USER MANUAL

RNAscope™ 2.5 LS and LSx Reagent Kits – BROWN

For use with BOND RX System, from Leica Biosystems

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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 E. Safety** in 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 2.5 LS and LSx Reagent Kits with the BOND RX Research Advanced Staining System, from Leica Biosystems. RNAscope 2.5 LS Assays are compatible with a variety of sample types.

Product description

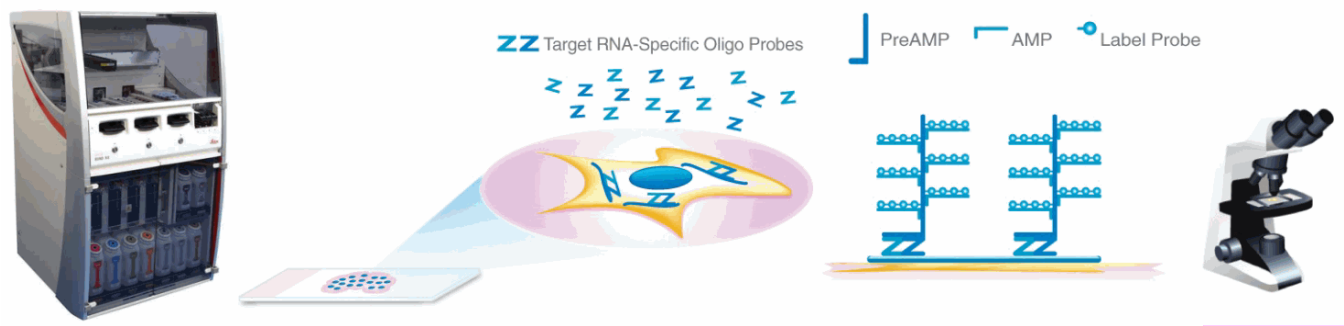
Background

The RNAscope 2.5 LS Assays use a novel and proprietary method of *in situ* hybridization (ISH) to visualize single RNA molecules per cell in formalin-fixed, paraffin-embedded (FFPE), fixed-frozen, and fresh-frozen tissue mounted on slides. The assays are based on Advanced Cell Diagnostic's patented signal amplification and background suppression technology and can detect RNA molecules in archival samples and partially degraded specimens. The RNAscope 2.5 LS Assay allows users to automate the highly sensitive RNAscope Assay using the BOND RX System.

Overview

Figure 1 illustrates the RNAscope 2.5 LS Assay procedure, which can be completed on the instrument in ~9–10 hours. Starting with properly prepared samples, sections are first pretreated, and then RNA-specific probes are hybridized to target RNA. The signal is amplified using multiple steps, followed by the hybridization of horseradish peroxidase (HRP)-labeled probes and detection using the 3,3'-diaminobenzidine (DAB) chromogenic substrate. Each single RNA transcript appears as a distinct dot of chromogen precipitate visible using a common bright-field microscope.

Figure 1. Procedure overview



1: Tissue section	2: Hybridize to target RNA	3: Amplify signal	4: Image
Start with properly prepared sections and load slides onto the instrument. Pretreat tissue to allow access to target RNA.	Hybridize gene-specific probe pairs to the target mRNA.	Apply a cascade of signal amplification molecules, culminating in binding of HRP-labeled probes. Add **DAB** substrate to detect target RNA.	Visualize target RNA using a standard bright field microscope .

Kit contents and storage

The RNAscope 2.5 LS Assay requires the RNAscope 2.5 LS Probes and the RNAscope 2.5 LS Reagents, available from Advanced Cell Diagnostics. The reagents are available in two different formats:

- RNAscope 2.5 LS Reagent Kit – BROWN (Cat. No. 322100)
- RNAscope 2.5 LSx Reagent Kit – BROWN (Cat. No. 322700) and RNAscope 2.5 LS Pro Reagents (Cat. No. 322020)

RNAscope 2.5 LS Probes

The RNAscope 2.5 LS Probes consist of the user-specified Target Probe and the Positive and Negative Control Probes. Visit **https://www.bio-techne.com/reagents/rnascope-ish-technology** to find a gene-specific Target Probe or control probes or order a custom probe.

RNAscope 2.5 LS singleplex assays can only be used with C1 probes. These probes either have a C1 designation in their name or no designation at all. A probe with a C2, C3 or C4 designation in its name is not compatible with this chemistry.

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

Target Probes					
☑	Reagent	Cat. No.	Content	Quantity	Storage
	RNAscope 2.5 LS Target Probe – *[species]* – *[gene]*	Various	Probe targeting specific RNA	16 mL x 1 bottle	2–8°C
Control Probes					
☑	Reagent	Cat. No.	Content	Quantity	Storage
	RNAscope 2.5 LS — Positive Control Probe – *[species]* – PPIB	Various	Probe targeting common housekeeping gene	16 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS — Negative Control Probe – dapB	312038	Probe targeting bacterial gene dapB	16 mL x 1 bottle	2–8°C

RNAscope 2.5 LS Reagents Kit – BROWN

The RNAscope 2.5 LS Reagent Kit – BROWN (Cat. No. 322100) contains 2 boxes (322025 and 322020) which include all the reagents needed to run the RNAscope 2.5 LS Assay on Leica Biosystems' BOND RX System, except for the RNA-specific probes. The kits provide enough reagents to stain ~60 standard slides.

The reagents are shipped in Ready-To-Use (RTU) bottles and must be transferred to BOND Open Containers by the user for use on the BOND RX instrument. Use the reagents in conjunction with the BOND Polymer Refine Detection (DS9800) Kit. The RNAscope reagents are stored as indicated in the following table:

RNAscope 2.5 LS Reagent Kit – BROWN (Cat. No. 322100)			
☑	Reagent	Quantity	Storage
	RNAscope 2.5 LS Hydrogen Peroxide	21 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS Protease III	21 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS PretreatPro™	28 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS AMP 1	21 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS AMP 2	21 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS AMP 3	21 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS AMP 4 – BROWN	21 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS AMP 5 – BROWN	21 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS AMP Pro™	21 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS AMP 6 – BROWN	21 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS Rinse	29 mL x 2 bottles	2–8°C
	RNAscope 2.5 LS Bluing Reagent*	21 ml x 1 bottles	2–8°C

* Bluing is optional.

IMPORTANT! Use only RNAscope 2.5 LS Probes. Do not substitute the reagent components of the RNAscope 2.5 LS Reagent Kit with those of any other RNAscope Reagent Kits.

RNAscope 2.5 LSx Reagent Kit – BROWN

The RNAscope 2.5 LSx Reagent Kit – BROWN (Cat. No. 322700 contains all the reagents needed to run the RNAscope 2.5 LS Assay on the BOND RX System, except for the RNA-specific probes and RNAscope 2.5 LS Pro Reagents (Cat. No. 322020). The kit includes two reagent trays consisting of containers pre-filled with the RNAscope reagents. The kits provide enough reagents to stain ~60 standard slides. The LSx BROWN Kit replaces the BOND Polymer Refine kit. To use the existing protease-free assay workflow, the RNAscope 2.5 LS Pro Reagents (Cat. No. 322020) kit must be transferred to BOND Open containers by the user.

Store the RNAscope 2.5 LSx Reagent Kit – BROWN at 2–8°C.

RNAscope 2.5 LSx Reagent Kit – BROWN (Cat. No. 322100)				
☑	Reagent Name	Tray Position	Volume (mL)	Storage
	RNAscope 2.5 LSx H_2O_2	Tray 2, position 7	9	2–8°C
	RNAscope 2.5 LSx Protease	Tray 2, position 8	12	2–8°C
	RNAscope 2.5 LSx AMP 1 DAB	Tray 2, position 1	18	2–8°C
	RNAscope 2.5 LSx AMP 2 DAB	Tray 2, position 2	18	2–8°C
	RNAscope 2.5 LSx AMP 3 DAB	Tray 2, position 3	18	2–8°C
	RNAscope 2.5 LSx AMP 4 DAB	Tray 2, position 4	18	2–8°C
	RNAscope 2.5 LSx AMP 5 DAB	Tray 2, position 5	18	2–8°C
	RNAscope 2.5 LSx AMP 6 DAB	Tray 2, position 6	18	2–8°C
	RNAscope 2.5 LSx Rinse	Tray 1, position 1-2	27 x 2	2–8°C
	RNAscope 2.5 LSx Bluing Reagent*	Tray 1, position 6	9	2–8°C
	RNAscope 2.5 LSx Hematoxylin	Tray 1, position 3	9	2–8°C
	RNAscope 2.5 LSx BROWN Part 1	Tray 1, position 4	1.2	2–8°C
	RNAscope 2.5 LSx BROWN Part B	Tray 1 position 5	22	2–8°C

* Bluing is optional.

IMPORTANT! Use only RNAscope 2.5 LS Probes. Do not substitute the reagent components of the RNAscope 2.5 LSx Brown Reagent Kit with those of any other RNAscope Reagent Kits.

Additional reagents required for the protease-free workflow

RNAscope 2.5 LS Pro Reagents Kit – (Cat. No.322020)			
☑	Reagent	Quantity	Storage
	RNAscope 2.5 LS PretreatPro	28 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS AMP Pro	21 mL x 1 bottle	2–8°C

Required materials from Leica Biosystems for the BOND RX.

The RNAscope 2.5 LS Assay requires specific materials and equipment available only from Leica Biosystems.

☑	Component	Cat. No.	Storage
	BOND Open Containers 30 mL	OP09700	Room temp (20–25°C)
	BOND Universal Covertiles (pack of 160)	S21.4611	Room temp (20–25°C)
	BOND Epitope Retrieval Solution 1-1L (RTU)	AR9961	2–8°C
	BOND Epitope Retrieval Solution 2-1L (RTU)	AR9640	2–8°C
	BOND Dewax Solution – 1L (RTU)	AR9222	2–26°C
	BOND Wash Solution 10X Concentrate – 1L	AR9590	2–8°C
	BOND Polymer Refine Detection (DAB)*	DS9800	2–8°C
	BOND Aspirating Probe Cleaning System	CS9100	2–8°C
	BOND Mixing Stations	S21.1971	Room temp (20–25°C)

* Do not substitute with any other chromogen kit.

Equipment

☑	Component	Cat. No.
	BOND RX System — automated slide stainer	—

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
	95% Ethanol (EtOH)	American Master Tech Scientific/MLS*	ALREA95
	Xylene	Fisher Scientific/MLS	X3P-1GAL
	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	—
	Cytoseal XYL xylene-based mounting medium	Richard-Allen Scientific/MLS	8312-4
	Tissue-Tek® Vertical 24 Slide Rack	American Master Tech Scientific/MLS	LWS2124
	Tissue-Tek Staining Dish (4 required)	American Master Tech Scientific/MLS	LWS20WH
	Tissue-Tek Clearing Agent Dish, xylene resistant (2 required)	American Master Tech Scientific/MLS	LWS20GR
	Cover Glass 24 x 50 mm	Fisher Scientific/MLS	12-545-F

☑	Description	Supplier	Cat. No.
	Distilled water	MLS	—
	Fume hood	MLS	—

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

2 Chapter 2. Before You Begin

Prior to running the RNAscope 2.5 LS Assay on your samples for the first time, we recommend that you:

- Become familiar with BOND RX Research Advanced Staining System. Refer to the *BOND RX 7 User Manual*.
- Run the assay on RNAscope Control Slides (Cat. No. 310045 for Human HeLa Cell Pellet, and Cat. No. 310023 for Mouse 3T3 Cell Pellet) using the RNAscope 2.5 LS Positive and Negative Control Probes.

Important procedural guidelines

- Start with properly fixed and prepared sections. Refer to **Chapter 3. Prepare Samples** for preparation of FFPE, fixed-frozen or fresh-frozen slides. For preparation of other sample types, contact **support.acd@bio-techne.com**.
- 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.
- Use good laboratory practices and follow all necessary safety procedures. Refer to **Appendix E. Safety** for more information.

3 Chapter 3. Prepare Samples

IMPORTANT! The following protocols describe formalin-fixed, paraffin-embedded (FFPE), fixed frozen and fresh frozen sample preparation. 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** 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 RNAscope 2.5 LS Assay.

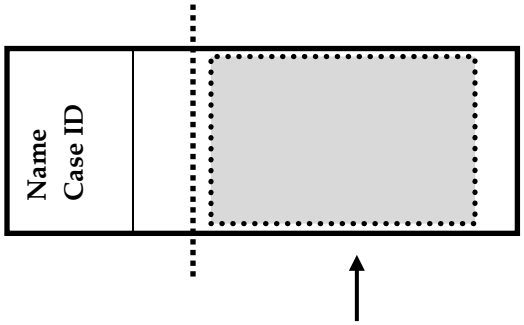
Dehydrate, embed, and cut the sample

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:



Tissue section location

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% alcohol (EtOH)
- Tissue-Tek Vertical 24 Slide Rack
- Tissue-Tek Staining Dishes
- Drying oven

Fix samples

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

Freeze tissues

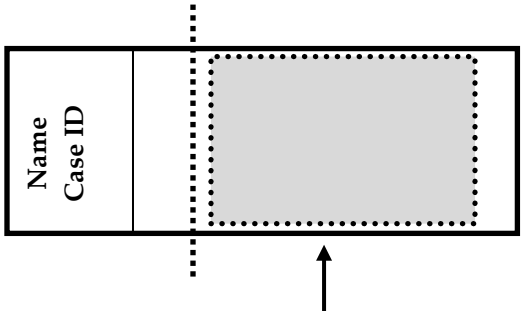
1. Immerse the tissue in 10% sucrose in 1X PBS at **4°C** until the tissue sinks to the bottom of the container (approximately **18 HRS** for brain tissue).
2. Repeat this step with 20% sucrose in 1X PBS, followed by 30% sucrose in 1X PBS, each time allowing the tissue to sink to the bottom of the container.

3. Freeze the tissue in Optimal Cutting Temperature (OCT) embedding media with dry ice or liquid nitrogen, and store it in an airtight container at **–80°C**.

Prepare sections

Before tissue sectioning, equilibrate the tissue blocks at **–20°C** for at least **1 HR** in a cryostat.

1. Section the blocks by cutting 7–15 µm thick sections. 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.

2. Air dry the slides for **60 –120 MIN** at **–20°C or overnight at –80°C**.

OPTIONAL STOPPING POINT (1). Use sectioned tissue within **3 MONTHS**. Store sections with desiccants at **–80°C**.

3. Wash the slides with 200 mL 1X PBS in a Tissue-Tek slide rack for **5 MIN** while moving the rack up and down to remove OCT.
4. Bake the slides for **60 MIN** at **60°C**.
5. Immediately post-fix the slides by immersing them in prechilled 10% NBF or 4% PFA in 1X PBS for **60 MIN** at **RT**.

Note: If you are experiencing issues with sample detachment, extending the post-fixation and baking times may be helpful.

Dehydrate and dry the sections

Reagents may 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. 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% alcohol (EtOH)
- Tissue-Tek Vertical 24 Slide Rack
- Tissue-Tek Staining Dishes

Prepare fresh frozen tissue sections

1. Remove tissue and cut to fit into cryomolds.

⚠ CAUTION! Handle biological specimens appropriately.

2. Freeze the specimen within **5 MIN** of tissue harvest.
3. 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.
4. 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**.

5. 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.
6. 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. Remove fresh-frozen tissue slides from **80°C** and place in a Tissue Tek Slide Rack.
2. *Immediately* immerse the slides in 200 mL of 10% NBF or freshly prepared 4% PFA.
3. 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 may 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.

Pretreat FFPE sections

Target retrieval

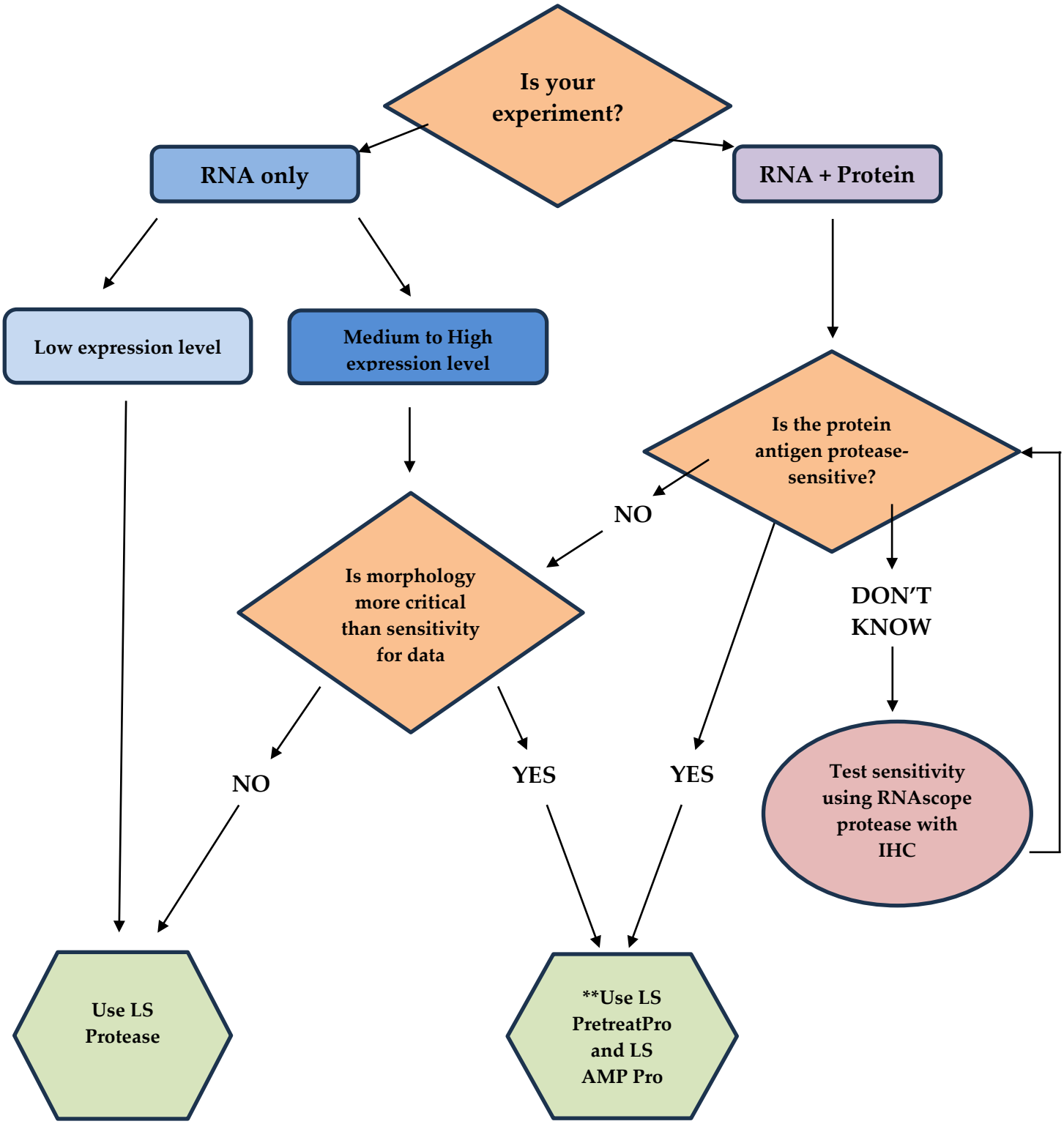
FFPE samples must be de-crosslinked with a target retrieval step. The RNAscope 2.5 LS Assays uses the BOND RX's ER2 solution exclusively for this step.

Permeabilization

Two options are available:

- Protease-based permeabilization is recommended for experiments that stain only RNA. This option uses LS Protease III or RNAScope 2.5 LSx Protease depending on the kit you are using.
- Protease-free permeabilization uses the LS PretreatPro reagent which is free of protease. 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 correct permeabilization option, please refer to the following flowchart:



** For optimizing ISH signal strength for low RNA expressors in the protease-free workflow.

Tissue pretreatment recommendations

Use these conditions as a starting point when tissues are prepared as described in Chapter 3. Depending on your tissue type, vary the amount of time for the ER2 and protease or LS PretreatPro until positive RNA control signal is maximized with minimal/no negative RNA control signal (see Appendix A for a list of tissues).

Reagent	Mild Protease Workflow	Standard Protease Workflow	Protease-Free Workflow
BOND ER2*	15 MIN at 88°C	15 MIN at 95°C	15 MIN at 95°C
LS PretreatPro†			30 MIN at 95°C
LS Protease III OR RNAscope 2.5 LSx Protease‡	15 MIN at 40°C	15 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. Protease/ LS PretreatPro incubation times can also be adjusted but is rarely needed. Contact ACD Support at **support.acd@bio-techne.com** if you need guidance on your tissue type.

† For ACD Control Cell pellets, we recommend starting with ER2 at 92°C for 15mins when using the LS PretreatPro workflow.

‡ Depending on which assay kit format you are using; user-filled OR pre-filled.

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 protease has been tested for use with fixed-frozen sections. Check with ACD Support for any updates.

Tissue pretreatment recommendations

Use these conditions as a starting point when tissues are prepared as described in **Chapter 3**. Depending on your tissue type, vary the amount of time for the ER2 and/or protease until positive control RNA signal is maximized with minimal or no negative RNA control signal (see **Appendix E** for details).

Reagent	Standard
BOND ER2*	5 MIN at 95°C
LS Protease III or RNAscope 2.5 LSx Protease†	15 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. Protease time can also be adjusted but is rarely needed. Contact ACD Support at **support.acd@bio-techne.com** if you need guidance on your tissue type.

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

Pretreat fresh-frozen sections

Target Retrieval

The retrieval requirements for fresh-frozen sections are determined by the permeabilization approach that is followed. Target retrieval is not needed when using protease. However, a short target retrieval is required to optimize the signal when using PretreatPro.

Permeabilization

ACD recommends starting with the PretreatPro workflow as all reagents are included in the kit purchases. If protease treatment is needed, then LS Protease IV needs to be purchased separately for optimal assay performance.

Tissue pretreatment recommendations

Use these conditions as a starting point when tissues are prepared as described in **Chapter 3**.

Protease-free workflow

Reagent	Standard
BOND ER2	5 MIN at 95°C*
LS PretreatPro*	30 MIN at 40°C

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

Protease workflow

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

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

5 Chapter 5. Staining Protocols and RNA Probes

Use the instructions in this chapter to set up the RNAscope 2.5 LS Assay using software slidesversion 6.0 and above.

IMPORTANT! BXD42 or higher is required to run the following setup on software version 6.0 and above. Please contact your Leica FAS to upgrade to BXD42 before starting the assay.

IMPORTANT! We strongly recommend you run the Control Slides (Cat. No. 310045 or Cat. No. 310023) using the RNAscope 2.5 LS positive and negative control probes along with your samples in every run.

RNA probe(s)

This workflow uses the standard BOND RX software setup. Probes are manually registered in the software as 'Probe RNA' (not as 'Ancillary'), and a pre-defined staining protocol will be selected as the default protocol for this probe. Follow the steps in this chapter to enable the workflow. Your ACD Field Application Specialist (FAS) can help you implement this procedure.

Staining protocols

Staining protocols have been pre-defined in the software (*) for the four different RNAscope BROWN assays, depending on the reagent kit and enzyme retrieval used. Following is a summary of each staining protocol:

- *ACD 2.5 DAB Rev B: This protocol has been pre-defined for use with:
  - RNAscope 2.5 LS Reagent Kit - BROWN (Cat. No. 322100) – user-filled
  - BOND Polymer Refine Detection (DS9800) DAB
  - Protease Enzyme Retrieval
- *ACD 2.5 DAB Rev B Protease-Free: This protocol has been pre-defined for use with:
  - RNAscope 2.5 LS Reagent Kit – BROWN (Cat. No. 322100) – user-filled
  - BOND Polymer Refine Detection (DS9800) DAB
  - PretreatPro (protease-free) Enzyme Retrieval
- *RNAscope 2.5 LSx DAB ISH: This protocol has been pre-defined for use with:
  - RNAscope 2.5 LSx Reagent Kit -BROWN (Cat. No. 322700)
  - Protease Enzyme Retrieval
- *RS 2.5 LSx DAB_Protease-Free: This protocol has been pre-defined for use with:
  - RNAscope 2.5 LSx Reagent Kit -BROWN (Cat. No. 322700)
  - PretreatPro (protease-free) Enzyme Retrieval

Slide setup and supporting protocols for HIER, EIER and Hybridization are described in more detail in **Chapter 6**.

Workflow

Edit *ACD 2.5 DAB Rev B Protocol (first use only)

⬇

Register the probes

⬇

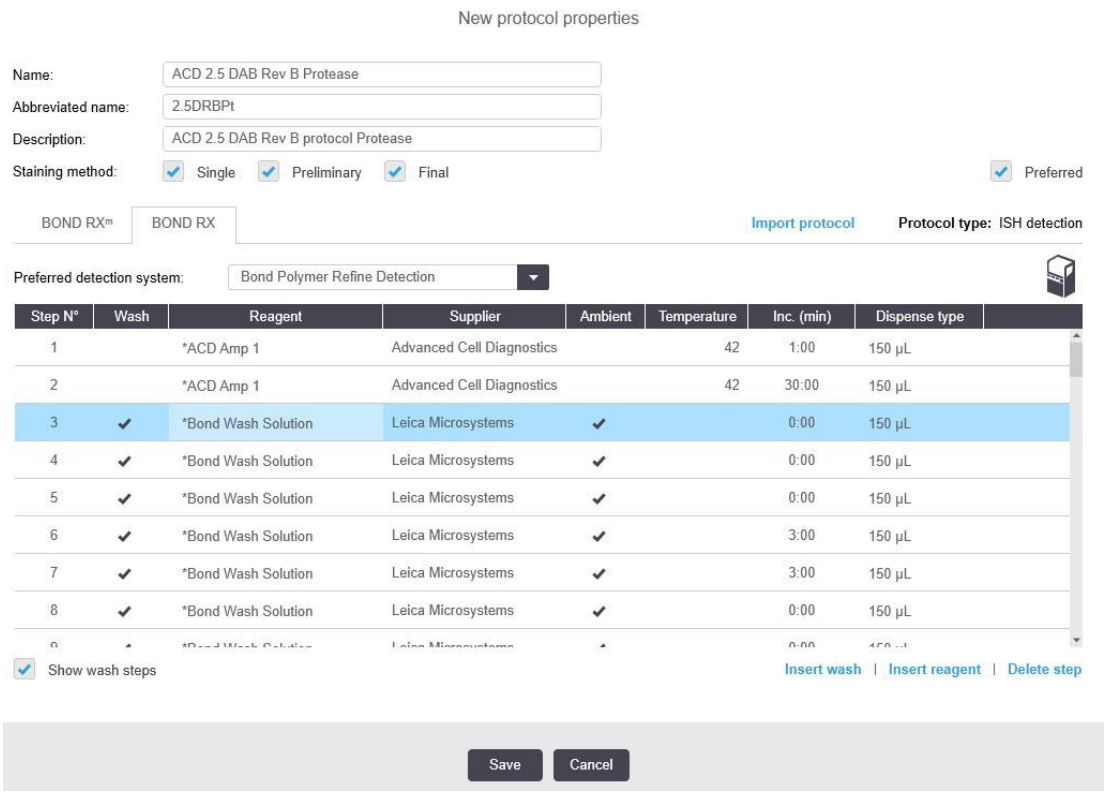
Select preferred DAB staining protocol

Edit *ACD 2.5 DAB Rev B (first use only)

This edit removes the first 14 reagent and wash steps that are no longer needed in the staining protocol when using software version 6.0 or greater.

1. In the Protocol setup screen, select **Staining** from the Protocol group menu.
2. Highlight the ***ACD 2.5 DAB Rev B** protocol**.** Select **Copy.**
3. Change the protocol name for your first probe to **ACD 2.5 DAB Rev B Protease** in the Name text box, **2.5DRBPt** in the Abbreviated name text box, and **ACD 2.5 DAB Rev B protocol Protease** in the Description text box.
4. Highlight the default probe reagent lines (Lines 1-3: *ACD 2.5 P1) and the following 11 wash steps and select **Delete step** at the bottom right of the window. Once this is complete, *ACD Amp1 should be the first step of the protocol.
5. Select **Preferred** in the bottom right corner of the window.
6. Select **Save**.
7. Click on **Next** to proceed. Acknowledge any pop-up warnings that may appear on the screen by clicking **OK**.

The following screenshot shows the protocol after editing. Moving forward, use this new protocol, which means you can deselect the ***ACD 2.5 DAB Rev B** protocol as the preferred protocol.

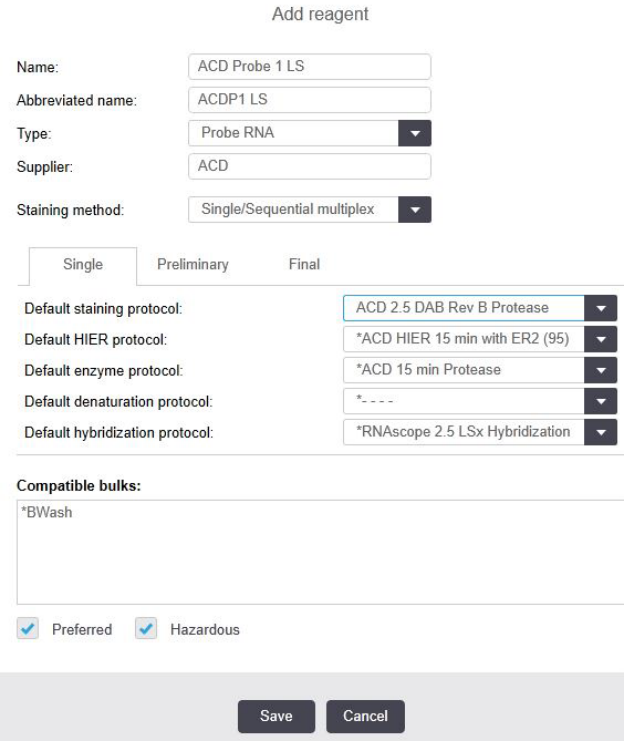


Create RNA probe(s)

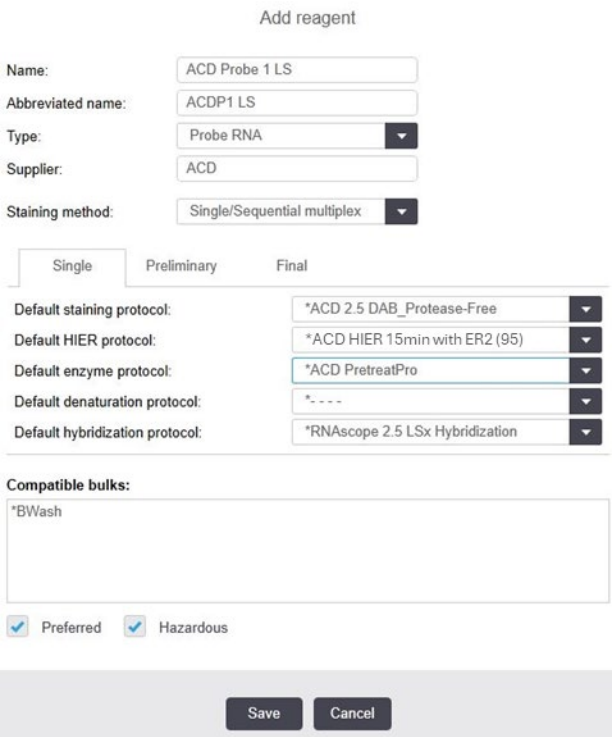
For each probe not already in the database, create it with the default workflow protocols that are appropriate for your needs. To ensure efficiency, select the protocols you anticipate using most frequently as your defaults. These defaults can be modified during slide setup if any slides require different protocols.

See the following workflow examples:

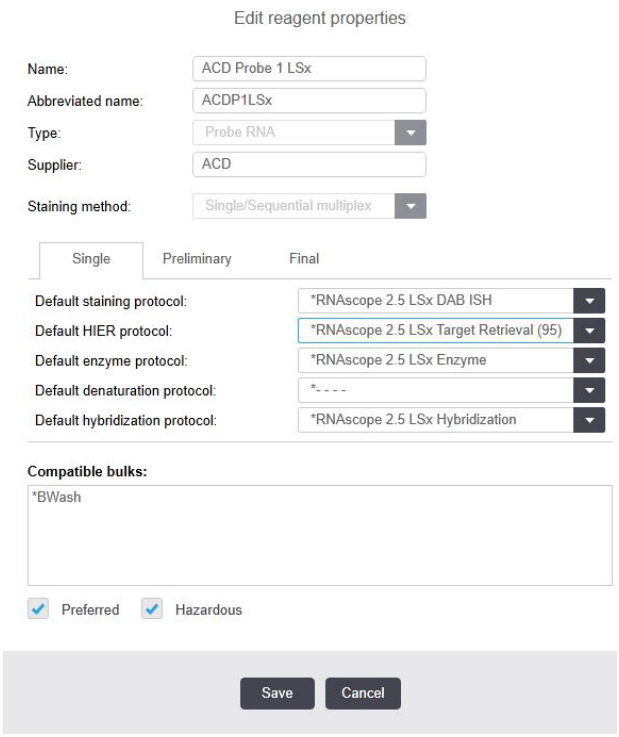
User-filled RNAscope 2.5 LS reagents using protease pretreatment



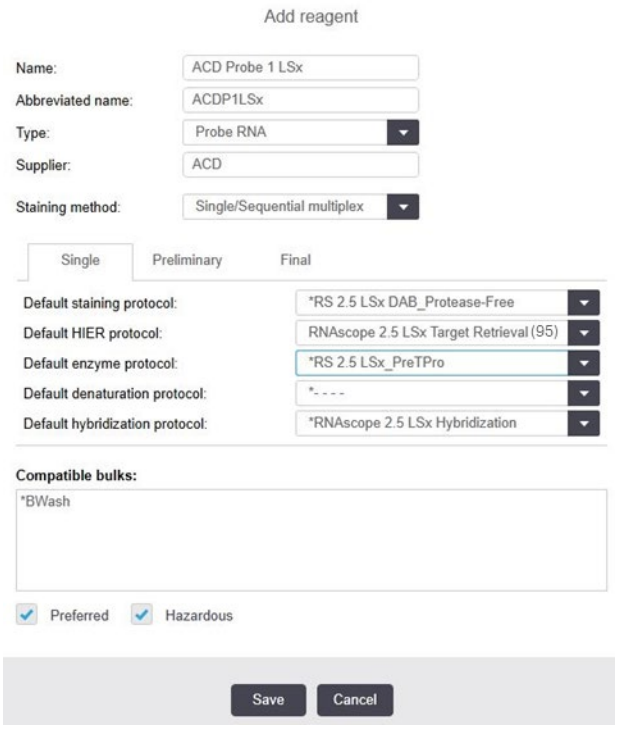
User-filled RNAscope 2.5 LS reagents using protease-free pretreatment



RNAscope 2.5 LSx Reagents using protease pretreatment



RNAscope 2.5 LSx Reagents using protease-free pretreatment



6 Chapter 6. Preparing the reagents

RNAscope 2.5 LS Reagent Kit reagents (Cat. No. 322100)

Materials required

Materials provided by Advanced Cell Diagnostics	Materials provided by Leica Biosystems	Materials provided by user
• RNAscope 2.5 LS Target Probe • RNAscope 2.5 LS Positive Control Probe • RNAscope 2.5 LS Negative Control Probe • RNAscope 2.5 LS Hydrogen Peroxide • RNAscope 2.5 LS Protease III • RNAscope 2.5 LS PretreatPro • RNAscope 2.5 LS AMP 1 • RNAscope 2.5 LS AMP 2 • RNAscope 2.5 LS AMP 3 • RNAscope 2.5 LS AMP 4 - BROWN • RNAscope 2.5 LS AMP 5 – BROWN • RNAscope 2.5 LS AMP 6 – BROWN • RNAscope 2.5 LS AMP Pro • RNAscope 2.5 LS Rinse • RNAscope 2.5 Bluing Reagent	BOND RX System • Stainer Bulk Reagents • BOND Wash Solution, 10X • BOND Dewax Solution • BOND Epitope Retrieval Solution 1 • BOND Epitope Retrieval Solution 2 Reagents • BOND Polymer Refine Detection	• Distilled water • Drying oven • Fume hood • Tissue-Tek Staining Dish • Tissue-Tek Clearing Agent Dish, xylene-resistant (2) • Tissue-Tek Vertical 24 Slide Rack • Drying oven • Cytoseal XYL Cover glass, 24 mm x 50 mm

Prepare the instrument reagents

1. Label the BOND Open Containers as shown in the following table.

Note: The table lists the required container names. If unsure, check your protocols to confirm the reagent names and label your containers accordingly.

RNAscope Reagents	BOND RX Container Name
RNAscope 2.5 LS Hydrogen Peroxide	*Open 0 Haz
RNAscope 2.5 LS Protease III (for protease retrieval only)	*ACD Enzyme
RNAscope LS PretreatPro (protease-free retrieval only)	*RNAscope LS PreteatPro
RNAscope 2.5 LS AMP 1	*ACD Amp 1
RNAscope 2.5 LS AMP 2	*ACD Amp 2
RNAscope 2.5 LS AMP 3	*ACD Amp 3
RNAscope 2.5 LS AMP 4	*ACD Amp 4

RNAscope Reagents	BOND RX Container Name
RNAscope 2.5 LS AMP 5 – BROWN (for protease assay only)	*ACD Amp 5 Brown
RNAscope 2.5 LS AMP Pro (For protease-free assay only)	*RNAscope 2.5 LS Amp Pro
RNAscope 2.5 LS AMP 6 – BROWN	*ACD Amp 6 Brown
RNAscope 2.5 LS Rinse	*LS Rinse
RNAscope 2.5 LS Bluing Reagent†	*ACD Blue
RNAscope 2.5 LS Target Probe	Name of your choice

†Bluing is optional

Note: Leica BOND Polymer Refine DAB Detection (DS9800) is a pre-filled BOND Detection System. The protocols use the DAB chromogen and Hematoxylin from this kit.

2. Carefully transfer all the RNAscope LS reagents into the labeled 30 mL BOND Open containers.
3. Determine the volume of probe reagent needed for the run.

Note: If using other open containers to deliver your probe, you must account for the dead volume required in each container. Suggested volumes can be found in the following table.

Open Container	Suggested Dead-Volume
30 mL	2.5 mL
7 mL	1 mL
6 mL	600 µL

4. Using the barcode scanner, scan the front barcode on the labeled 30 mL BOND Open container. A window will appear.
5. From the drop-down menu, select the corresponding name of the reagent as shown in the table above under **BOND RX Container Name**:
6. Enter the RNAscope 2.5 LS Reagent Kit lot number and the expiration date in their respective fields. Select **OK**.
7. Scan the two side barcodes on the tray for BOND Polymer Refine Detection. When the window opens, select **OK** to register the kit.

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.

RNAscope 2.5 LSx Reagent Kit-BROWN reagents (Cat. No. 322700)

Materials required

Materials provided by Advanced Cell Diagnostics	Materials provided by Leica Biosystems	Materials provided by user
• RNAscope 2.5 LS Target Probe • RNAscope 2.5 LS Positive Control Probe • RNAscope 2.5 LS Negative Control Probe • RNAscope 2.5 LS PretreatPro • RNAscope 2.5 LS AMP Pro • RNAscope 2.5 LSx Reagent Kit - BROWN	BOND RX System • Stainer Bulk Reagents • BOND Wash Solution, 10X • BOND Dewax Solution • BOND Epitope Retrieval Solution 1 • BOND Epitope Retrieval Solution 2	• Distilled water • Drying oven • Fume hood • Tissue-Tek Staining Dish • Tissue-Tek Clearing Agent Dish, xylene-resistant (2) • Tissue-Tek Vertical 24 Slide Rack • Drying oven • Cytoseal XYL • Cover glass, 24 mm x 50 mm

Prepare the instrument reagents

1. Label the BOND Open Containers for your RNA Probes (including controls).

Note: The table lists the required container names. If unsure, check your protocols to confirm the reagent names and label your containers accordingly.

RNAscope Reagents	BOND RX Container Name
RNAscope 2.5 LS Target Probe	Name of your choice
RNAscope LS PretreatPro (for protease-free enzyme retrieval only)	*RNAscope LS PretreatPro
RNAscope 2.5 LS AMP Pro (for protease-free assay only)	*RNAscope 2.5 LS AMP Pro

2. Determine the volume of probe reagent needed for the run.

Note: If using other open containers to deliver your probe, you must account for the dead volume required in each container. Suggested volumes can be found in the following table.

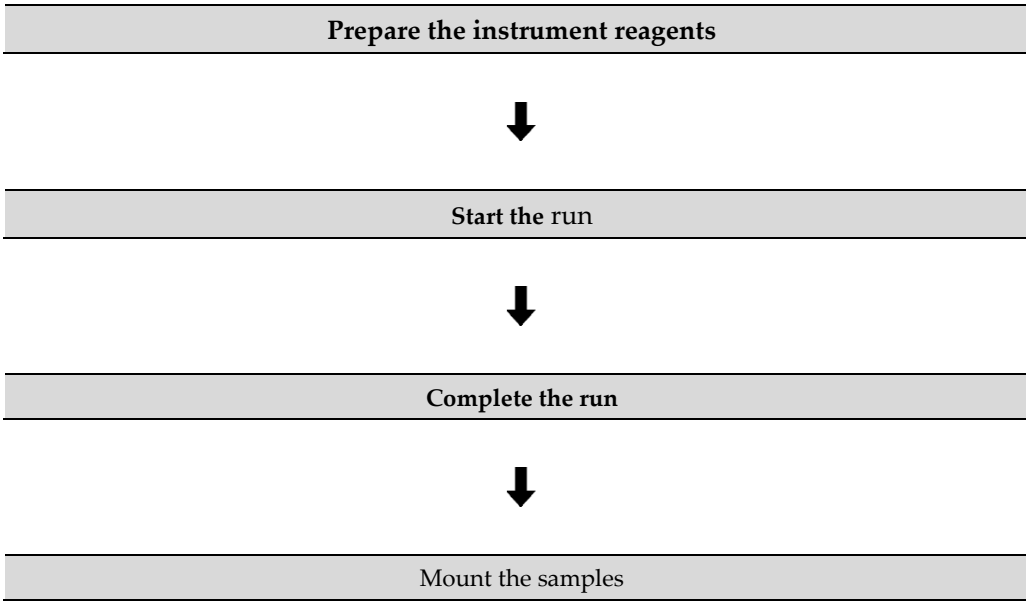
Open Container	Suggested Dead-Volume
30 mL	2.5 mL
7 mL	1 mL
6 mL	600 µL

3. Using the barcode scanner, scan the front barcode on the labeled 30 mL BOND Open container. A window will appear.

4. From the drop-down menu, select the corresponding name of the RNA probe created in Chapter 5. Enter in a Lot# and expiration date in their respective fields. Select **OK**.
5. If running the protease-free assay, label BOND Open Containers for PretreatPro and AMP Pro. Scan the front barcode on these containers and select the corresponding name from the drop-down list. Select **OK**.
6. Transfer the RNA Probes, (and if running the protease-free assay) the LS PretreatPro and LS 2.5 AMP Pro reagents into the corresponding BOND Open Containers.
7. Scan the two side barcodes on Tray #1 for the RNAscope 2.5 LSx Reagent Kit-BROWN kit. When the window opens, select **OK** to register the tray.
8. Next scan the front barcode for the first reagent in Tray #2 (ie, AMP reagents). Select **OK** and then repeat for all containers in Tray #2.

7 Chapter 7. Running the Assay

Workflow



Prepare the instrument

1. Fill the large containers located in the bottom of the instrument with the BOND RX bulk reagents. Dilute Bond Wash Solution 10X Concentrate 1:10, with DI Water to make the 1X Bond Wash Solution (working solution).

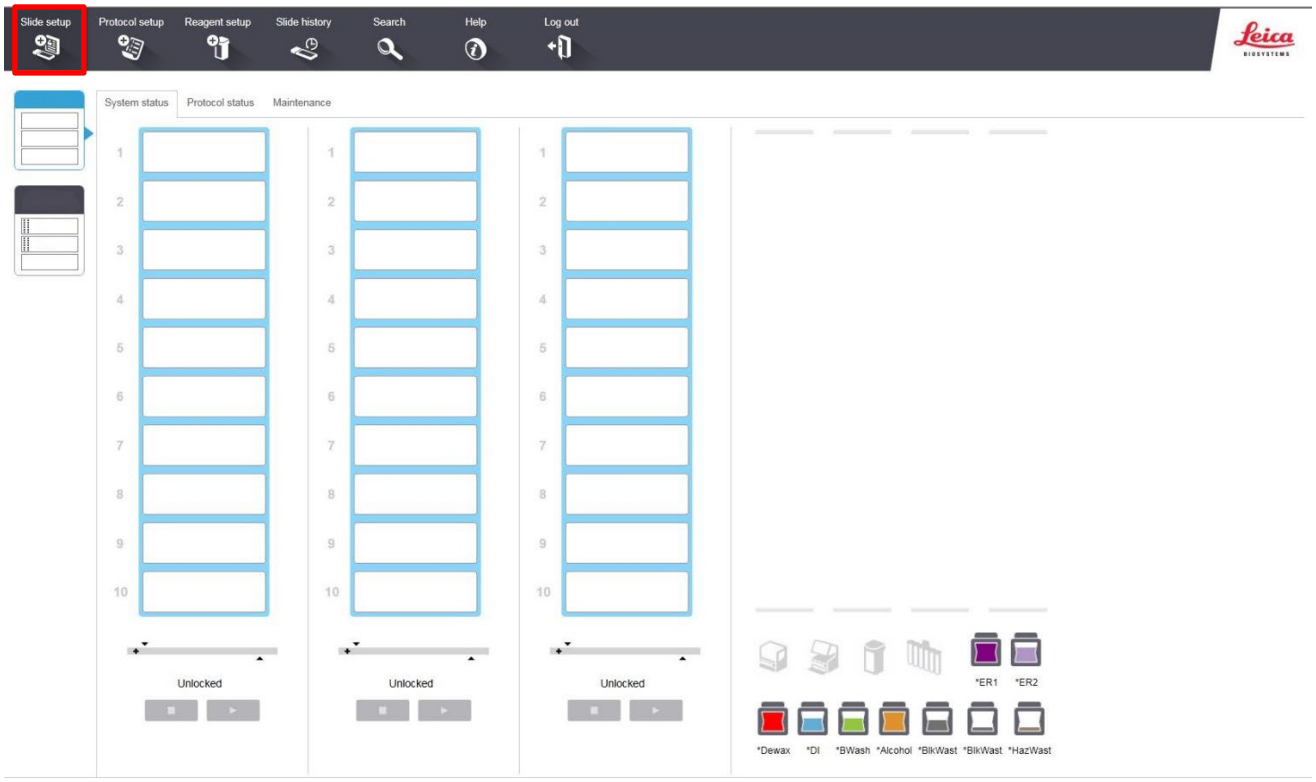
Note: Insufficient bulk reagent volumes may lead to run failure.

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.

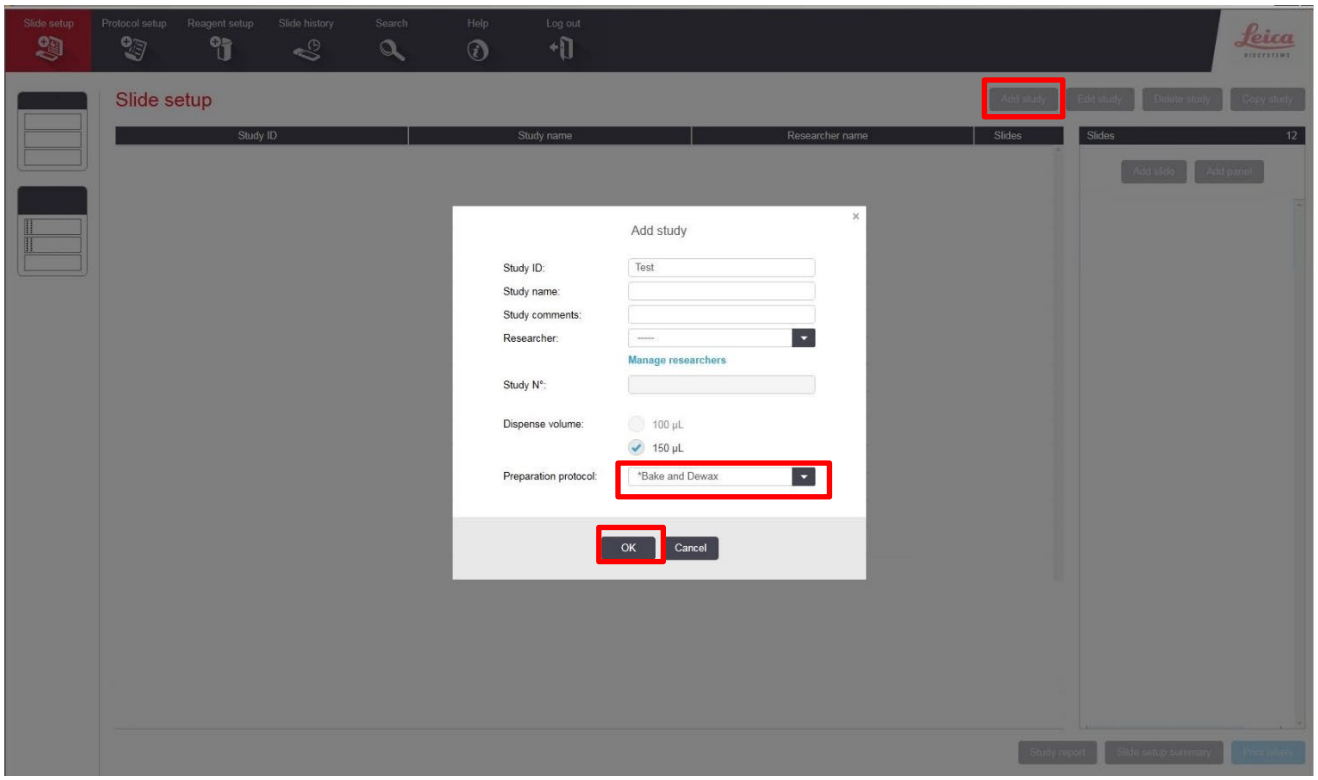
2. Use clean, dry covertiles for every run. Clean used covertiles with water, bleach, and ethanol. Air dry before reuse. See Leica Biosystems documentation for details.
3. Before starting a run, empty bulk waste containers. Discard waste according to all local, state/provincial, and/or national regulations.

Set up a study

1. To build a study, 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 alternative tissue preps, see **Appendix D**).
4. Select **OK**.

Set up slides

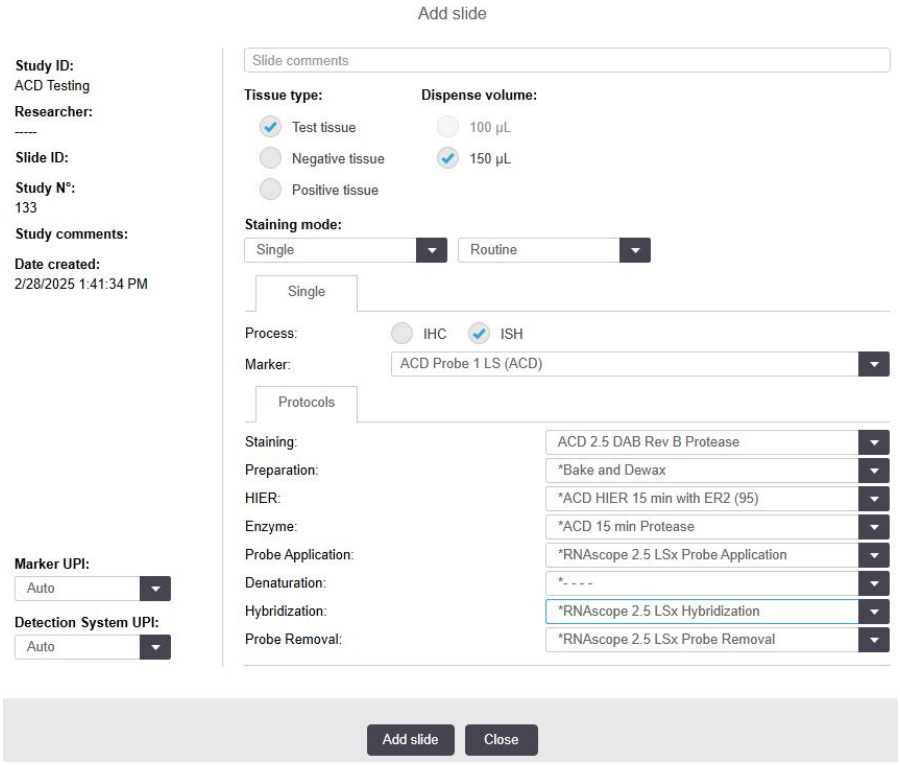
The slide setup differs based on the assay (protease or protease-free) and the reagents (user-filled RNAscope 2.5 LS reagents or RNAscope 2.5 LSx Reagents – BROWN kit).

The following screen shots indicate the protocols to select for the slide setup for each of the assay and reagent variables. Ensure you select the correct protocols.

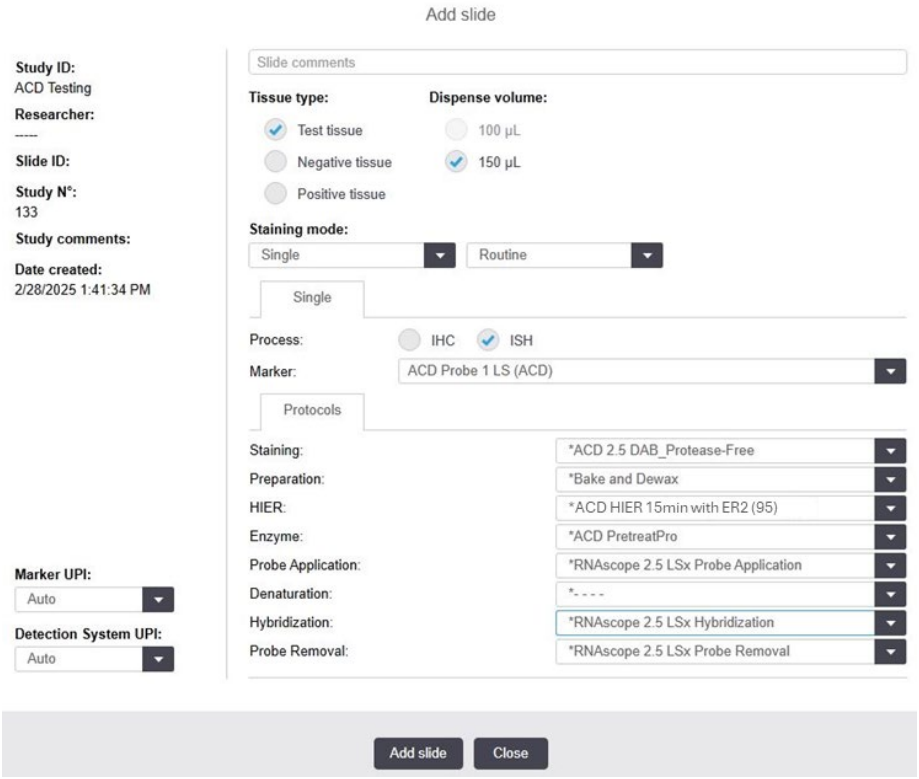
1. Select **Add slide** to assign a protocol to each slide.
2. Enter the tissue type and probe name in the Comments field.
3. Choose the Staining, HIER, Enzyme and Hybridization protocols depending on the reagent kit and enzyme method you are using. Ensure you change Probe Application and Probe Removal protocols from *Default to the *RNAscope 2.5 LSx choice.

See the following four options.

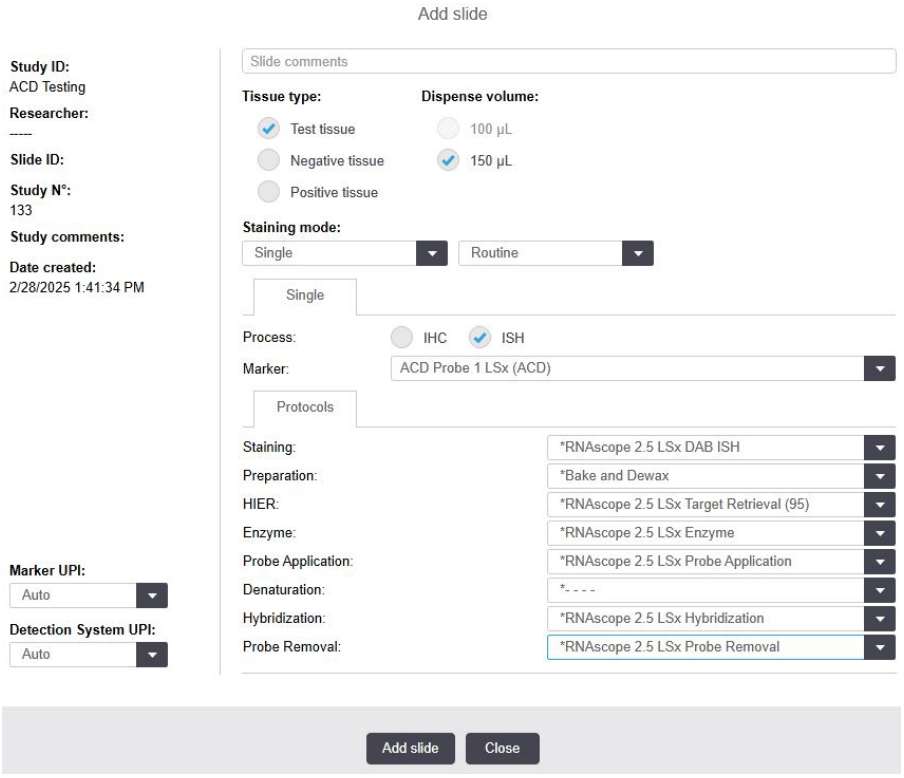
User-filled RNAscope 2.5 LS reagents using protease pretreatment



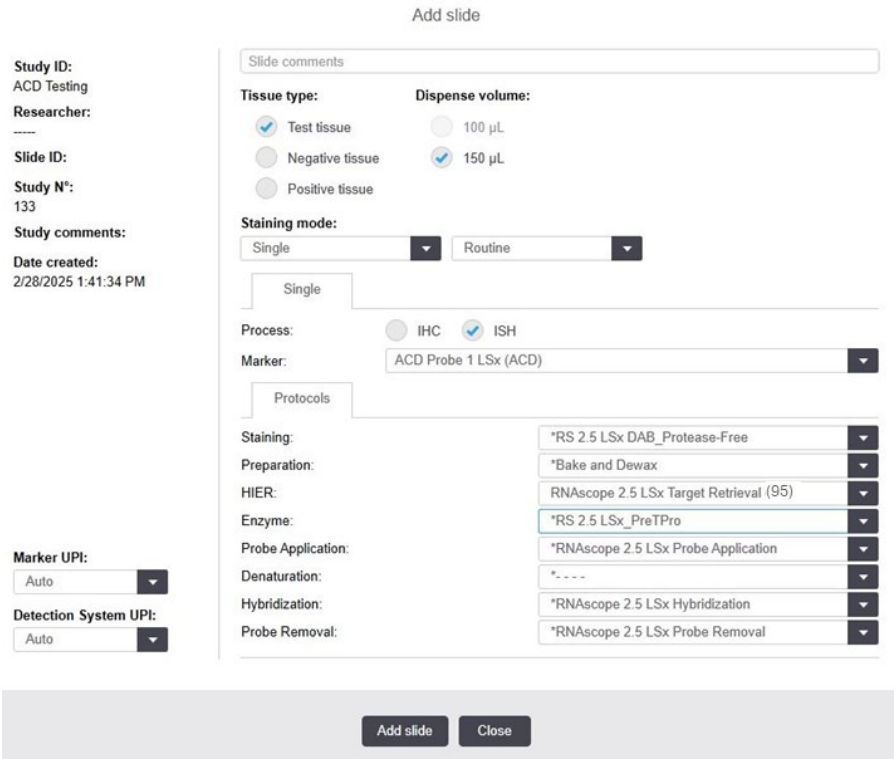
User-filled RNAscope 2.5 LS reagents using protease-free pretreatment



RNAscope 2.5 LSx Reagents – BROWN kit using protease pretreatment

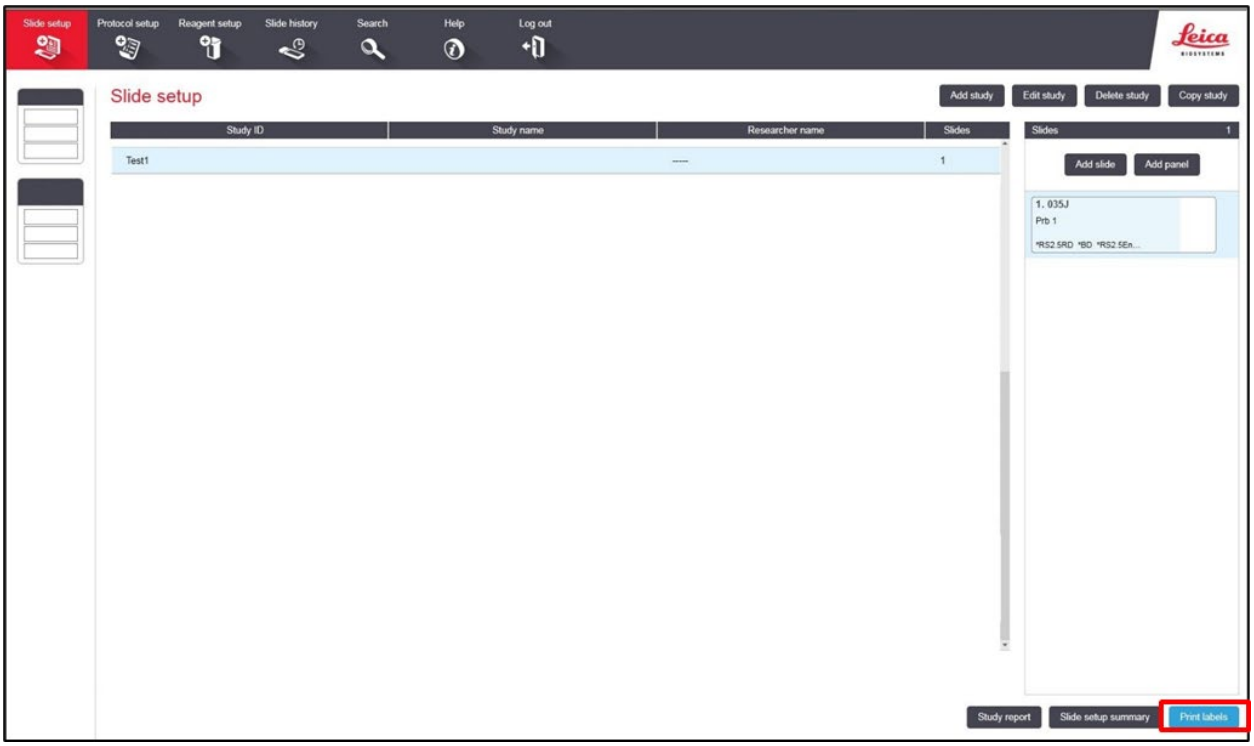


RNAscope 2.5 LSx Reagents – BROWN kit using protease-free pretreatment



Note: HIER protocol time and temperature varies depending on tissue type.

4. Select **Add slide**.
5. After adding all the slides to the study, select **Close** to return to the Slide setup screen.
6. Select **Print labels** to print barcodes to attach to the slides.



Start the run

1. Attach the barcode labels to the slides and add the slides to the slide tray with the label sides facing up.

Note: Add a covertile on top of each slide. The rectangular-shaped neck of the covertile should fit into the groove of the slide tray. Verify placement and seating of covertile.

2. Place the tray in the BOND RX and press the button to load the tray onto the machine.
3. Once the slides have been scanned, select the **PLAY** (triangular) button on the screen located under the start tray to start the run. Alternatively, right-click on scanned label images and select **Delayed Start** to start the run at a future time.

IMPORTANT! Before leaving the instrument unattended, ensure that the instrument is running successfully. In the event of a problem, please contact **support.acd@bio-techne.com** or your Field Application Scientist.

Complete the run

1. After the run is complete, press the button on the front of the instrument to unload the slides.
2. Place the slides onto the Tissue-Tek Slide Rack and move the rack into a staining dish containing distilled water.

3. Flush the slides with flowing tap water for a few minutes.
4. (Optional) Perform offline bluing of choice or use the RNAscope 2.5 LS Bluing Reagent included in the kit by pipetting a small amount onto each slide for **1–2 MIN** followed by rinsing.

Dehydrate the slides

1. Move the Tissue-Tek Slide Rack into the staining dish containing 70% Ethanol in the fume hood for **2 MIN**. Agitate the slides by occasionally lifting the slide rack up and down.
2. Move the slide rack into a second staining dish containing 95% Ethanol for **2 MIN** with occasional agitation.
3. Move the slide rack into a third staining dish containing 95% Ethanol for **2 MIN** with occasional agitation.
4. Move the Tissue-Tek Slide rack into a clearing agent dish containing xylene for **5 MIN** with occasional agitation.

Mount the samples

1. Remove the slides from the Tissue-Tek Slide Rack and lay flat with the sections facing up in the fume hood.
2. Mount one slide at a time by adding **1 DROP** of Cytoseal or other xylene-based mounting medium to each slide and carefully placing a 24 mm x 50 mm coverslip over the section. Avoid trapping air bubbles.
3. Air dry slides for **5 MIN**.
4. Proceed to Error! Reference source not found..

8 Chapter 8. Evaluate the Results

Examine tissue sections under a standard bright field microscope at 20–40X magnification:

- Assess tissue and cell morphology.
- Assess positive control signal strength. Positive control signal should be visible as punctate dots within the cell at 20–40X magnification.
- Assess negative control background. One dot to every 10 cells displaying background staining per 20X microscope field is acceptable.
- Evaluate target probe signal using the scoring guidelines in the next section.

Scoring guidelines

The RNAscope 2.5 LS Assay enables a semi-quantitative 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 RNAscope staining intensity is presented below for a gene with expression level varying between 1 to > 10 copies per cell.

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

Categorize staining into five grades: **0, 1+, 2+, 3+, and 4+** according to the following table:

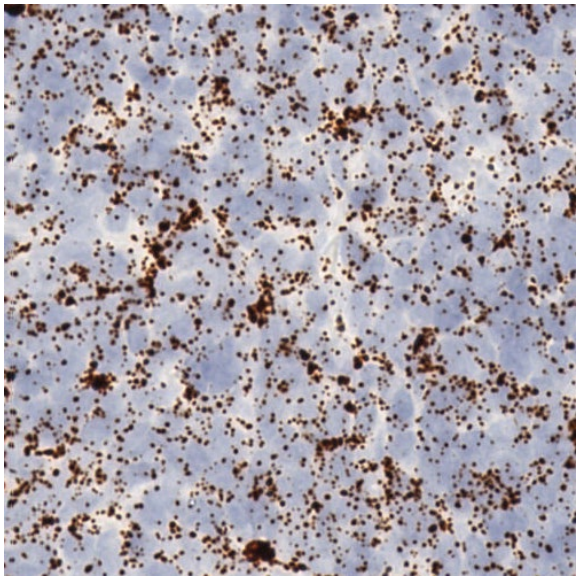
Staining Score	Microscope Objective Scoring*
0	No staining or less than 1 dot for every 10 cells (40X magnification)
1	1–3 dots/cell (visible at 20–40X magnification)
2	4–9 dots/cell. No or very few dot clusters (visible at 20–40X magnification)
3	10–15 dots/cell and/or < 10% positive cells have dot in clusters (visible at 20X magnification)
4	>15 dots/cell and/or >10% positive cells have dot in clusters (visible at 20X magnification)

* Discount cells with artificially high nuclear background staining.

Control example

If the assay is successful, the staining should look like the following image:

Figure 2. RNAscope 2.5 Assay detection of PPIB mRNA in lymph node FFPE tissue



Troubleshooting

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

- For the protease assay, if you observe the presence of background staining, increase the Epitope Retrieval 2 (ER2) in increments of five minutes and/or increase the protease time in increments of ten minutes (see **Appendix B** and **C** for instructions on editing protocols).
- For the protease-free assay, if you observe lower signal intensity, increase the ER2 temperature from 88°C to 92°C for cell pellets or from 95°C to 100°C for tissues to obtain better signal intensity.
- Use the previous process for over-fixed tissues.
- The RNAscope 2.5 LS RED and LS BROWN assays use Leica Biosystems' BOND Polymer Refine Detection and BOND Polymer Refine Red Detection kits, respectively. Do not use 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.
- Do not alter the staining protocol in any way except for the hematoxylin incubation time. The parameters in the staining protocol have been optimized to run the RNAscope assay on the instrument.

For troubleshooting information, please contact technical support at **support.acd@bio-techne.com**.

A Appendix A. Pretreatment Guidance for FFPE Samples

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

- Any new or previously untested FFPE tissue types
- 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

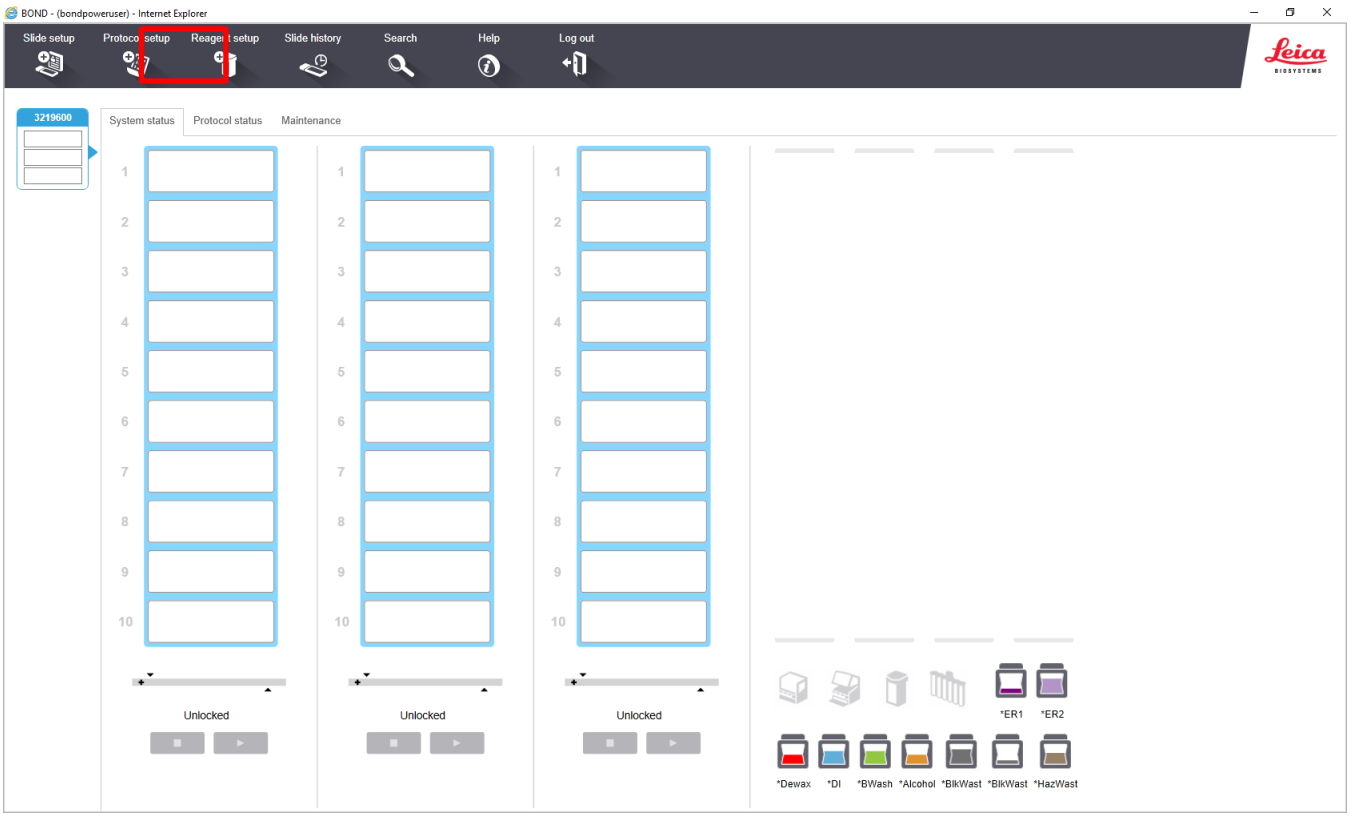
Species	Tissue Type	Pathology	Pretreatment Condition
Dog	Spleen	Normal	Mild
	Lymph Node	Mild	Mild
	Tonsil	N.A.	N.A.
	Thymus	Mild	Mild
	Retina	Mild	Mild
	Prostate Gland	Mild	Mild
	Epididymis	Mild	Mild
	Testis	Mild/Standard	Mild/Standard
	Ovary	Mild/Standard	Mild/Standard
	Duodenum	Normal	Mild
	Jejunum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

B Appendix B. Edit a Heat Treatment Protocol

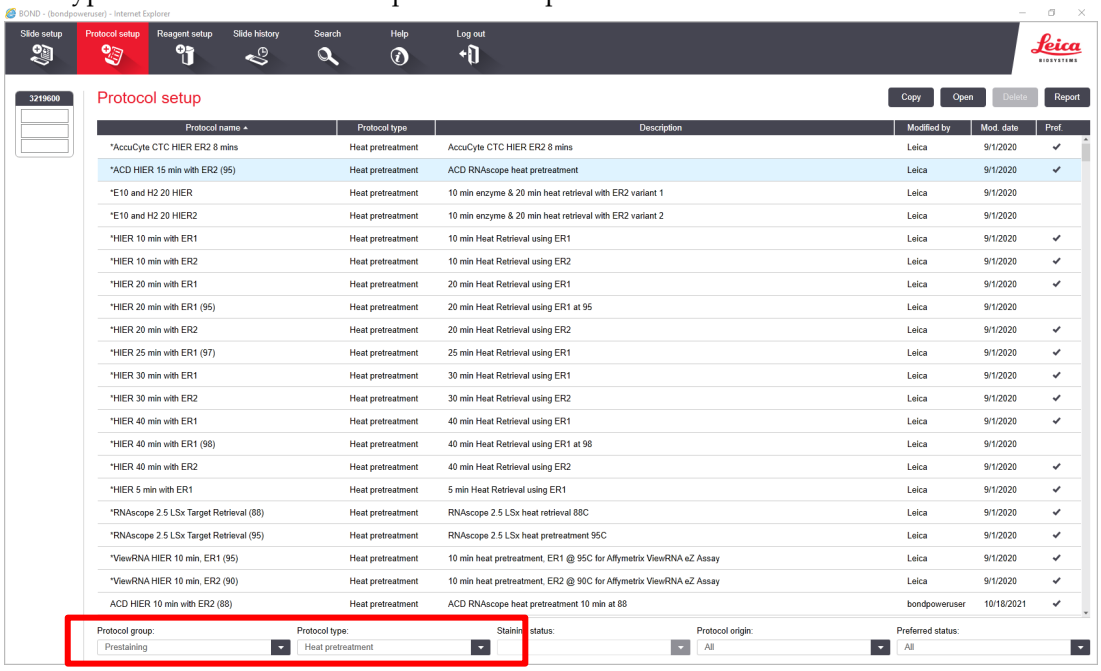
The following example shows how to edit the Epitope Retrieval procedure from within the software.

Create a prestaining protocol

1. Open the Leica BOND software and click on the **Protocol setup icon** as shown.



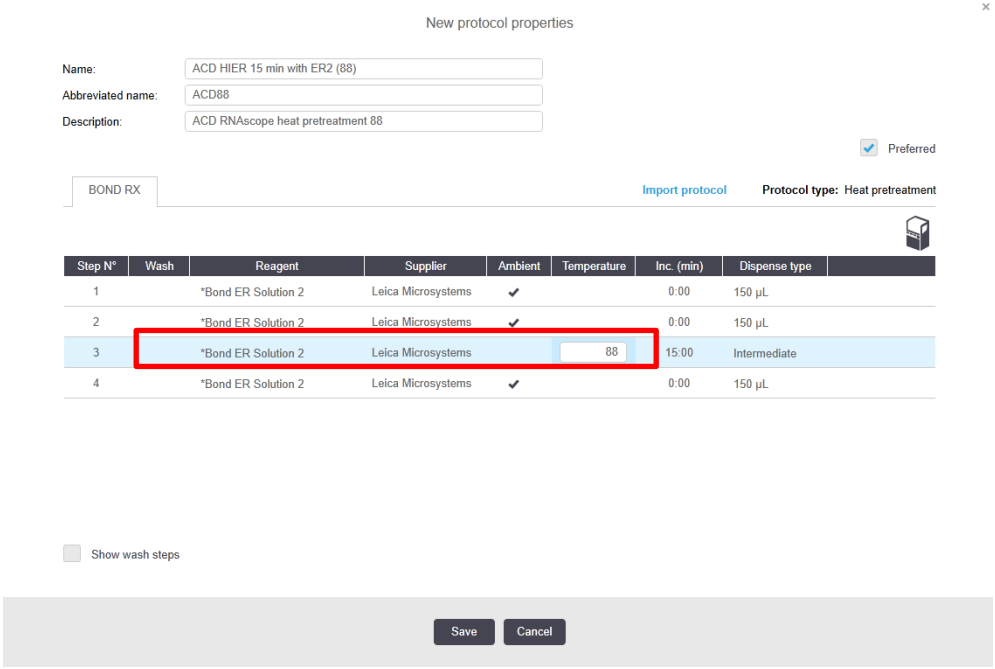
2. Select **Prestaining** under the Protocol group menu and **Heat pretreatment** under the Protocol type menu to access the heat pretreatment protocols.



3. Highlight a protocol, for example, ***ACD HIER 15 min with ER2 (95).*** Select **Copy.**

Note: ER2 = Epitope Retrieval 2.

4. Rename the protocol as **ACD HIER 15 min with ER2 (88)**.
5. Rename the Abbreviated name as **ER2-88**.
6. Rename the Description to **ACD RNAscope heat pretreatment 88**.
7. Highlight the third *BOND ER Solution 2 step (see above) and change temperature to **88°C.**

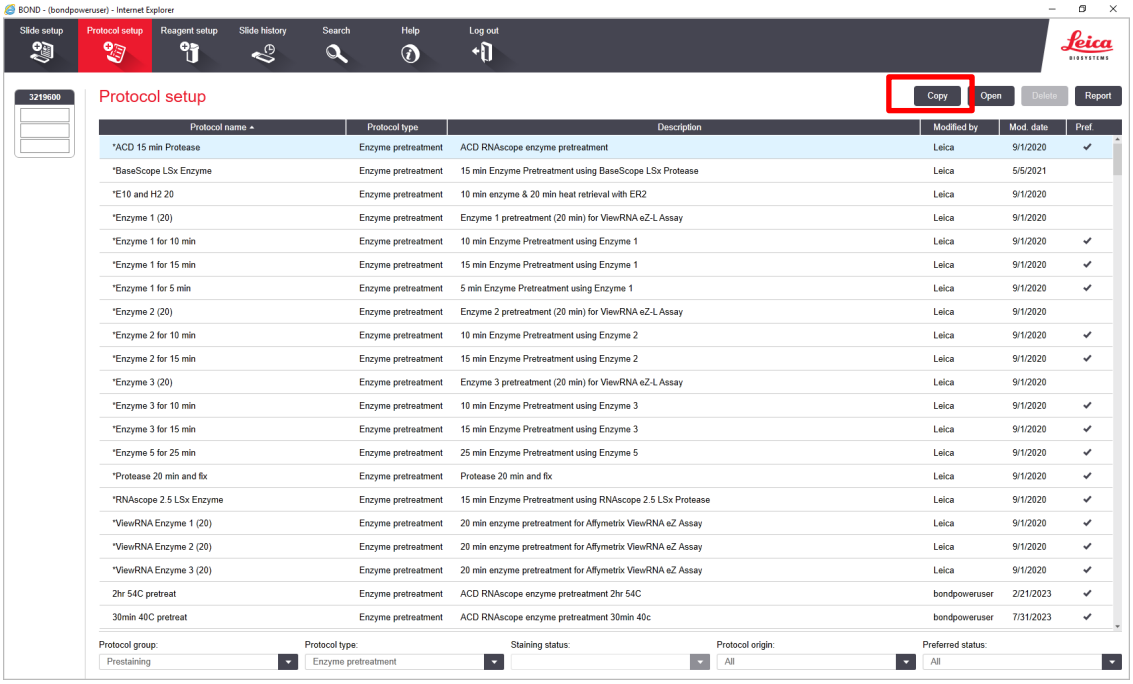


8. For RNAscope, ER 2 temperature varies between **95°C** and **88°C** depending on the tissue type used. Please see **Appendix A** for a list of tissues.
9. Select **Save** to create a protocol for ER2 pretreatment at **88°C**.
10. If needed, repeat Steps 1–8 to create new heating protocols for different incubation times (for example, ACD 25minER2).

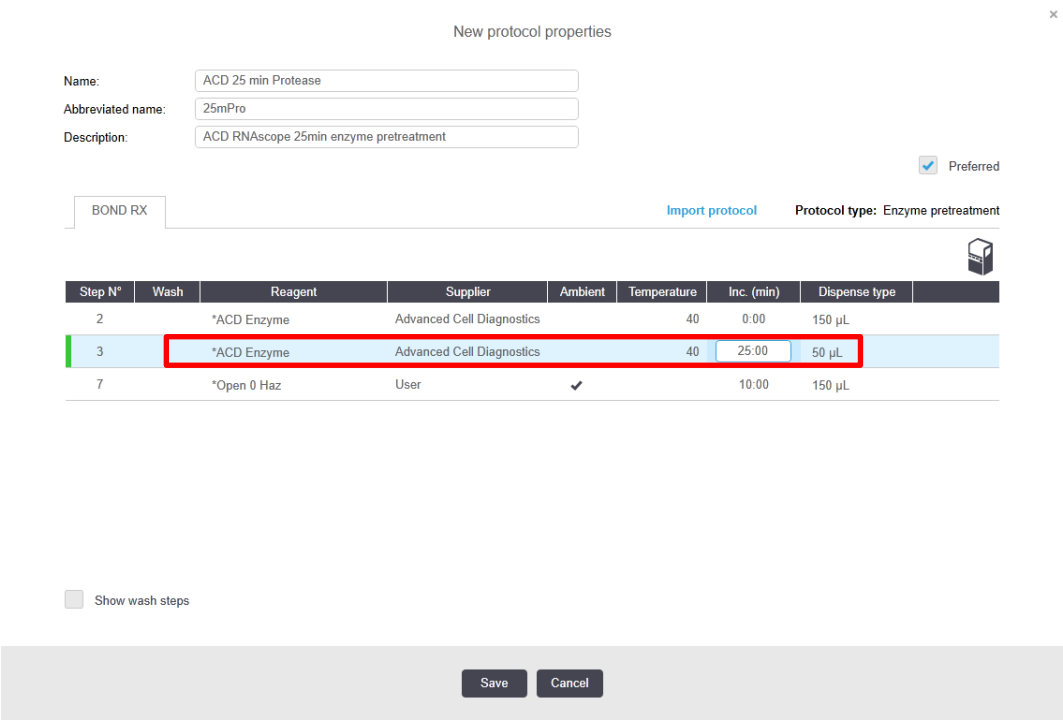
Appendix C. Edit an Enzyme Pretreatment Protocol

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**.
5. 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).
6. A similar workflow can be used to edit the *ACD PretreatPro and *RS 2.5 LSx PreTreatPro protocols if needed.

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

D Appendix D. Slide Setup for Additional Tissue Types

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

Note: The following guidelines are not for use with the older mock probe workflow. If you need to use the older workflow and need assistance, please contact **support.acd@bio-techne.com** or your ACD FAS.

Fixed-frozen tissues

As described in **Chapter 4**, fixed-frozen tissues have only been optimized using the protease method. Start with the following conditions:

- ER2 retrieval of 5 minutes at 95°C
- 15 minutes of ACD's protease treatment at 40°C

1. Confirm that you have a heat pretreatment available for the 5-minute treatment. See **Appendix B** to create this protocol if needed.
2. In Slide setup, select the following:
   a. Staining: Choose the appropriate staining protocol for the chemistry and workflow you are using; LS 2.5 or LSx assay kits
   b. Preparation: Select ***----**.
   c. HIER: Choose **ACD HIER 5 min with ER2 (95)** or an edited **RNAscope 2.5 LSx Target Retrieval** protocol.
   d. Enzyme: Select the appropriate protocol for the chemistry and workflow you are using; ***ACD 15min Protease** or **RNAscope LSx Enzyme**.
   e. Probe Application: Select ***RNAscope 2.5 LSx Probe Application**.
   f. Denaturation: Choose ***….**
   g. Hybridization: Choose the appropriate protocol for the chemistry and workflow you are using; **RNAscope 2.5 LSx Hybridization**.
   h. Probe Removal: Select ***RNAscope 2.5 LSx Probe Removal**.

Note: 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.

Start your run immediately after setting it up. Do not use a delayed start. This causes poor protease spreadability and negatively impacts results.

Fresh-frozen tissues

As described in Chapter 4, there are 2 options for processing Fresh-frozen tissues on the BOND RX: a protease-free option and a protease-based option that requires RNAscope LS Protease IV

Cat. No. 322140 which must be ordered separately. See below for both slide set ups. We recommend starting the protease-free workflow.

Protease-free workflow

With this method, a short retrieval step is applied followed by treatment with LS PretreatPro.

1. In Slide setup, please skip the Bake and Dewax steps
   a. Staining: Select the appropriate protocol depending on the kit format you have: LS 2.5 or LSx assay kits.
   b. Preparation: Choose ***----**.
   c. HIER: Choose **ACD HIER 5 min with ER2 (95)** or edited **RNAscope 2.5 LSx Target Retrieval (for 5mins)** protocol depending on the kit format you have (See **Appendix B** to create this protocol if needed)
   d. Enzyme: *ACD PretreatPro OR *RS 2.5 LSx PreTPro depending on the kit format you have
   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**.

Note: Start your run immediately after setting it up. Do not use a delayed start. This causes poor protease spreadability and negatively impacts results.

Protease workflow

With this method tissues do NOT need a target retrieval. Instead, permeabilize the tissue at ambient temperature with a stronger protease; RNAscope LS Protease IV Cat. No. 322140

1. In Slide setup, please skip the following steps: 1) Bake or Bake and Dewax 2) Heat retrieval. Choose the following instead:
   a. Staining: Select the appropriate protocol depending on the kit format you have: LS 2.5 or LSx assay kits.
   b. Preparation: Choose ***----**.
   c. HIER: Choose ***----**.
   d. Enzyme: Create and select a protocol with the following conditions **ACD 30min RT with LS Protease IV** (see **Appendix C**. Edit an Enzyme Pretreatment Protocol
   e. Probe Application: Select ***RNAscope 2.5 LSx Probe Application**.
   f. Denaturation: Select ***….**
   g. Hybridization: Choose ***RNAscope 2.5 LSx Hybridization**.
   h. Probe Removal: Select ***RNAscope 2.5 LSx Probe Removal**.

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 which can result in poor permeabilization.

E Appendix E. 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) before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see **http://www.acdbio.com/technical-support/user-manuals**
- 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), found at: **www.access.gpo.gov/nara/cfr/waisidx_01/%2029cfr1910a_01.html**
- 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, found at: **http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/**
- Information about the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) can be found at: **http://echa.europa.eu/regulations/reach**

Documentation and Support

Obtaining SDSs

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

Obtaining support

For the latest support information, go to: **https://www.bio-techne.com/resources** or Contact us at **https://www.bio-techne.com/support/contact-us**.

Contact information

Advanced Cell Diagnostics, Inc.

7707 Gateway Blvd

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: **order.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 Bio-techne website at **https://www.bio-techne.com/terms-and-conditions**. If you have any questions, please contact Advanced Cell Diagnostics at **https://www.bio-techne.com/support/contact-us**

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