10. Open ***ACD Amplification 6** protocol found in **ISH staining** protocol type.



11. Rename the protocol with a name and abbreviated name of your choice.
12. Select your previously created BOND Research Detection System from the **Preferred Detection System** drop-down.
13. Ensure the **Preferred** box is selected.
14. On the instrument, delete protocol steps from 72 to 203 and change reagent in steps 44 and 45 to *Multiomic LS HRP C6 from your copied and renamed 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. Assay with ProximityScope Primary Antibodies

This assay is a two-protocol 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 1–7 from the "ProximityScope with Secondary Antibodies" protocol procedure to create the modified *ACD Antibody protocol.
2. Delete steps 8–26 from your copied and renamed protocol. (see **Appendix B** for all the steps in this protocol)

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

3. Save the protocol.
4. Follow steps 10–14 from the "ProximityScope with Secondary Antibodies" protocol procedure to create the modified *ACD Amplification 6 protocol. (see **Appendix B** for all the steps in this protocol)

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 C6	*MO-HRPC6
*RNAscope Multiomic LS HRP Blocker	*HRPBK
*Multiomic TSA-F1	*MO-TSAF1
*Co-Detection Antibody 1 (for the user-supplied primaries when using ProximityScope secondary antibodies)	*Cd-D Ab1
*Co-Detection Antibody 2 (for ProximityScope secondary antibodies)	*Cd-D Ab2
*Co-Detection Antibody 3 (for ProximityScope primary antibodies)	*Cd-D Ab3
*RNAscope LS PretreatPro	*PretPro
*DAPI	*DAPI
*LS Rinse	*LS Rinse
*Open 1	*Open1
*10% Neutral Buffered Formalin	*10% NBF
*Open 0 Haz	*Open0H

4. Select **Save**.

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.

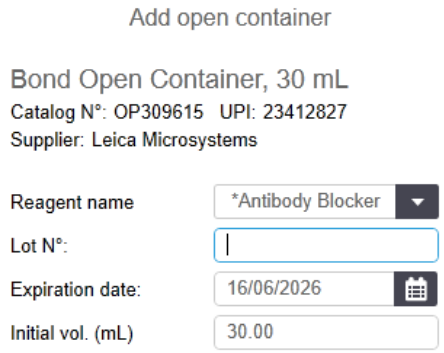
Table 1. Reagent volumes

Reagent Name	Number of dispenses/volume required per slide for each assay	
	ProximityScope with secondary antibodies	ProximityScope with primary antibodies
*RNAscope LS PretreatPro	2/300 µL	2/300 µL
*Open 0 Haz/ RNAscope Multiomic LS Hydrogen Peroxide	1/150 µL	1/150 µL
*Open 1	2/300 µL	2/300 µL

Reagent Name	Number of dispenses/volume required per slide for each assay	
	ProximityScope with secondary antibodies	ProximityScope with primary antibodies
*10% Neutral Buffered Formalin	1/150 µL	1/150 µL
*Co-Detection Antibody 1	2/300 µL	—
*Co-Detection Antibody 2	2/300 µL	—
*Co-Detection Antibody 3	—	2/300 µL
*LS Rinse	6/900 µL	6/900 µL
*RNAscope Multiomic LS AMP 1	2/300 µL	2/300 µL
*RNAscope Multiomic LS AMP 2	2/300 µL	2/300 µL
*RNAscope Multiomic LS AMP 3	2/300 µL	2/300 µL
*RNAscope Multiomic LS HRP C6	2/300 µL	2/300 µL
*RNAscope Multiomic LS HRP Blocker	2/300 µL	2/300 µL
*Multiomic TSA-F1	2/300 µL	2/300 µL
*DAPI	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 300 µL reagent per slide.

2. Carefully transfer all other RNAscope Multiomic LS kit reagents ***except for the TSA buffer and the Multiomic Antibody Diluent*** 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 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 (user-supplied) antibody mix	*Co-Detection Antibody 1	• When using ProximityScope Secondary Antibodies • Dilute to user defined concentration in Multiomic Antibody Diluent
Secondary conjugated antibody mix	*Co-Detection Antibody 2	• When using ProximityScope Secondary Antibodies • 1:25 dilution in Multiomic Antibody Diluent recommended
Primary conjugated antibody mix	*Co-Detection Antibody 3	• When using ProximityScope Primary Antibodies • 1:50 dilution in Multiomic Antibody Diluent recommended
Salmon sperm DNA	*Open 1	• 500 µg/mL, in Multiomic Antibody Diluent

   b. If setting up ProximityScope with Secondary Antibodies workflow, dilute raw primary antibodies together into one tube using the Multiomic Antibody Diluent. In a separate tube, dilute proximity secondary antibodies using the Multiomic Antibody Diluent
   c. If setting up ProximityScope with Primary Antibodies workflow, dilute the primary antibody conjugates using the Multiomic Antibody Diluent.
   d. Add diluted antibody conjugates and ancillaries to the labeled BOND open containers.

IMPORTANT! Do not pool secondaries with primary antibody conjugates.

4. 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.

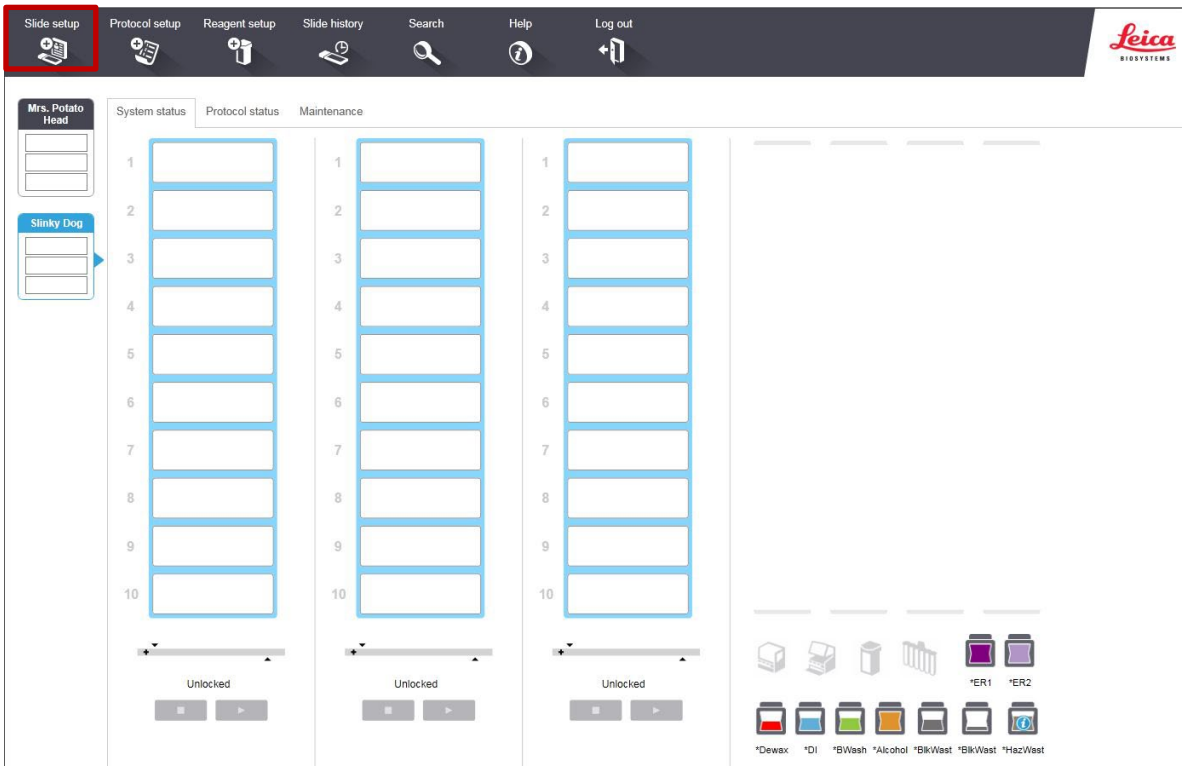
Assay	Recommended TSA dye and relative intensity	Recommended dilution range
ProximityScope secondary antibodies	Opal 520 (Highest)* Opal 570 (Medium)*	1:1000 – 1:3000
ProximityScope primary antibodies	Opal 520 (Highest)* Opal 570 (Medium)*	1:500 – 1:1000

*Refer to manufacturer's website for reconstitution instruction for Opal fluorophore stock preparation.

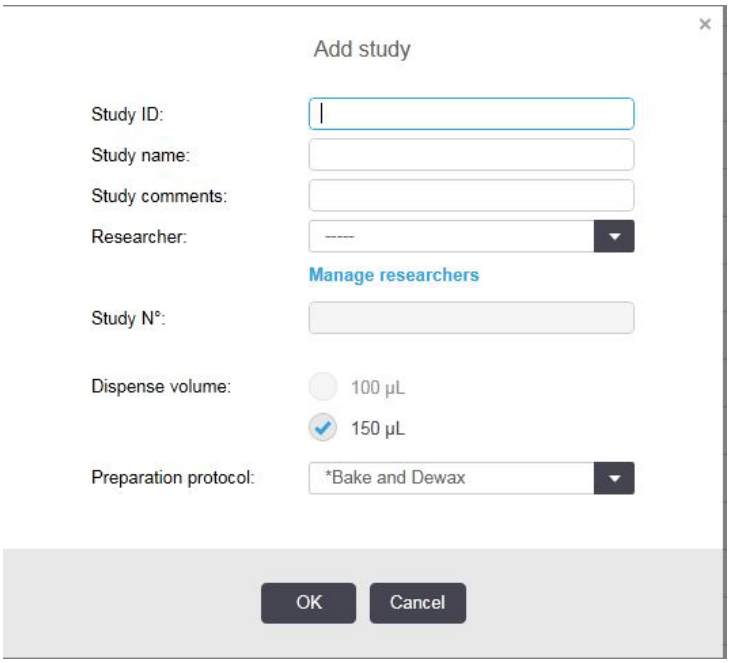
Setting up the study and slides

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).



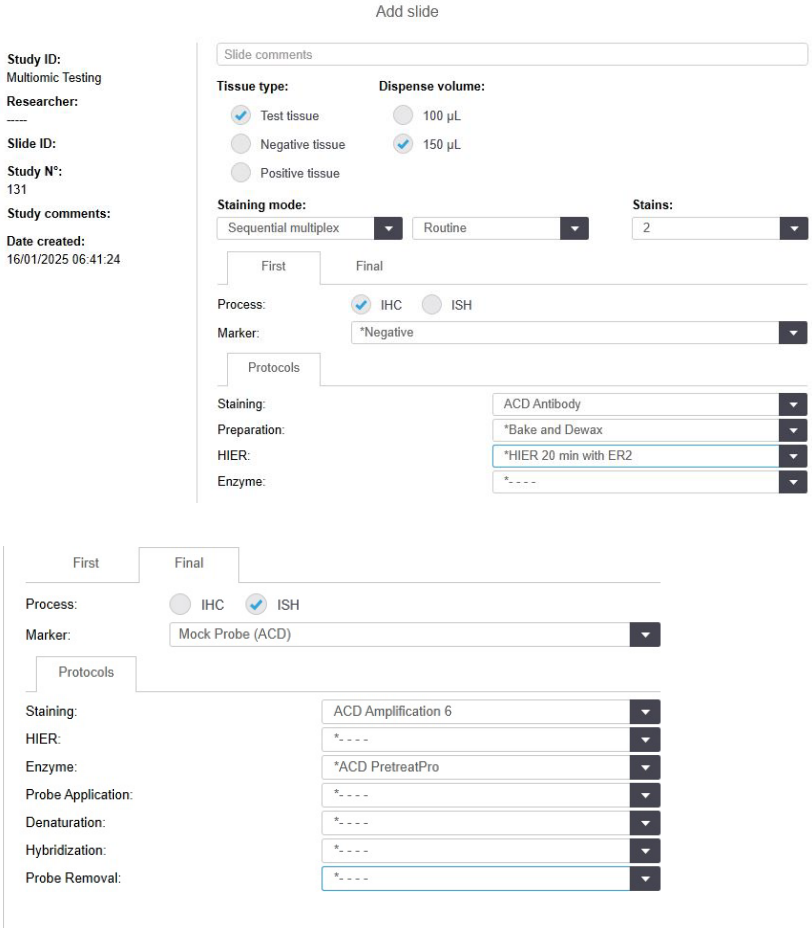
3. For FFPE tissues, select ***Bake and Dewax** as the Preparation protocol.
4. Select **OK**.

Creating the slides

1. Set up the slides for Assay 1 with Proximity 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:
      1. Process = IHC
      2. Marker = *Negative
      3. Staining protocol = your 'First' protocol created above for Assay 1 (modified from ACD Antibody)
      4. Preparation = *Bake and Dewax
      5. HIER = *HIER 20 min with ER2
      6. Enzyme = *----
   f. On the **Final** tab, select the following:
      1. Process = ISH
      2. Marker = Mock Probe (ACD)
      3. Staining protocol = your ISH protocol created above (modified from ACD Amplification 6)
      4. HIER = *----

5. Enzyme = *ACD PretreatPro
6. Probe Application = *----
7. Denaturation = *----
8. Hybridization = *----
9. Probe Removal = *----

g. Select **Add slide** 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.



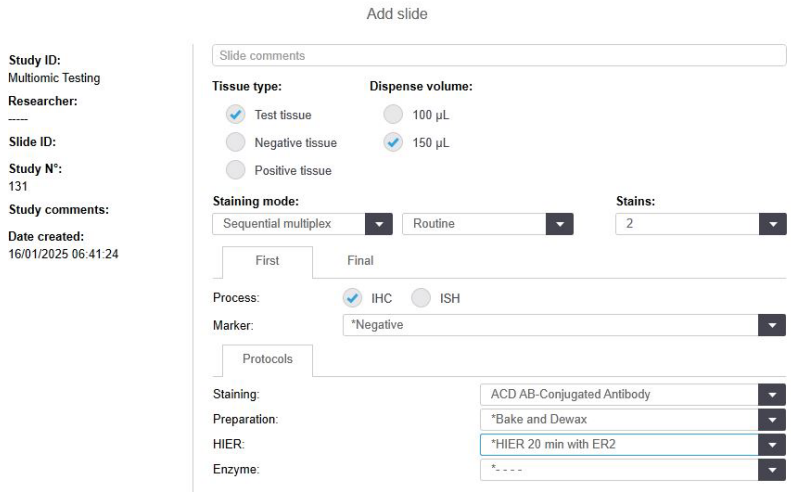
2. Set up the slides for Assay 2 with Proximity primary antibodies:
   a. Select **Add slide** to assign a protocol to each slide.
   b. Enter the tissue type and probe name under the Comments field.
   c. Select **Sequential Multiplex** as default from the Staining mode drop-down menu.
   d. Select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

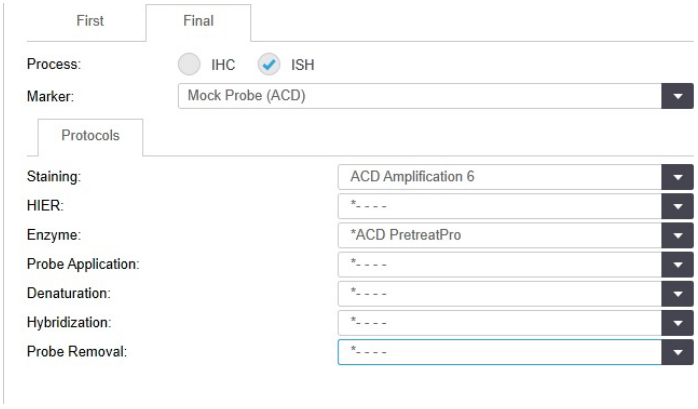
f. On the **Final** tab, select the following:
   7. Process = ISH
   8. Marker = Mock Probe (ACD)
   9. Staining protocol = your ISH protocol created above (modified from ACD Amplification 6)
   10. HIER = *----
   11. Enzyme = *ACD PretreatPro
   12. Probe Application = *----
   13. Denaturation = *----
   14. Hybridization = *----
   15. Probe Removal = *----

g. Select **Add slide** 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.





6 Chapter 6. Run the ProximityScope Protein Assay

Prepare the instrument

1. Fill the large containers located at 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.
   - Proximity-only assay = 12 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. Slides must be unloaded from the instrument within 30 min of run completion.
2. After the run is complete, press the button on the front of the instrument to unlock the slide trays.

3. Remove the slide trays, followed by the covertile and slides.
4. Add a drop of ProLong Gold Antifade Mountant to each slide. Avoid introducing bubbles.
5. Carefully place a glass coverslip on the slides, and dry overnight in the dark.
6. 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. Set the light source and exposure time of image acquisition to acceptable background levels.
- Assess positive control signal strength using the same exposure time as negative control.

Example assay images

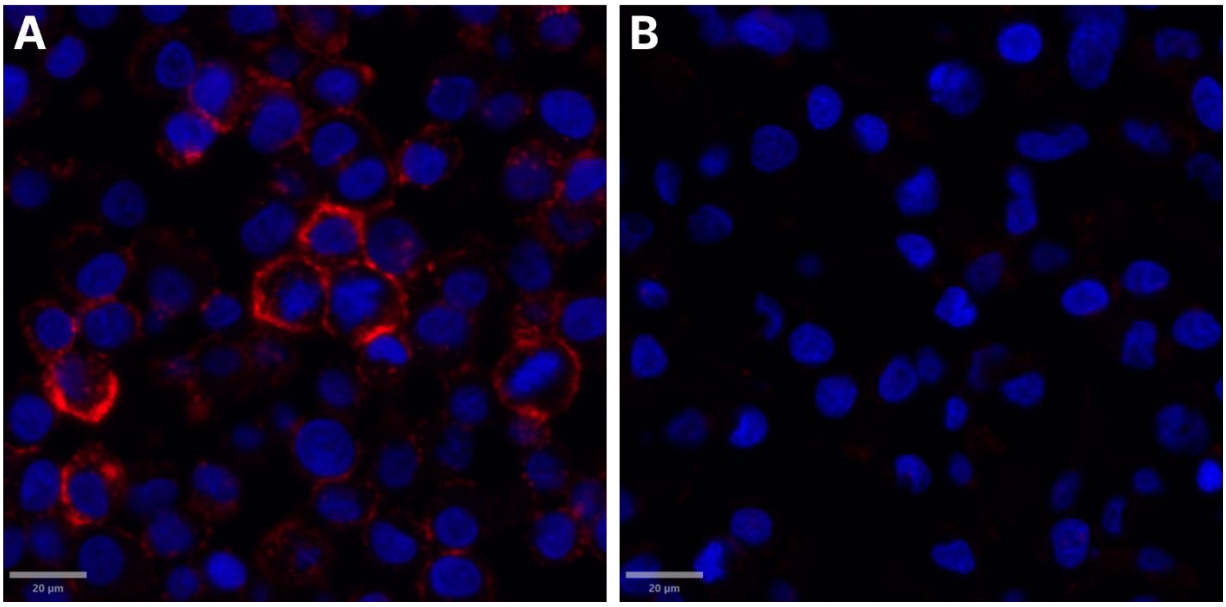


Figure 3. EGFR-HER2 heterodimer detection (red) using ProximityScope anti-rabbit and anti-mouse secondary antibodies in (A) SK-BR-3 and (B) MDA-MB-231 on FFPE cell pellets. (Blue: Nuclear counter stain)

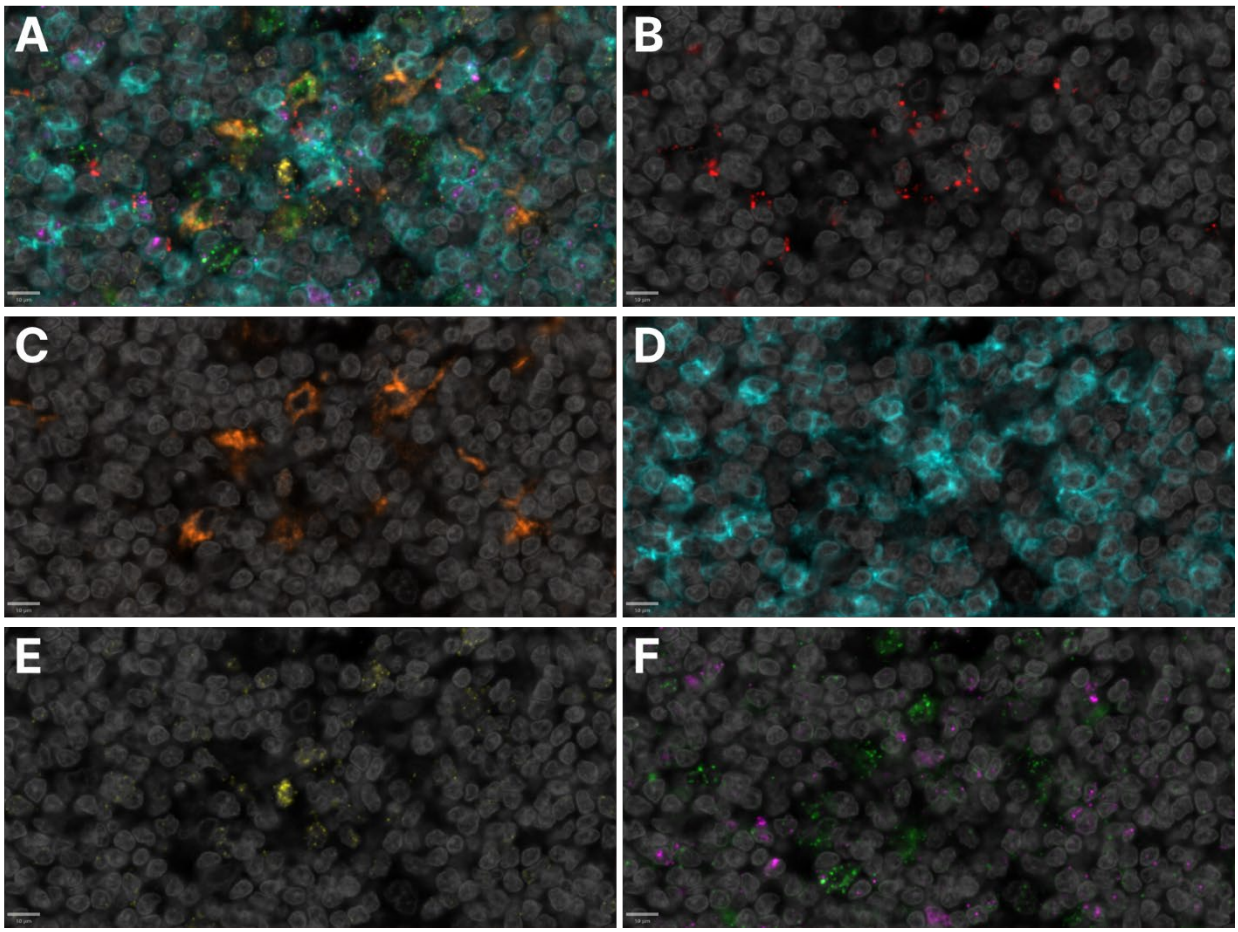


Figure 4. Detection of PD1/PD-L1 proximity along with 2 proteins and 3 mRNA target in Hodgkin's Lymphoma FFPE section using RNAscope Multiomic LS Assay: (A) overlay of 6 targets, (B) PD1/PD-L1 proximity (red), (C) CD68 detected with RNAscope anti-mouse-C2 secondary antibody (orange), (D) CD4-C3 (cyan), (E) *IFNG* (yellow) and (F) *PDCD1* (green) and *CD274* (magenta). For additional information on visualizing additional RNA and protein targets on the same section, refer to UM 323175 RNAscope Multiomic LS assay user manual.

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 ProximityScope signal cannot be distinguished from autofluorescence in tissues with high autofluorescence, increase the fluorophore concentration.
- For optimal assay sensitivity and specificity, include both technical and biological controls.
    - When using ProximityScope secondary antibodies, perform technical negative controls by omitting one primary antibody. For biological controls, include samples known to be either positive or negative for the protein proximity of interest.
    - For assays involving ProximityScope human PD-1/PD-L1 antibodies, use human tonsil tissue as a biological positive control, available by contacting your ACD representative.
- If you observe the presence of background staining with the ProximityScope signal, try the following:
    - When using ProximityScope secondaries, reduce the raw primary antibody concentration. Excessive primary antibody levels can cause non-specific background staining.
    - Decrease the ProximityScope antibody concentration to 0.5X and/or shorten the incubation time to 15 minutes. This may reduce non-specific background without significantly reducing signal intensity.
    - Limit image acquisition sensitivity 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 ProximityScope Protein 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. ProximityScope Secondary Antibody Protocol

The ***ACD Antibody** and ***ACD Amplification 6** template protocols are pre-programmed into BOND software versions 6.0 and higher on BXD 42 and above. The template should be truncated to include just the steps in the following protocol.

Reagent	Container name	Details (concentration, dilution)
Salmon sperm DNA	*Open 1	500 µg/mL in multiomic antibody diluent
10% NBF	*10% Neutral Buffered Formalin	None
Primary raw antibody mix	*Co-Detection Antibody 1	Multiomic antibody diluent (user defined)
Secondary conjugated antibody mix	*Co-Detection Antibody 2	Multiomic antibody diluent (1:25 recommended)

First staining 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
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

Step No.	Reagent	Step type	Incubation time	Temperature
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	*LS Rinse	Reagent	5 MIN	Ambient
21	*LS Rinse	Reagent	5 MIN	Ambient
22	*Bond Wash Solution	Wash	0 MIN	Ambient
23	*Bond Wash Solution	Wash	0 MIN	Ambient
24	*Bond Wash Solution	Open Wash	0 MIN	Ambient
25	*Bond Wash Solution	Wash	0 MIN	Ambient
26	*10% Neutral Buffered Formalin	Reagent	30 MIN	Ambient
27	*Bond Wash Solution	Wash	0 MIN	Ambient
28	*Bond Wash Solution	Wash	0 MIN	Ambient
29	*Bond Wash Solution	Wash	0 MIN	Ambient
30	*Bond Wash Solution	Wash	3 MIN	Ambient
31	*Bond Wash Solution	Wash	3 MIN	Ambient
32	*Bond Wash Solution	Wash	0 MIN	Ambient
33	*Bond Wash Solution	Wash	0 MIN	Ambient
34	*Bond Wash Solution	Wash	0 MIN	Ambient

Final staining protocol

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

Step No.	Reagent	Step type	Incubation time	Temperature†
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
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 C6	Reagent	1 MIN	42°C
45	*RNAscope Multiomic LS HRP C6	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

Step No.	Reagent	Step type	Incubation time	Temperature†
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
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 LS DAPI‡	Reagent	10 MIN	Ambient
73	*Bond Wash Solution	Wash	0 MIN	Ambient
74	*Bond Wash Solution	Wash	0 MIN	Ambient
75	*Bond Wash Solution	Wash	0 MIN	Ambient

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

‡ The standard protocol uses DAPI. Use BOND Wash instead of DAPI, if you are using DAPI offline.

B Appendix B. ProximityScope Primary Antibody Protocol

The ***ACD Antibody** template protocol is pre-programmed into BOND software versions 6.0 and higher on BXD 42 and 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:

Reagent	Container name	Details (concentration, dilution)
Salmon sperm DNA	*Open 1	500 µg/ml, in multiomic antibody diluent
10% NBF	*10% Neutral Buffered Formalin	None
Antibody mix	*Co-Detection Antibody 3	Multiomic antibody diluent (1:50 recommended)

First staining 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 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	Wash	0 MIN	Ambient
12	*Bond Wash Solution	Wash	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

Step No.	Reagent	Step type	Incubation time	Temperature
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
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	Wash	0 MIN	Ambient
29	*Bond Wash Solution	Wash	0 MIN	Ambient

Final staining protocol

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 C6	Reagent	1 MIN	42°C
45	*RNAscope Multiomic LS HRP C6	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 LS DAPI‡	Reagent	10 MIN	Ambient
73	*Bond Wash Solution	Wash	0 MIN	Ambient
74	*Bond Wash Solution	Wash	0 MIN	Ambient
75	*Bond Wash Solution	Wash	0 MIN	Ambient

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

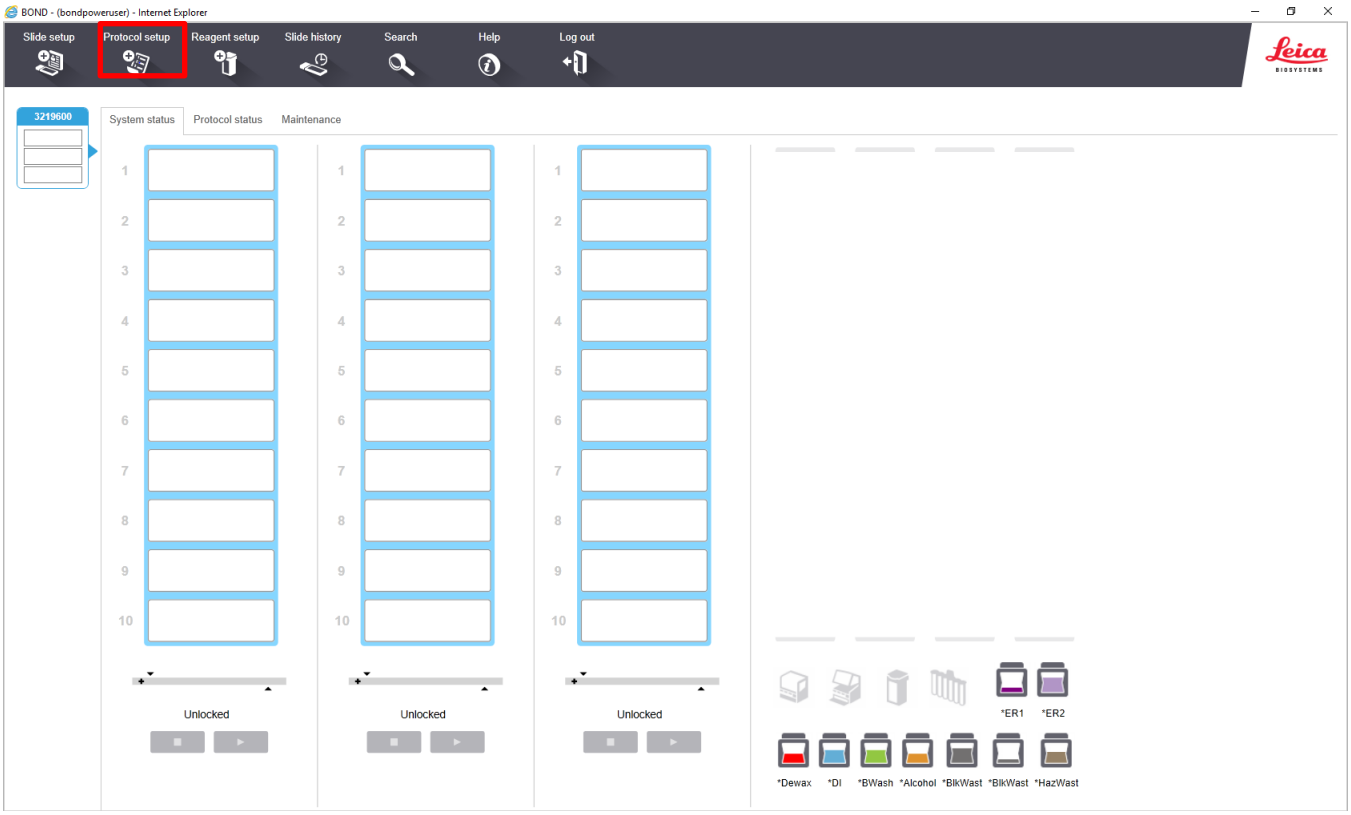
‡ The standard protocol uses DAPI. Use BOND Wash instead of DAPI, if you are using DAPI offline.

Appendix C. Edit the Epitope Retrieval Protocol

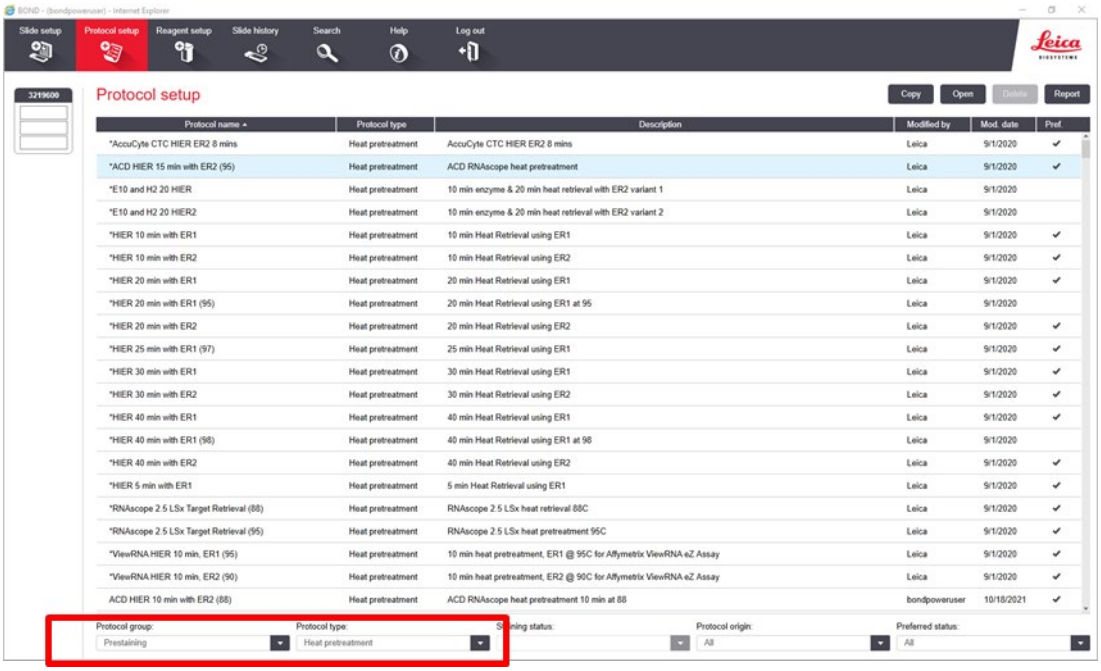
The following example shows how to edit the Epitope Retrieval procedure from within the software. For mild pretreatment with PretreatPro, 92°C heat retrieval is recommended.

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.



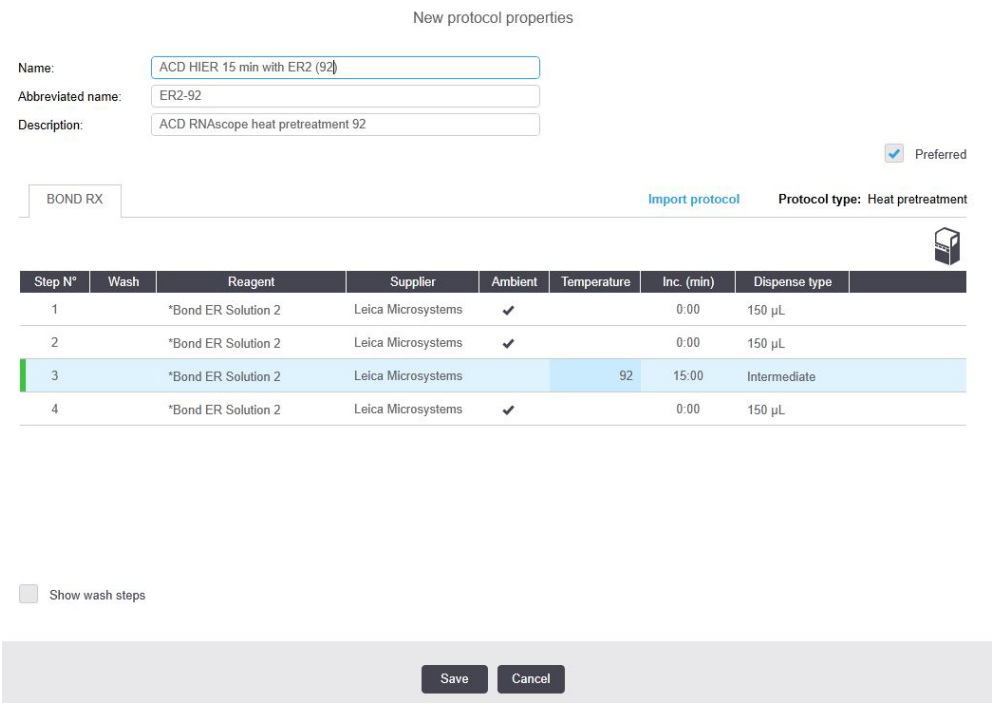
3. Select **Prestaining** under the Protocol group menu and **Heat pretreatment** under the Protocol type menu to access the heat pretreatment protocols.



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

Note: ER2 = Epitope Retrieval 2.

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



10. Select **Save**.
11. If needed, repeat Steps 1–8 to create new heating protocols for different incubation times and temperatures (for example, ACD 25min ER2).

D Appendix D. 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**.

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