a bio-techne brand

USER MANUAL

RNAscope™ 2.5 LS Reagent Kit – BROWN User Manual

For use with Leica Biosystems' BOND RX System

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

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Citing RNAscope in Publications

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

Disclaimers

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Contents

1 Chapter 1. Product Information

Before using this product, read and understand the information in **Appendix F. Safety** on page **57** 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 Reagent Kit for use with Leica Biosystems' BOND RX Research Advanced Staining System. RNAscope 2.5 LS Assays are compatible with a variety of sample types.

Product description

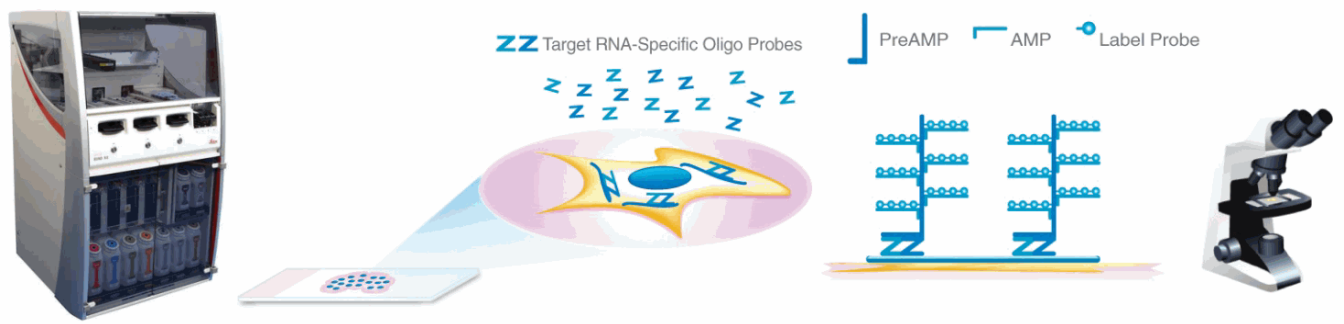
Background

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 Leica Biosystems' BOND RX System.

Overview

Error! Reference source not found. 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.

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 **www.acdbio.com/products/target-probes/search-product** to find a gene-specific Target Probe or order a custom probe. Visit **www.acdbio.com/products/target-probes/controls-housekeeping** to order appropriate Control Probes.

Each probe is sufficient for staining ~30 standard slides. The probes have a shelf life of two years from the 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

The RNAscope 2.5 LS Reagent Kit – BROWN (Cat. No. 322100) contains 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 Ready-To-Use (RTU) and 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	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 bottle	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

Required materials from Leica 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	Op309700	Room temp (20–25°C)
	BOND Universal Covertiles 100 pack	S21.2001	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	2–8°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.
	Leica Biosystems' 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
	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 Leica Biosystems' BOND RX Research Advanced Staining System. Refer to the *Leica Biosystems' BOND RX System Instructions For Use*.
- 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. 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 F. Safety** for more information.

3 Chapter 3. Prepare Samples

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

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

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.

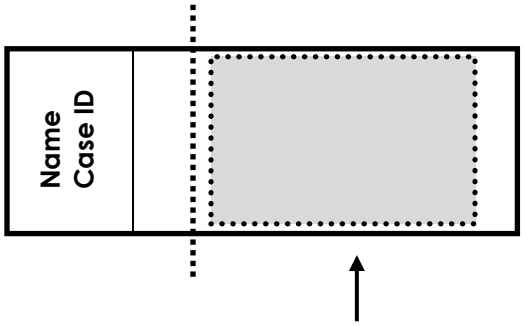
Dehydrate, embed, and cut the sample

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

Bake and fix the sections

1. Remove fixed-frozen tissue slides from **-80°C** and place in a Tissue Tek Slide Rack. Bake in drying oven for **60 MIN** at **60°C**.
2. *Immediately* immerse the slides in prechilled 10% NBF or 4% PFA for **15–60 MIN** at **4°C.**

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

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**.
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

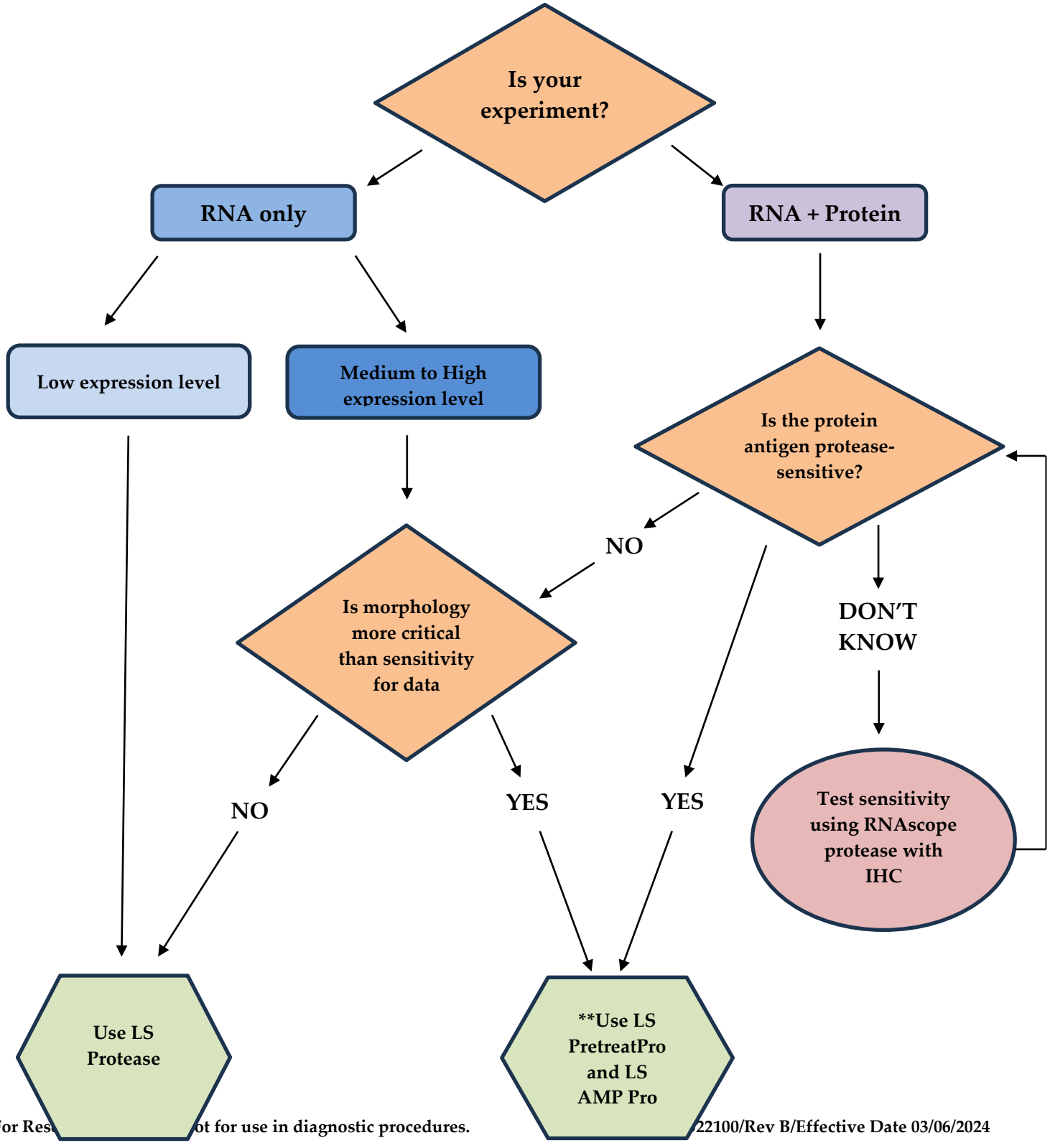
FFPE samples must be de-crosslinked with a target retrieval step. The RNAscope 2.5 LS Assay uses the BOND RX's ER2 solution exclusively for this step.

Permeabilization

Two options are available:

- Protease-based permeabilization is recommended for experiments that stain only RNA. This option uses LS Protease III.
- Protease-free permeabilization uses the LS PretreatPro reagent which is free of protease enzyme. This allows co-detection of RNA and proteins that were previously incompatible with protease on the same tissue section using immunohistochemistry (IHC). Please refer to **Appendix D**. How to use LS PretreatPro to implement the use of LS PretreatPro reagent on the BOND Rx.

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, please refer to the **Optimization Note** in **Appendix D** on page 53

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 LS Protease III, 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	Standard
BOND ER2	15 MIN @88C	15 MIN @95C
LS Protease III	15 MIN @40C	15 MIN @40C
OR		
LS PretreatPro* (see Appendix D)	30 MIN @40C	30 MIN @40C

Sample types, such as certain xenografts and cell pellets, might require shorter incubation time. For these tissue types, reduce the BOND ER2 incubation time. LS Protease III and LS PretreatPro incubation times can also be adjusted but is rarely needed. If you have a tissue type not listed, contact ACD Support at **support.acd@bio-techne.com.**

Pretreat fixed-frozen sections

Target Retrieval

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

Permeabilization

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

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 LS Protease III until positive control RNA signal is maximized with minimal or no negative RNA control signal (see **Appendix E**. Slide Setup for Additional Tissue Types for details).

Reagent	Standard
BOND ER2	5 MIN @95C*
LS Protease III†	15 MIN @40C

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

†Sample types, such as certain xenografts and cell pellets, might require shorter incubation time. For these tissue types, reduce the BOND ER2 incubation time. LS Protease III time can also be adjusted but is rarely needed.

Pretreat fresh-frozen sections

Target Retrieval

Fresh-frozen sections do not need target retrieval.

Permeabilization

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

Tissue pretreatment recommendations

Use 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 **Protease** IV until positive RNA control signal is maximized with minimal/no negative RNA control signal (see **Appendix E**. Slide Setup for Additional Tissue Types for details).

Reagent	Standard
LS Protease IV (ACD Part Number 322140)	30 MIN @Ambient*

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

5 Chapter 5. Set Up Software Version 5.2

Use the instructions in this chapter to set up the RNAscope 2.5 LS Assay using software version 5.2. To set up the assay using software version 6.0, proceed to **Chapter 6**.

IMPORTANT! BXD11 or higher is required to run the following setup on software version 5.2. Please contact your Leica FAS to upgrade to BXD11 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.

This workflow is a "workaround" in the existing 5.2 software to accommodate the RNAscope 2.5 LS Assay. It performs probe hybridization during the staining protocol to enable the use of a high probe volume required for optimal assay performance. In summary, a probe is created as an ancillary reagent and added to the staining protocol. A 'Mock Probe' and mock one-minute hybridization protocol are also created to fulfill BOND RX software requirements when performing any staining run. Follow the steps in this chapter to enable the workflow. Your ACD Field Application Specialist (FAS) can help you implement this procedure.

Workflow

Register the reagents

↓

Create a one-minute probe hybridization protocol (ONE TIME ONLY)

↓

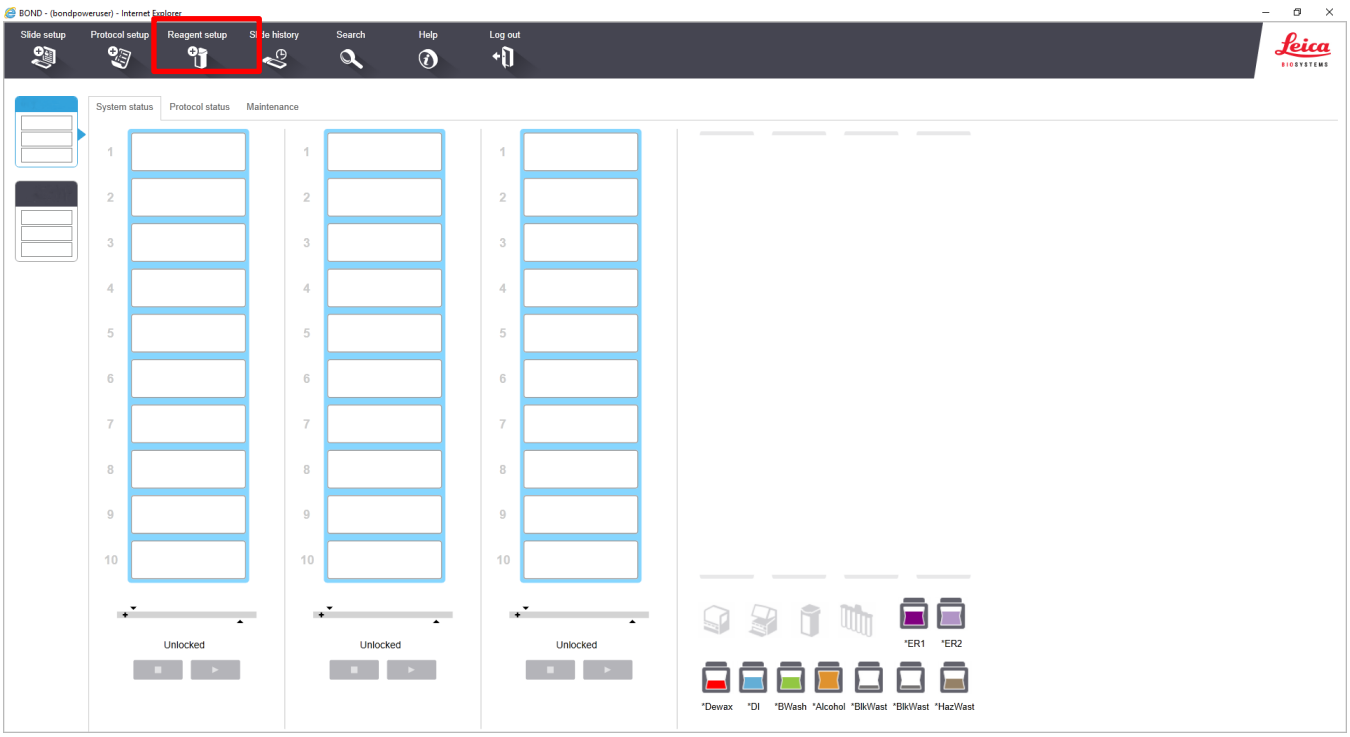
Create a staining protocol (FIRST TIME FOR EACH PROBE)

↓

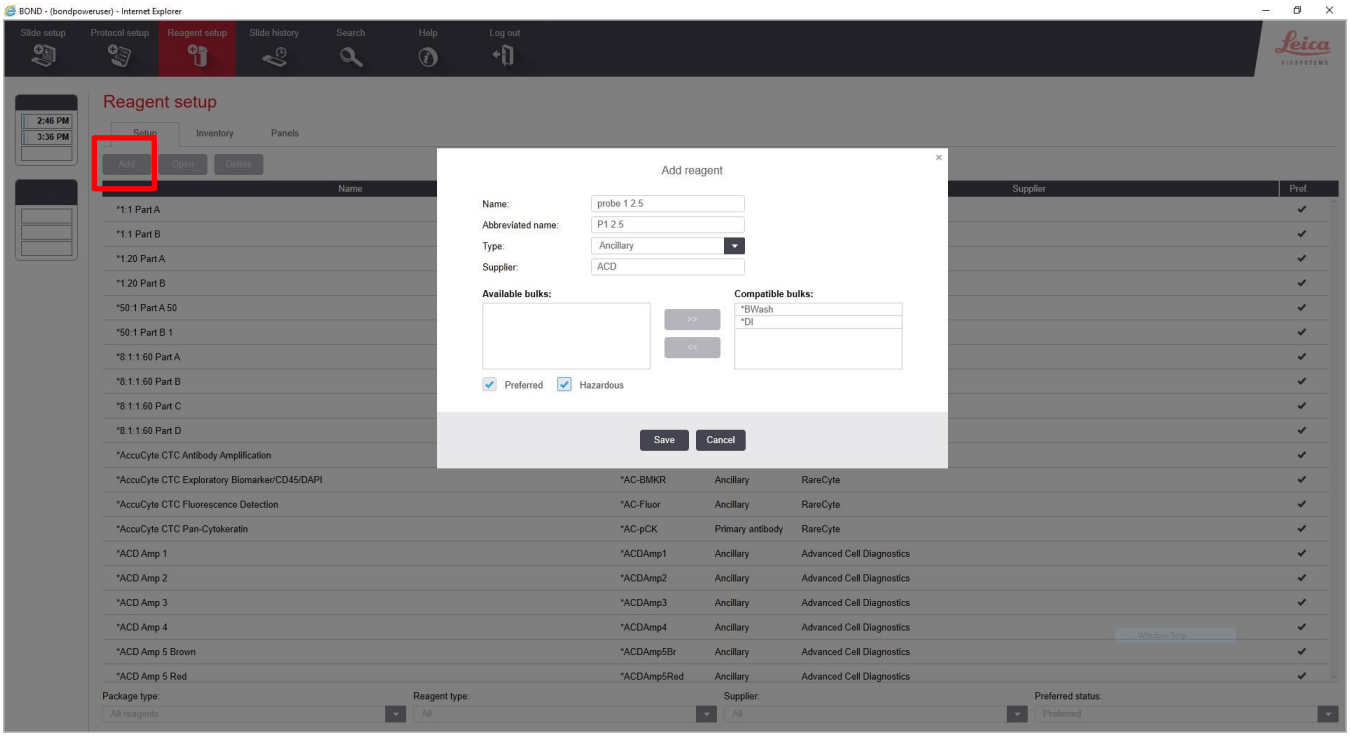
Register the mock probe (ONE TIME ONLY)

Register the reagents

1. Select the **Reagent Setup** icon at the top of the screen.



2. Select **Add** to enter reagent information.



3. Enter a reagent name (for example, probe1 2.5) in the Name text box.
4. Enter **P1 2.5** (for example) in the Abbreviated name text box.
5. Select **Ancillary** in the Type drop-down menu.
6. Enter **ACD** in the Supplier text box.

7. Check both the **Preferred** and **Hazardous** boxes (for all probe reagents).
8. Select **Save**.

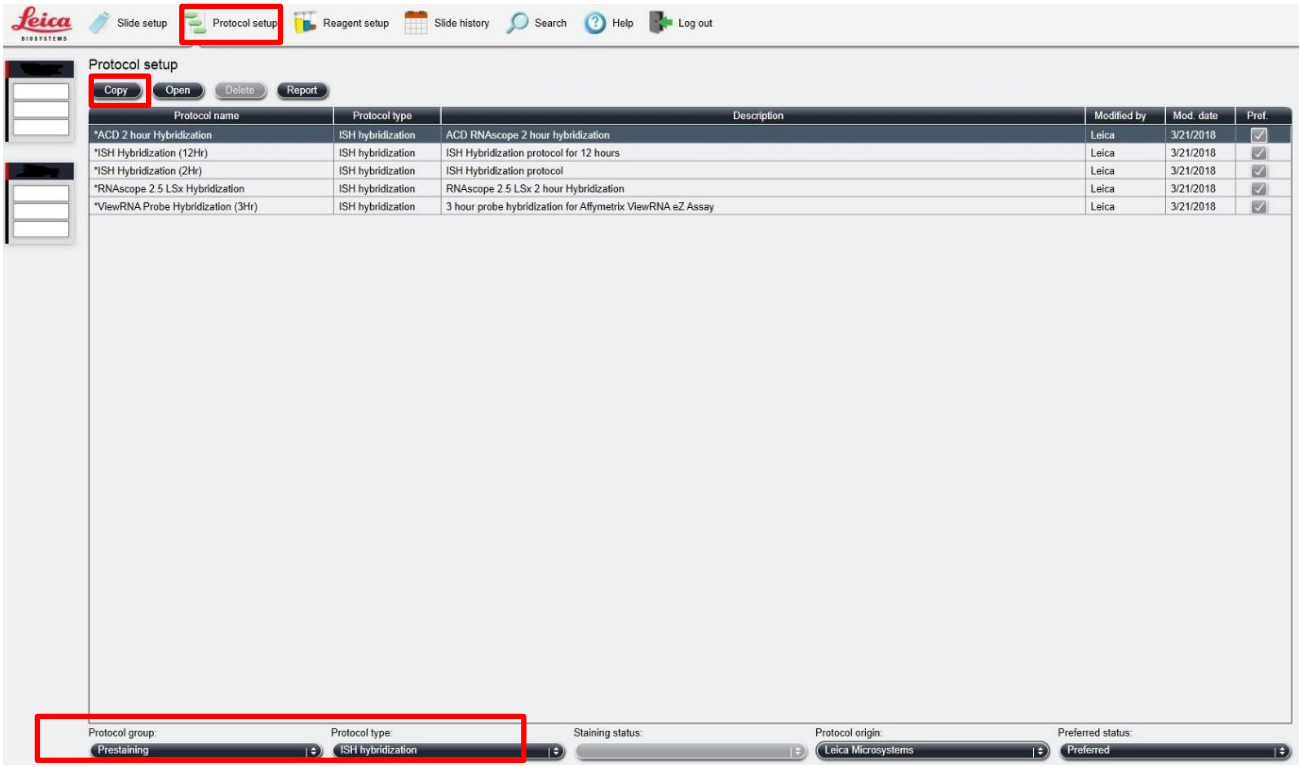
Note: Standard Amp reagents are pre-loaded into the database for this chemistry and do not need to be registered.

Create a one-minute probe hybridization protocol (one time only)

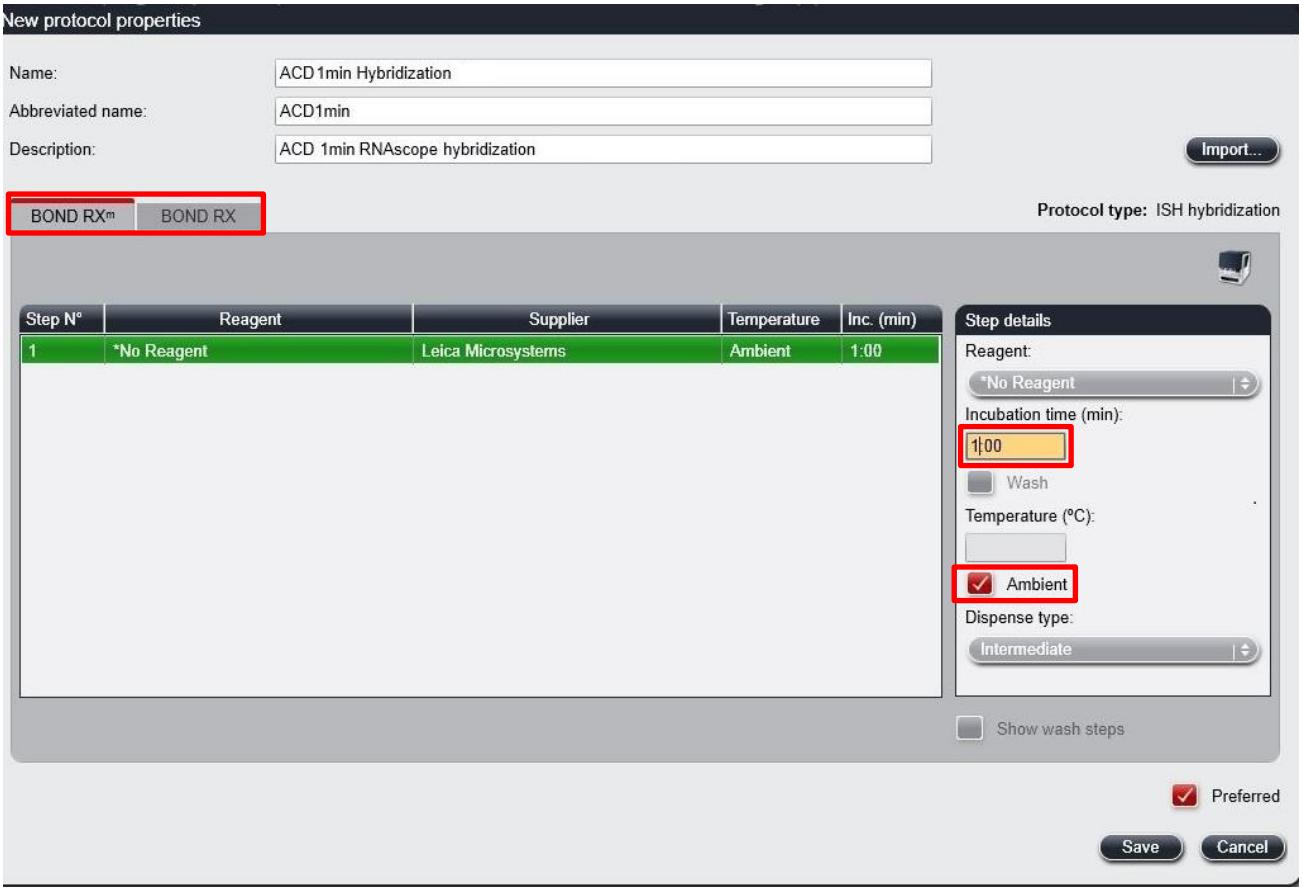
A mock probe hybridization protocol must be created as part of the version 5.2 software workaround for the RNAscope 2.5 LS assay. The following example copies the existing two-hour hybridization protocol and changes the incubation time to one minute and the temperature to ambient.

If an existing one-minute probe hybridization protocol already exists, skip to the next section.

1. In the Protocol setup screen, select **Prestaining** from the Protocol group menu and **ISH Hybridization** from the Protocol type menu.
2. Highlight the ***ACD 2 hour Hybridization** protocol. Select **Copy.**



3. When using 5.2 software ensure that you have the correct instrument tab highlighted before making the following edits.
4. Change the Name to **ACD 1min Hybridization**, the Abbreviated Name to **ACD1min**, and the Description to **ACD 1min RNAscope hybridization**.

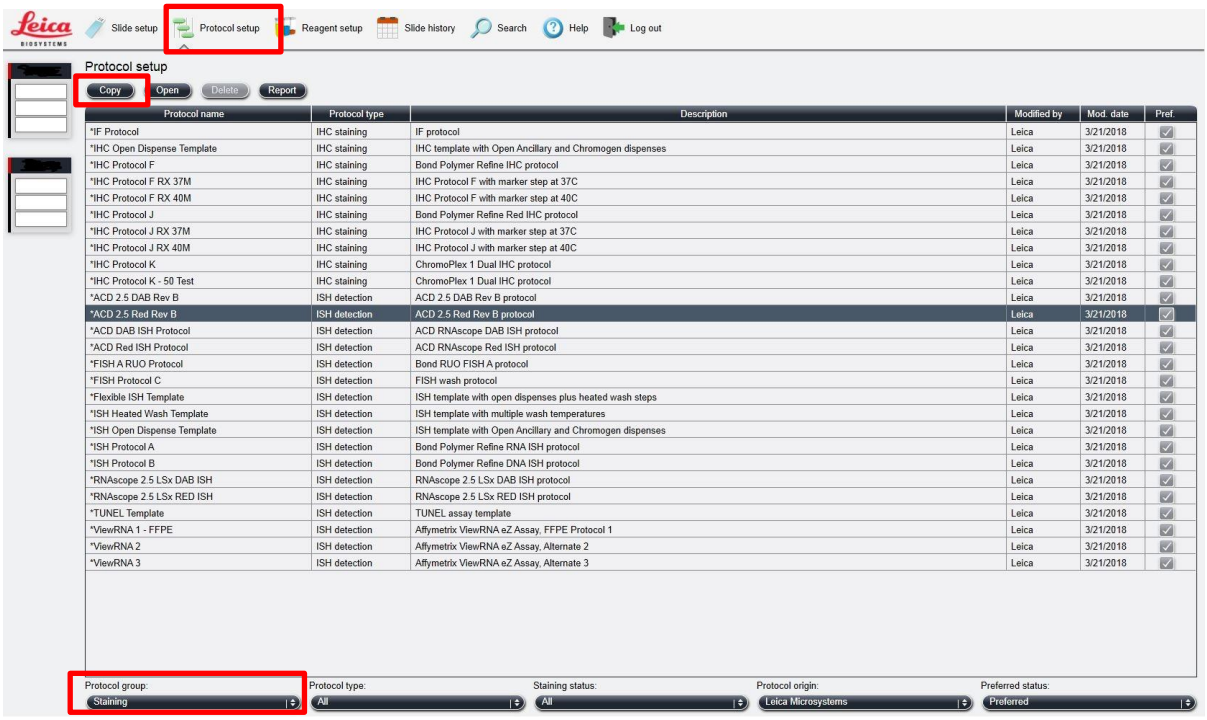


5. Highlight the ***No Reagent** step.
6. Change the incubation time to **1 MIN** and select **Ambient** as Temperature (°C).
7. Select **Save**.

Create a staining protocol

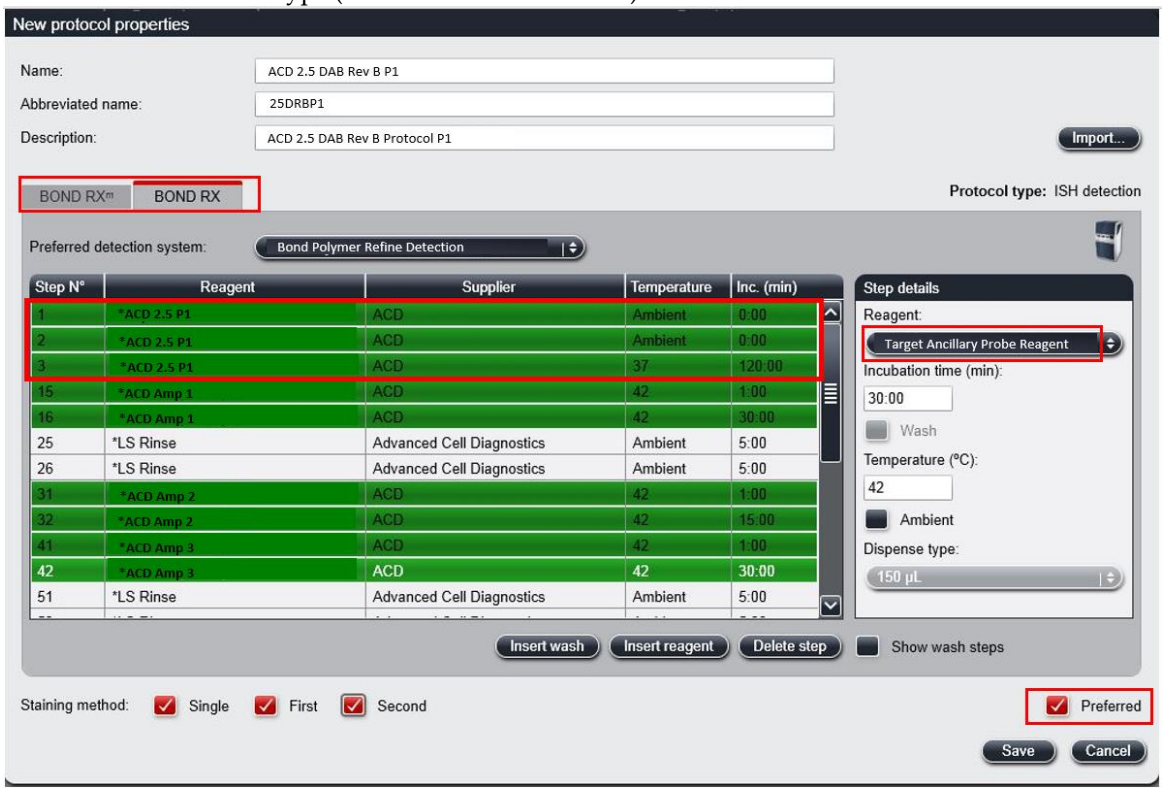
Due to the 5.2 software workaround for the RNAscope 2.5 LS assay, a unique staining protocol *must be created for each probe*.

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.**



Protocol name	Protocol type	Description	Modified by	Mod. date	Pref.
*IF Protocol	IHC staining	IF protocol	Leica	3/21/2018	
*IHC Open Dispense Template	IHC staining	IHC template with Open Ancillary and Chromogen dispenses	Leica	3/21/2018	
*IHC Protocol F	IHC staining	Bond Polymer Refine IHC protocol	Leica	3/21/2018	
*IHC Protocol F RX 37M	IHC staining	IHC Protocol F with marker step at 37C	Leica	3/21/2018	
*IHC Protocol F RX 40M	IHC staining	IHC Protocol F with marker step at 40C	Leica	3/21/2018	
*IHC Protocol J	IHC staining	Bond Polymer Refine Red IHC protocol	Leica	3/21/2018	
*IHC Protocol J RX 37M	IHC staining	IHC Protocol J with marker step at 37C	Leica	3/21/2018	
*IHC Protocol J RX 40M	IHC staining	IHC Protocol J with marker step at 40C	Leica	3/21/2018	
*IHC Protocol K	IHC staining	ChromoPlex 1 Dual IHC protocol	Leica	3/21/2018	
*IHC Protocol K - 50 Test	IHC staining	ChromoPlex 1 Dual IHC protocol	Leica	3/21/2018	
*ACD 2.5 DAB Rev B	ISH detection	ACD 2.5 DAB Rev B protocol	Leica	3/21/2018	
*ACD 2.5 Red Rev B	ISH detection	ACD 2.5 Red Rev B protocol	Leica	3/21/2018	
*ACD DAB ISH Protocol	ISH detection	ACD RNAscope DAB ISH protocol	Leica	3/21/2018	
*ACD Red ISH Protocol	ISH detection	ACD RNAscope Red ISH protocol	Leica	3/21/2018	
*FISH A RUO Protocol	ISH detection	Bond RUO FISH A protocol	Leica	3/21/2018	
*FISH Protocol C	ISH detection	FISH wash protocol	Leica	3/21/2018	
*Flexible ISH Template	ISH detection	ISH template with open dispenses plus heated wash steps	Leica	3/21/2018	
*ISH Heated Wash Template	ISH detection	ISH template with multiple wash temperatures	Leica	3/21/2018	
*ISH Open Dispense Template	ISH detection	ISH template with Open Ancillary and Chromogen dispenses	Leica	3/21/2018	
*ISH Protocol A	ISH detection	Bond Polymer Refine RNA ISH protocol	Leica	3/21/2018	
*ISH Protocol B	ISH detection	Bond Polymer Refine DNA ISH protocol	Leica	3/21/2018	
*RNAscope 2.5 LSx DAB ISH	ISH detection	RNAscope 2.5 LSx DAB ISH protocol	Leica	3/21/2018	
*RNAscope 2.5 LSx RED ISH	ISH detection	RNAscope 2.5 LSx RED ISH protocol	Leica	3/21/2018	
*TUNEL Template	ISH detection	TUNEL assay template	Leica	3/21/2018	
*ViewRNA 1 - FFPE	ISH detection	Affymetrix ViewRNA eZ Assay, FFPE Protocol 1	Leica	3/21/2018	
*ViewRNA 2	ISH detection	Affymetrix ViewRNA eZ Assay, Alternate 2	Leica	3/21/2018	
*ViewRNA 3	ISH detection	Affymetrix ViewRNA eZ Assay, Alternate 3	Leica	3/21/2018	

3. Change the protocol name for your first probe to (i.e. **ACD 2.5 DAB Rev B P1)** in the Name text box, **25DRBP1** in the Abbreviated name text box, and **ACD 2.5 DAB Rev B protocol P1** in the Description text box OR use your own naming convention.
4. When editing the staining protocol, make sure to select the correct tab that matches your instrument type (BOND RX^{m} or BOND RX).



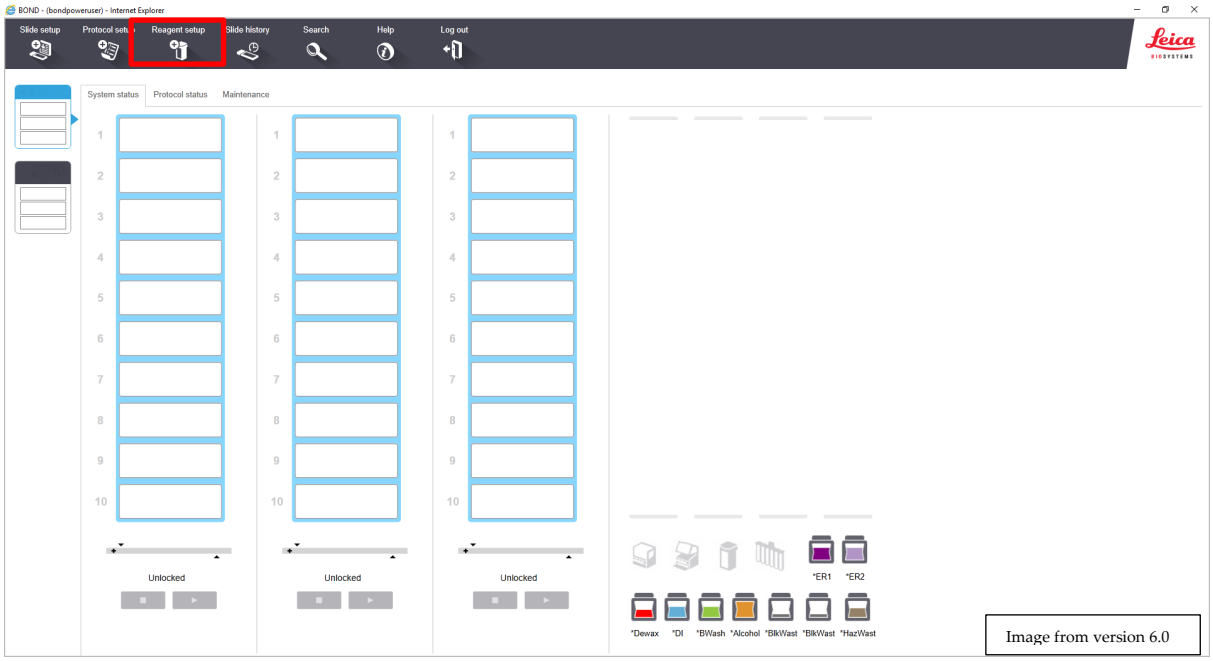
Step N°	Reagent	Supplier	Temperature	Inc. (min)
1	*ACD 2.5 P1	ACD	Ambient	0:00
2	*ACD 2.5 P1	ACD	Ambient	0:00
3	*ACD 2.5 P1	ACD	37	120:00
15	*ACD Amp 1	ACD	42	1:00
16	*ACD Amp 1	ACD	42	30:00
25	*LS Rinse	Advanced Cell Diagnostics	Ambient	5:00
26	*LS Rinse	Advanced Cell Diagnostics	Ambient	5:00
31	*ACD Amp 2	ACD	42	1:00
32	*ACD Amp 2	ACD	42	15:00
41	*ACD Amp 3	ACD	42	1:00
42	*ACD Amp 3	ACD	42	30:00
51	*LS Rinse	Advanced Cell Diagnostics	Ambient	5:00

5. Next highlight the default probe reagent lines (Lines 1-3: *ACD 2.5 P1) and choose the appropriate Ancillary probe reagent created earlier from the Reagent drop-down list on the right.
6. Select **Preferred** in the bottom right corner of the window.
7. Select **Save**.
8. Click on **Next** to proceed. Ignore any pop-up warnings that may appear on the screen.

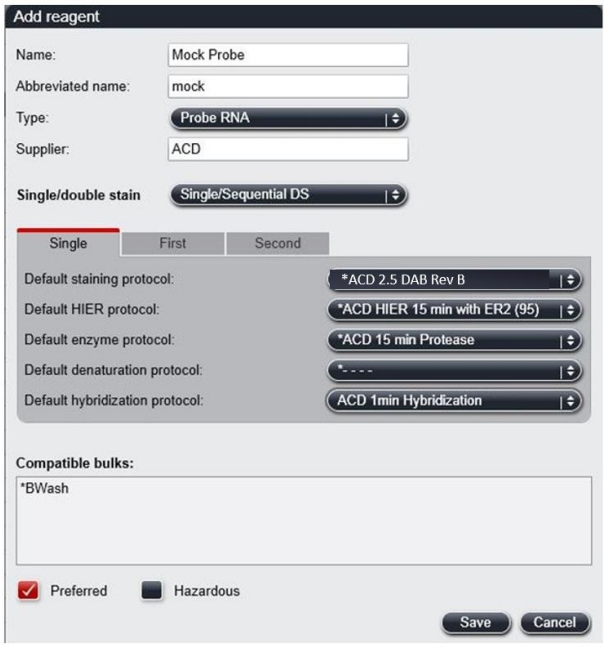
Register the mock probe (one time only)

Create a mock probe in the reagent set up.

1. Click on the **Reagent setup** icon.

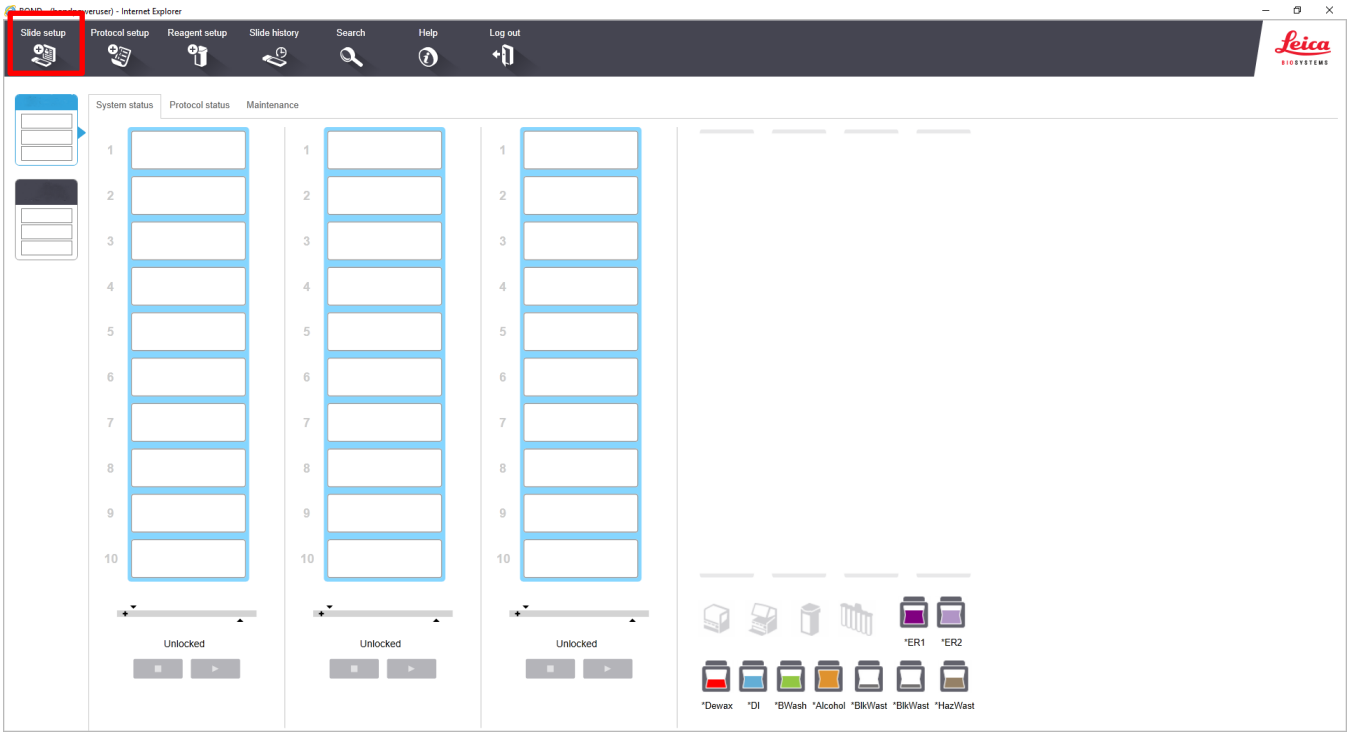


2. Select **Add**.
3. Enter **Mock Probe** in the Name and Abbreviated name text boxes.
4. Select **Probe RNA** in the Type drop-down menu.
5. Enter **ACD** in the Supplier text box.
6. For Single/Double Stain: Select **Single/Sequential DS**
7. Select a commonly used ACD staining protocol as the Default staining protocol. During slide set up, you can select other protocols.
8. Select ***ACD HIER 15min with ER2 (95)** or as the Default HIER protocol.
9. Select ***ACD 15min Protease** as the Default enzyme protocol.
10. Leave the Default denaturation protocol blank.
11. Select **ACD 1 min Hybridization** as the Default hybridization protocol.
12. Select **Preferred**, then select **Save**.

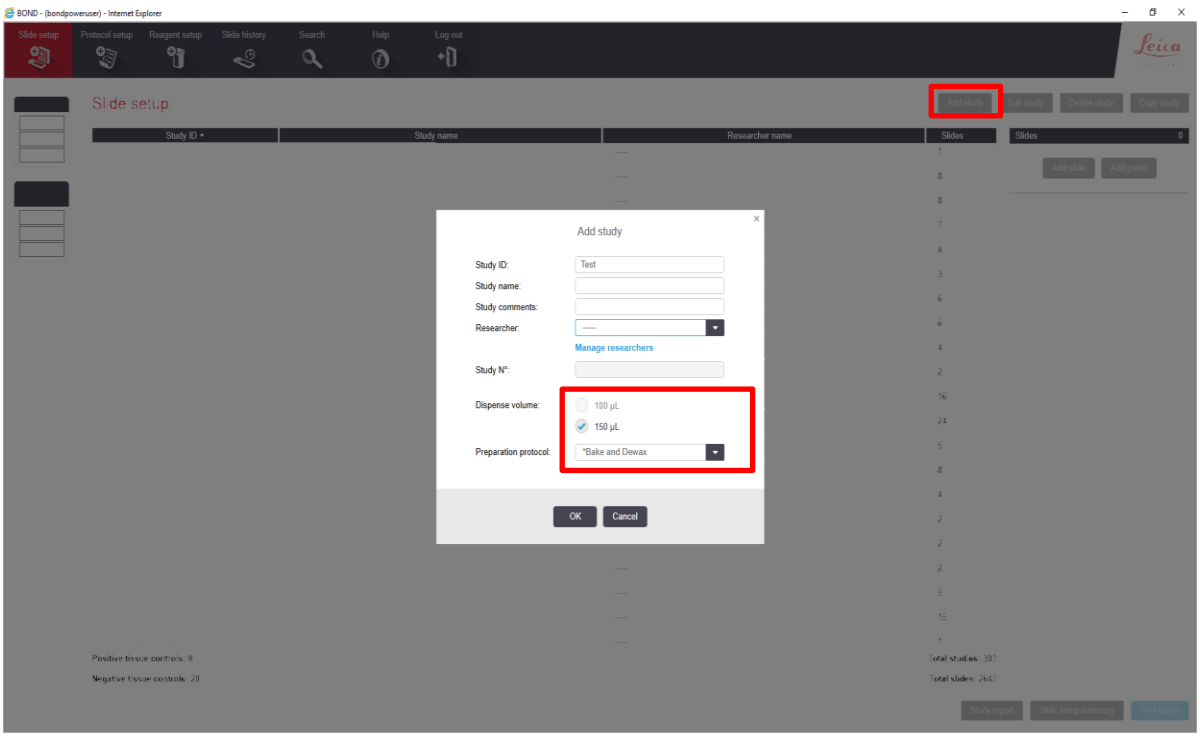


Set up a study

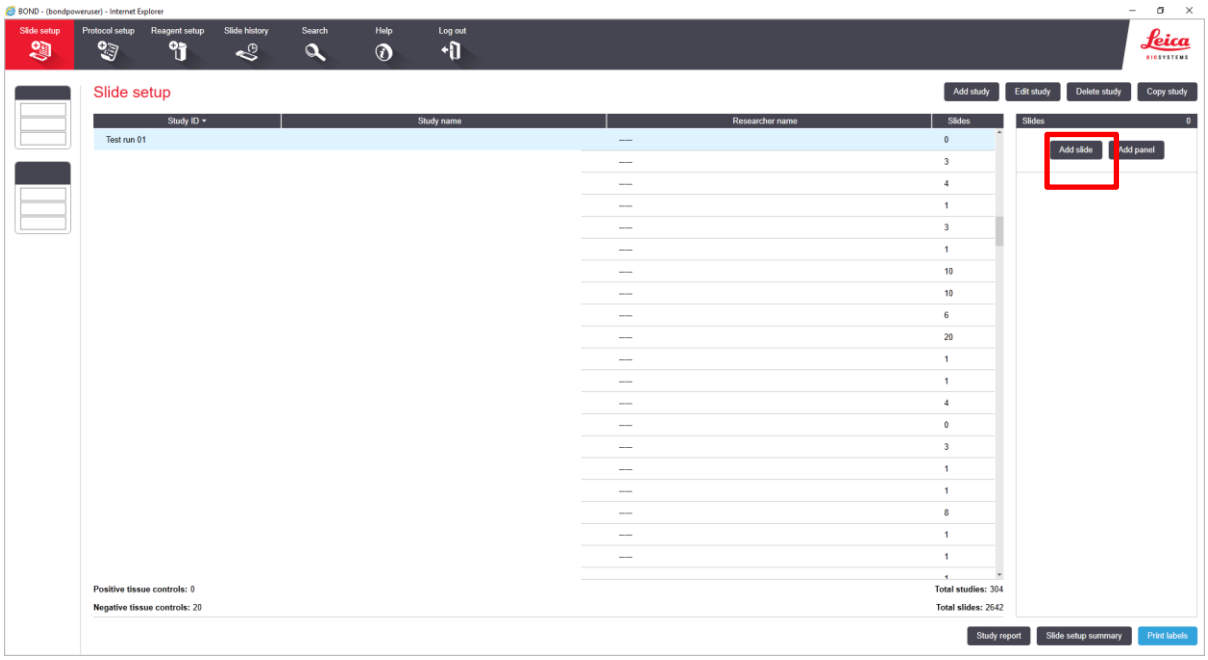
1. To build a study, select the **Slide setup** icon at the top of the screen.

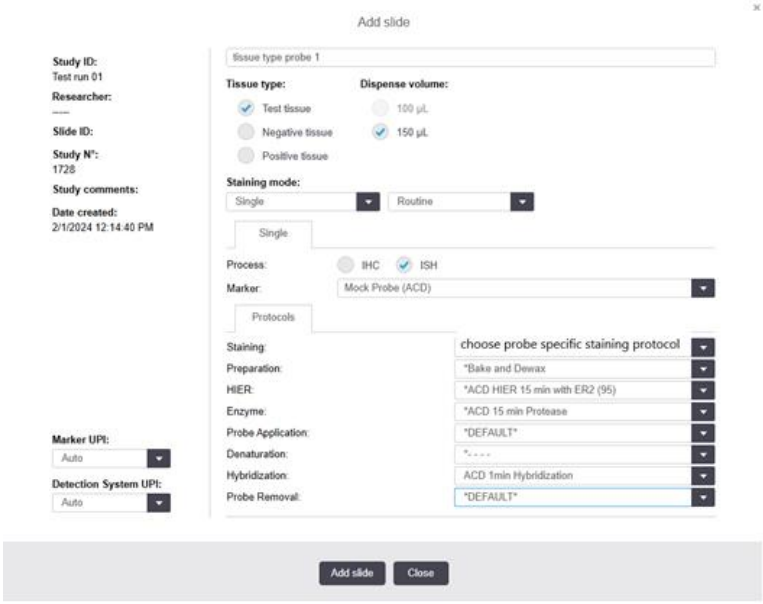


2. Select **Add study** and enter a name in the Study ID field (keep the Dispense volume at 150 µl as shown).



3. For FFPE tissues, select ***Bake and Dewax** as the Preparation protocol. For alternative tissue preps, see **Appendix E**. for details).
4. Select **OK**.
5. Select **Add slide** to assign a protocol to each slide.





6. Enter the tissue type and probe name in the Comments field.
7. Keep **Single** as default from the Staining mode drop down menu.
8. Select **ISH** for Process and **Mock Probe (ACD)** from the Marker drop down menu.
9. In the **Protocols** tab, do the following:
    a. For each distinct probe, select a different protocol from the Staining drop down menu (for example, ACD 2.5 DAB Rev B P1).
    b. For FFPE tissues, select the protocol ***Bake and Dewax** from the Preparation drop down menu.

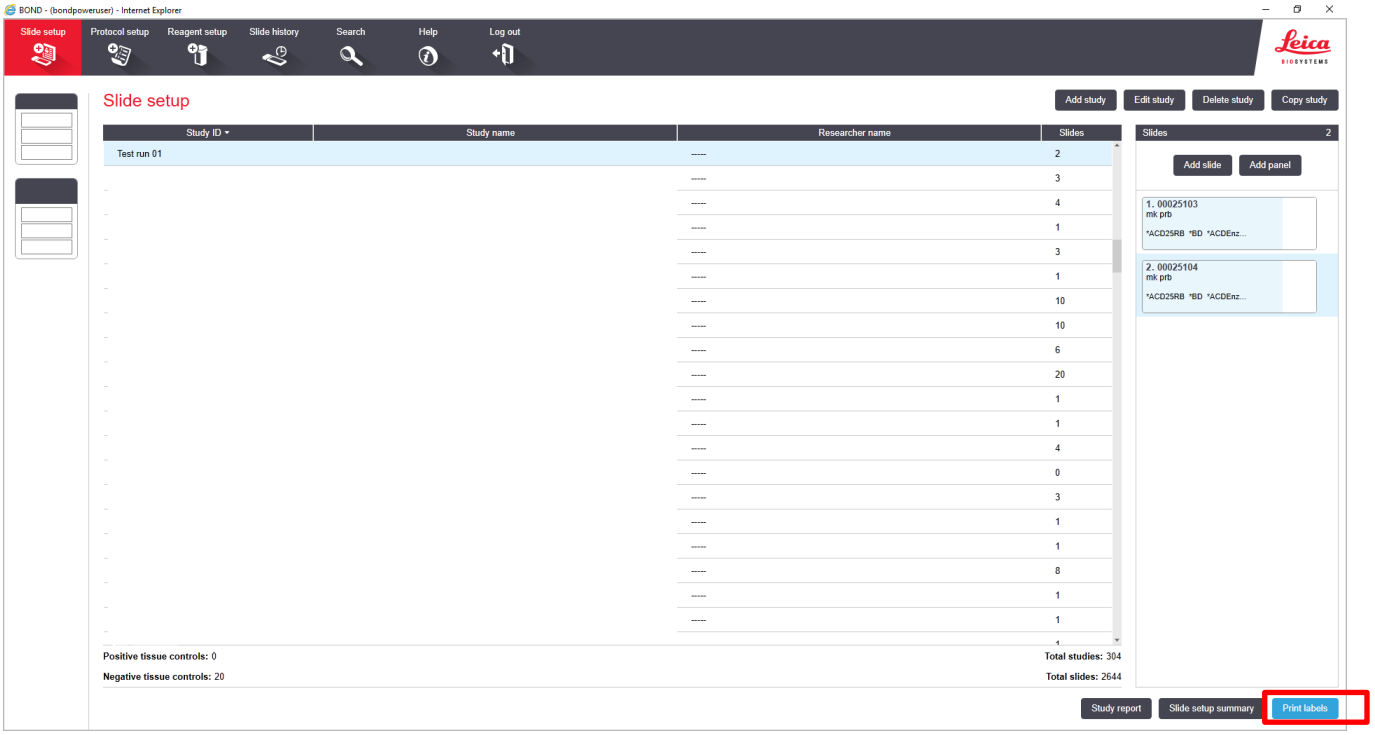
c. For FFPE tissues, select ***ACD HIER 15 min with ER2 (95)** as the HIER protocol.

d. For FFPE tissues, select ***ACD 15 min Protease** for Enzyme, or the appropriate enzyme protocol for your tissue (see **Appendix C** to edit the enzyme treatment protocol).

e. Select **ACD 1 min Hybridization** for Hybridization.

Note: HIER protocol time and temperature varies depending on the tissue type (see **Appendix A**).

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



13. Proceed to **Chapter 7. Run RNAscope 2.5 LS Assay**

6 Chapter 6. Set Up Software Version 6.0 or higher

Use the instructions in this chapter to set up the RNAscope 2.5 LS Assay using software version 6.0. To set up the assay using software version 5.2, see **Chapter 5**.

IMPORTANT! BXD20 or higher is required to run the following setup on software version 6.0. Please contact your Leica FAS to upgrade to BXD20 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.

This workflow uses the standard software setup for the BOND RX instruction control software. Probes are registered in the software as Probe RNA and a universal staining protocol is created and used for all staining runs of this type. Follow the steps in this chapter to enable the workflow. Your ACD Field Application Specialist (FAS) can help you implement this procedure.

Workflow

Register the probes

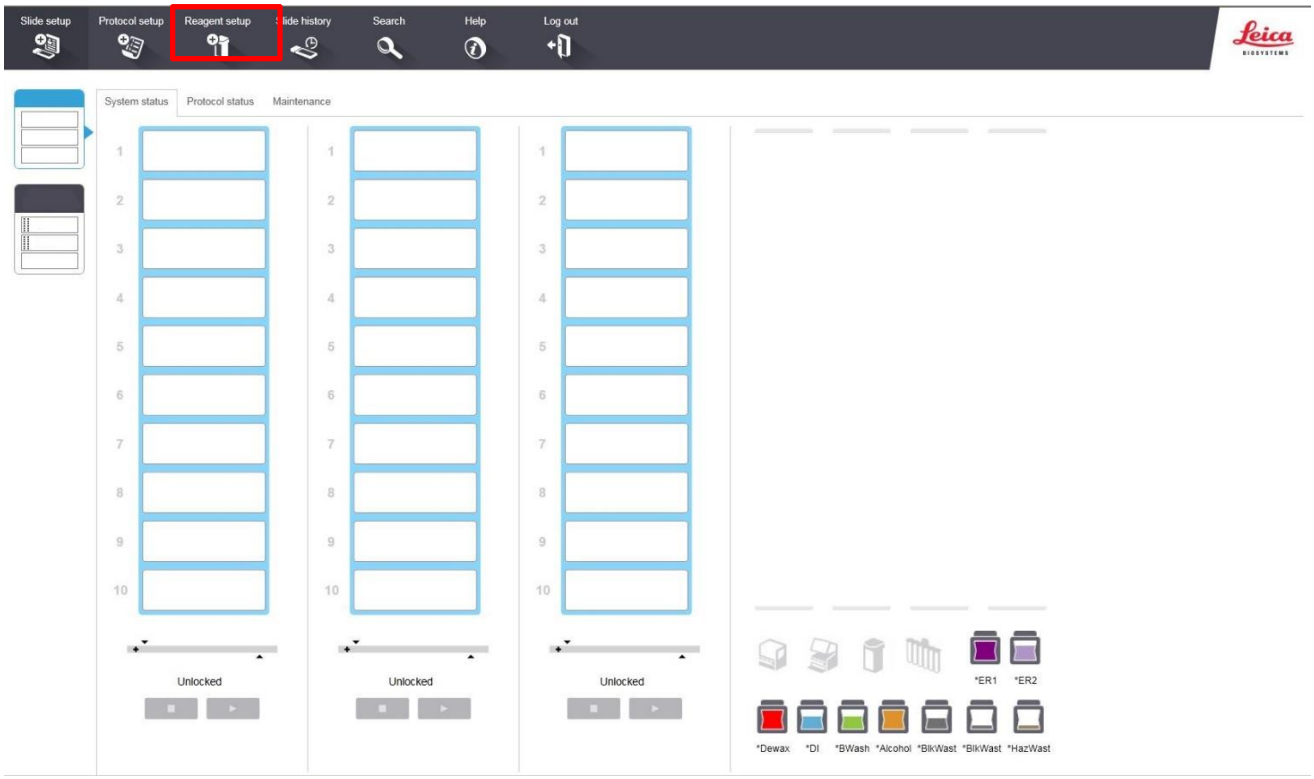
⬇

Create a Universal DAB staining protocol (ONE TIME ONLY)

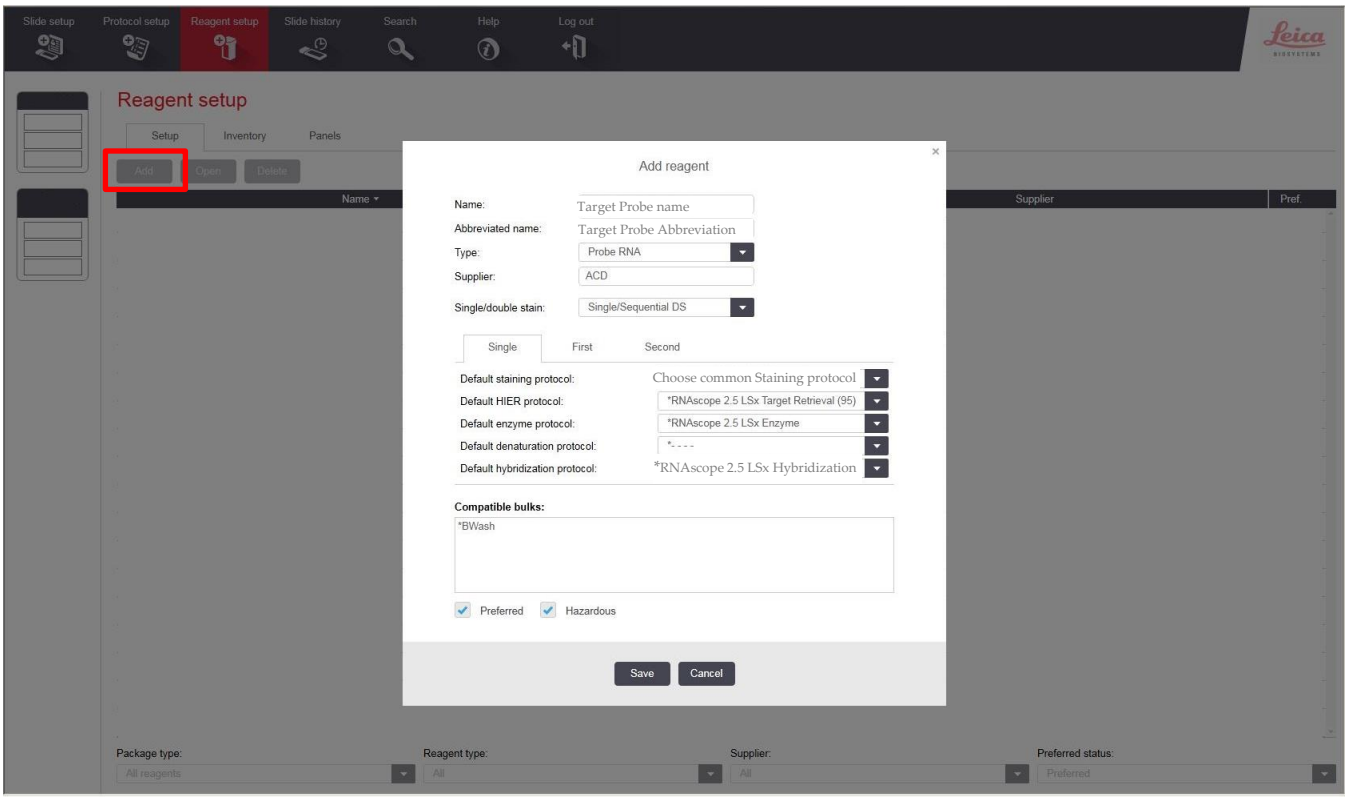
Register the probes

If you have software version 6.0, you can register the probes as Probe RNA. Contact your ACD Field Application Scientist (FAS) to assist you if needed.

1. Select **the Reagent Setup** icon at the top of the screen.



2. Select **Add** to enter reagent information.

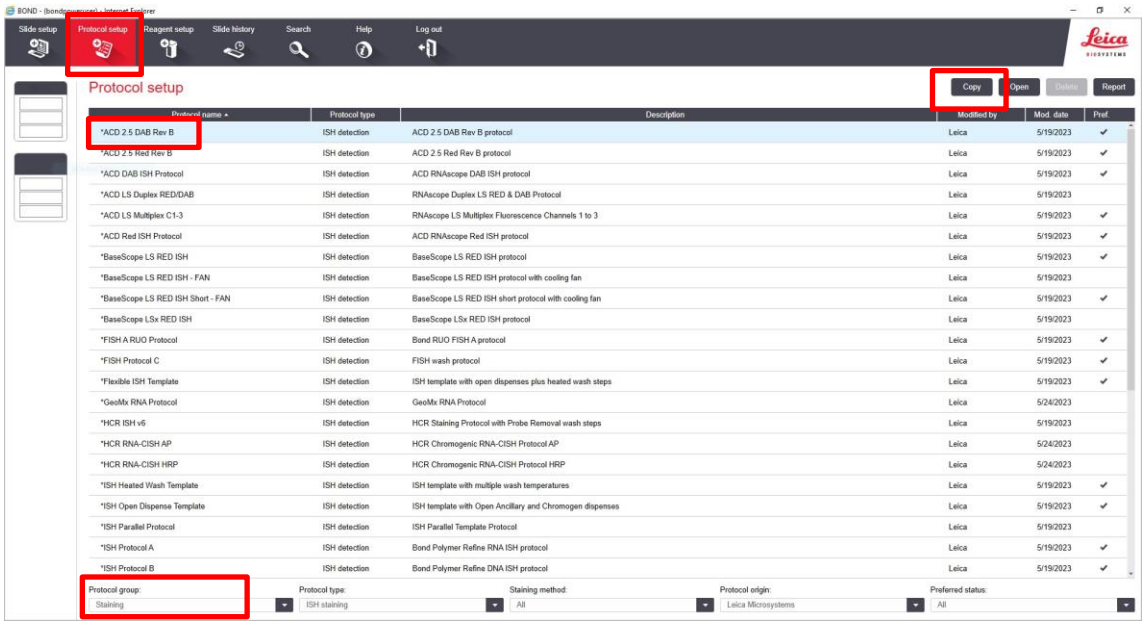


1. Enter **Target Probe 1** (for example) in the Abbreviated name text box.
2. Select **Probe RNA** in the Type drop-down menu.
3. Enter **ACD** in the Supplier text box.
4. Enter **Single/Sequential DS** for Single/double stain
5. Select *** ACD 2.5 DAB Rev B or other common protocol** as the Default Staining protocol.
6. Select ***RNAscope 2.5 LSx Target Retrieval (95)** as the Default HIER protocol.
7. Select ***RNAscope 2.5 LSx Enzyme** as the Default enzyme protocol.
8. Leave the Default denaturation protocol blank.
9. Select ***RNAscope 2.5 LSx Hybridization** as the Default hybridization protocol.
10. Check both the **Preferred** and **Hazardous** boxes.
11. Select **Save**.

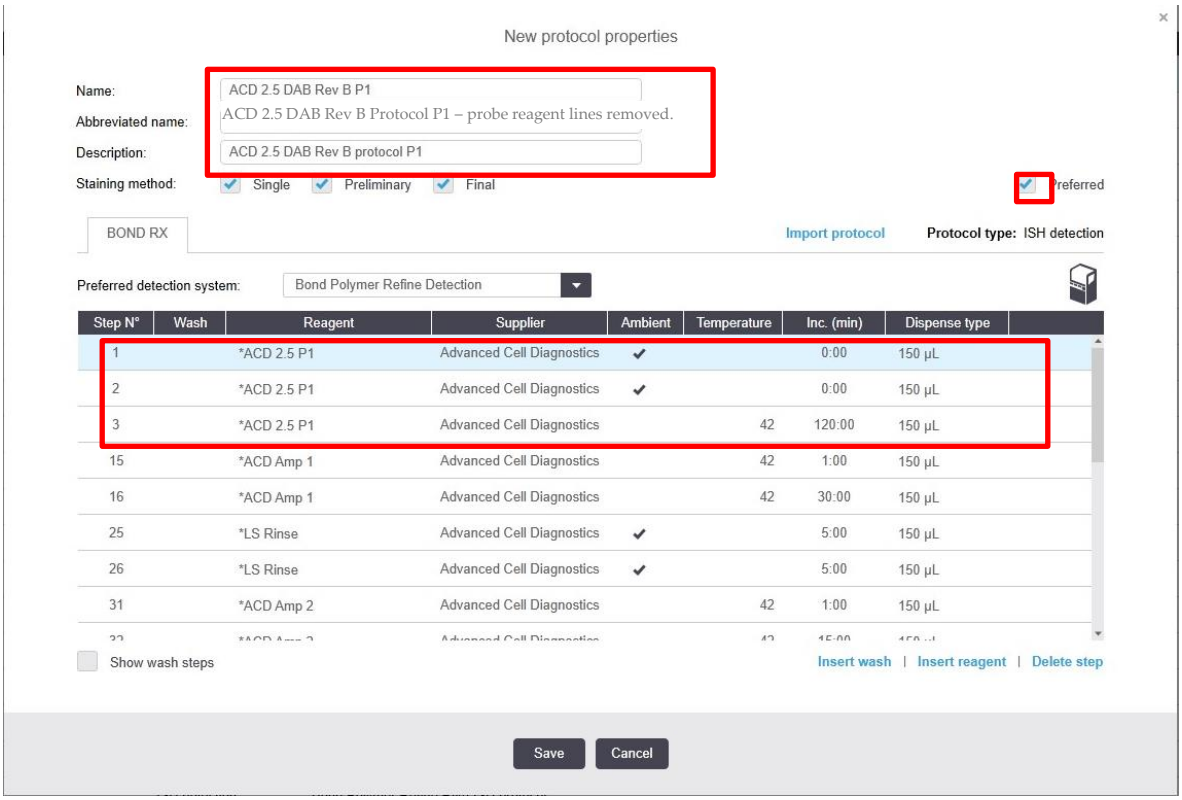
Create the universal staining protocol (one time only)

For software version 6.0 or higher, you can use the same staining protocol for all RNAscope probes but first the *ACD 2.5 DAB Rev B protocol must be edited.

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 to **ACD 2.5 DAB Rev B P1 (for example)** in the Name text box, **25DRBP1** in the Abbreviated name text box, and **ACD 2.5 DAB Rev B protocol P1 – probe reagent lines removed** in the Description text box.



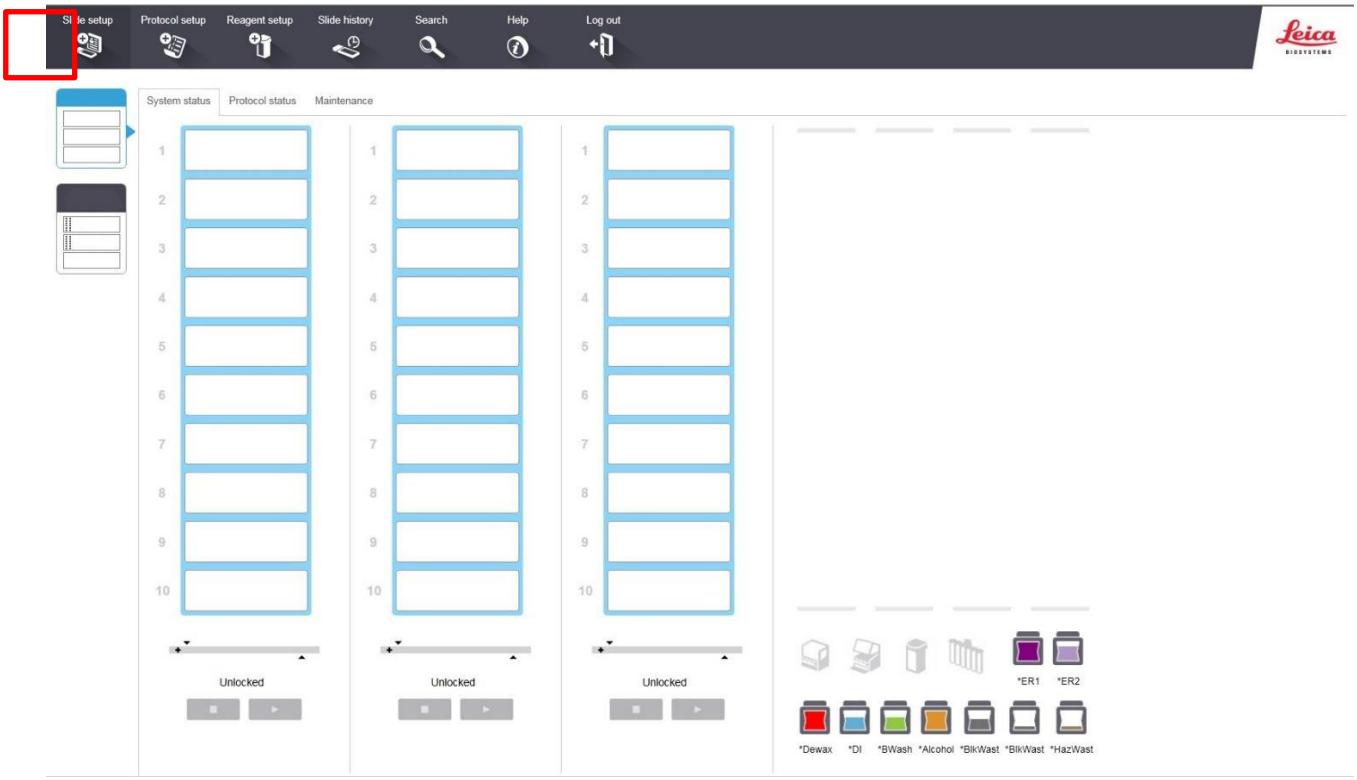
4. Next highlight the default probe reagent lines (Lines 1-3: *ACD 2.5 P1) and delete those steps.

5. Select **Preferred** in the top right corner of the window.
6. Select **Save**.
7. Click on **Next** to proceed. Ignore any pop-up warnings that may appear on the screen.

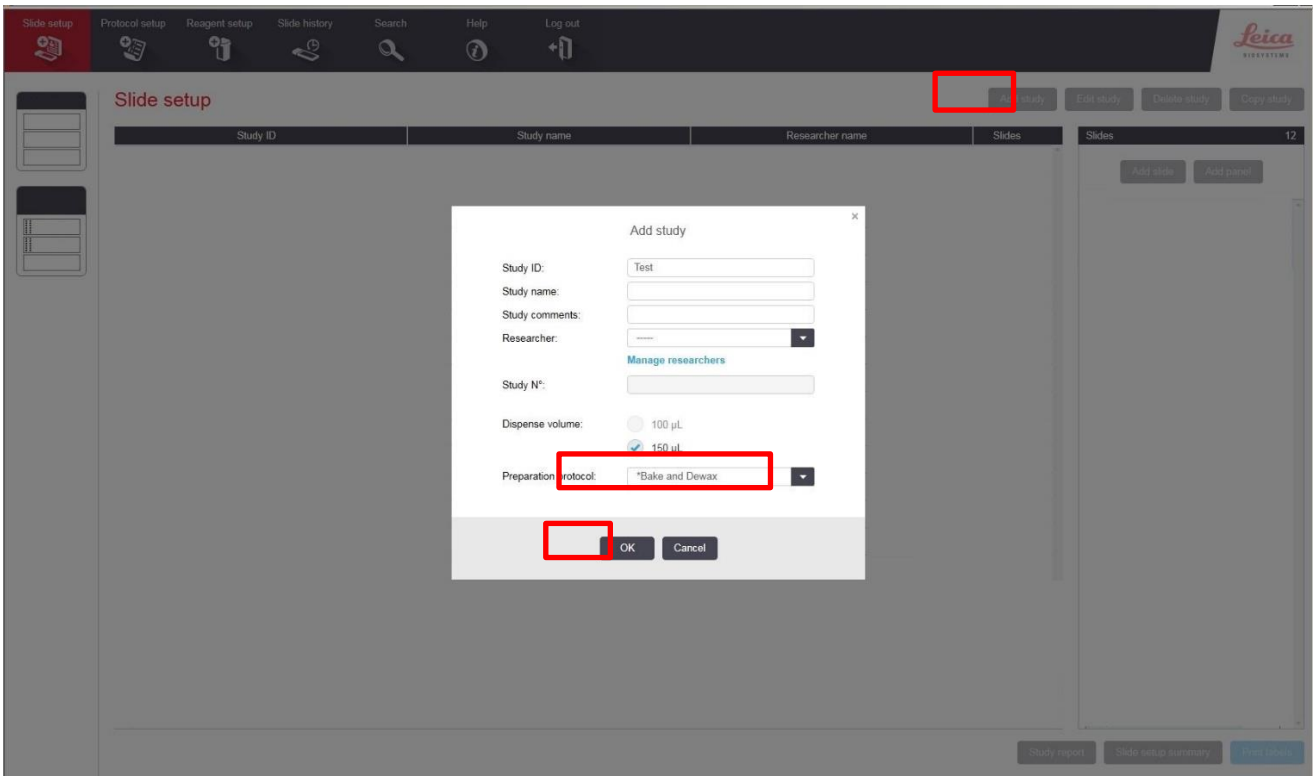
Note: Use this protocol to perform all RNAscope DAB assays.

Set up a study

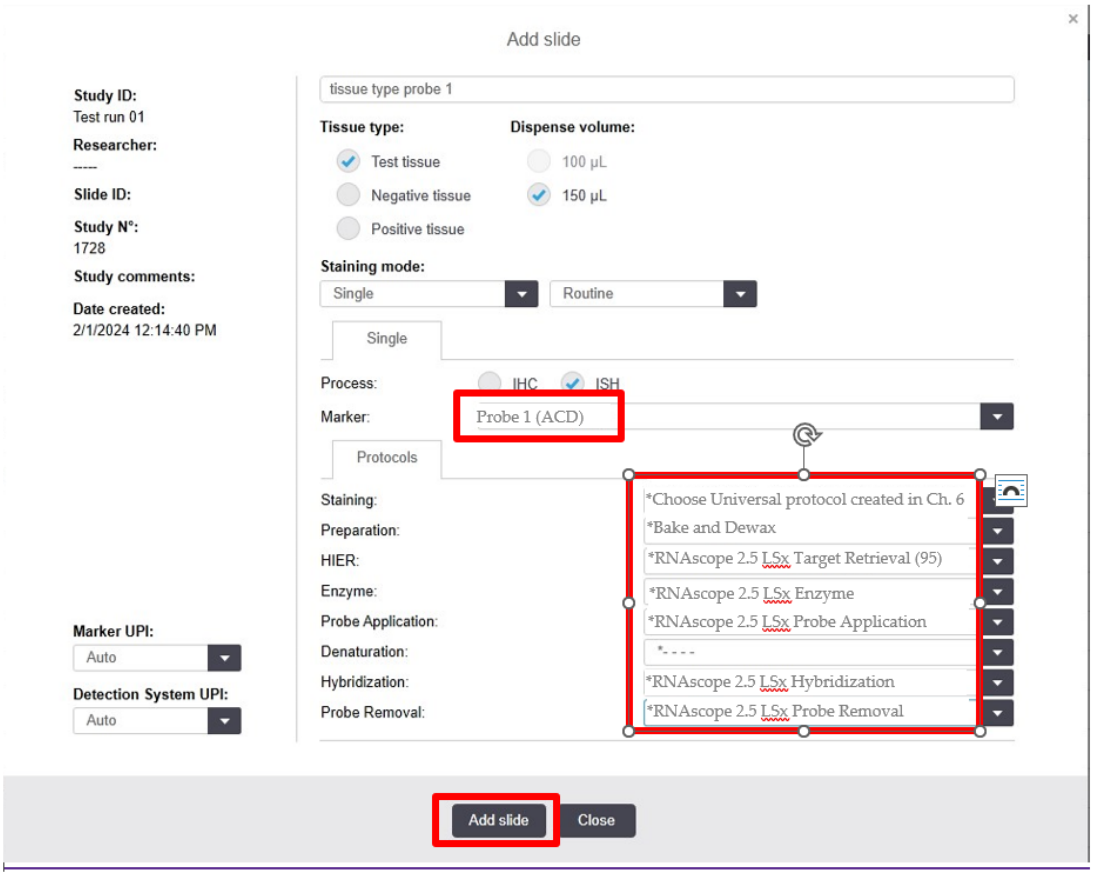
1. To build a study, select the **Slide setup** icon at the top of the screen.



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 E**).
4. Select **OK**.
5. Select **Add slide** to assign a protocol to each slide.
6. Enter the tissue type and probe name in the Comments field.

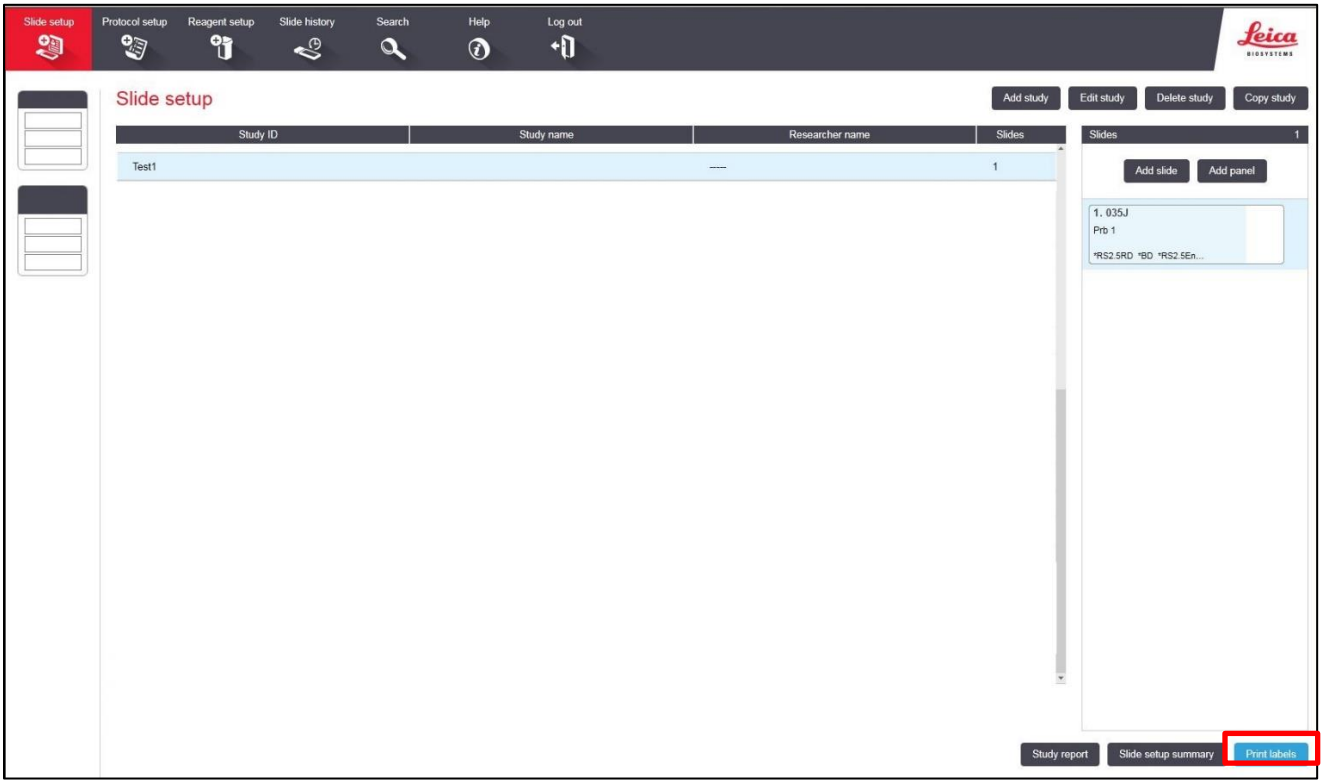


7. Keep **Single** as default from the Staining mode drop down menu.
8. Select **ISH** for Process and the appropriate Marker from the drop-down menu.
9. For RNAscope 2.5 LS assays, under the **Protocols** tab, do the following:
   a. Staining: Select the universal protocol created in the previous step.
   b. Preparation: For FFPE tissues, select ***Bake and Dewax.** For alternative tissue preps, see **Appendix E**.
   c. HIER: For FFPE tissues, select ***RNAscope 2.5 LSx Target Retrieval (95)** or **(88).** (See Appendix A.) For alternative tissue preps, see **Appendix E**.
   d. Enzyme: For FFPE tissues, select ***RNAscope 2.5 LSx Enzyme.** For alternative tissue preps, see **Appendix E**.
   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: HIER protocol time and temperature varies depending on tissue type.

10. Select **Add slide.**
11. After adding all the slides to the study, select **Close** to return to the Slide setup screen.

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



13. Proceed to **Chapter 7**. Run the **RNAscope 2.5 LS Assay.**

7 Chapter 7. Run the RNAscope 2.5 LS Brown Assay

Workflow

Fill the large containers located in the bottom of the instrument with the Leica BOND RX bulk reagents. 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.

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

Prepare the instrument reagents

Start the run

Complete the run

Dehydrate the slides

Mount the samples

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 • 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 LS Bluing Reagent	Leica Biosystems' 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 (DAB)	• Distilled water • 95% Ethanol (EtOH) • Xylene • Drying oven • Fume hood • Tissue-Tek Staining Dish • Cytoseal or Pertex • Tissue-Tek Clearing Agent Dish, xylene-resistant (2) • Tissue-Tek Vertical 24 Slide Rack • Cover Glass, 24 mm x 50 mm

Prepare the instrument

1. Fill the large containers located in the bottom of the instrument with the Leica BOND RX bulk reagents. 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.

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

Prepare the instrument reagents

1. If using the mock probe workflow from **Chapter 5**, obtain one empty 30 mL Open BOND container and label it as **Mock Probe**.
2. Fill the **Mock Probe** container with Leica Biosystems 1X BOND Wash.
3. Carefully transfer all the RNAscope LS reagents into the empty 30 mL BOND Open containers.
4. 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

5. Using the Barcode Scanner, scan the front barcode on the 30 mL Open BOND Open container. A window will appear.
6. From the drop-down menu, select the corresponding name of the reagent as shown in the following table under **Container Name**:
7. Below is a summary of the container names needed based on the different software workflows. If you are unsure, open the protocols you are using and confirm the reagents names the protocol calls for then register your containers to match.

Reagents	Version 6.0/7.0 Container Names	Version 5.2 Container Names
RNAscope 2.5 LS Hydrogen Peroxide	*RNAScope 2.5 LSx Hydrogen Peroxide	*Open 0 Haz
RNAscope 2.5 LS Protease III or LS PretreatPro	*RNAScope 2.5 LSx Enzyme	*ACD Enzyme
RNAscope 2.5 LS AMP 1	*ACD Amp 1	*ACD Amp 1
RNAscope 2.5 LS AMP 2	*ACD Amp 2	*ACD Amp 2
RNAscope 2.5 LS AMP 3	*ACD Amp 3	*ACD Amp 3
RNAscope 2.5 LS AMP 4	*ACD Amp 4	*ACD Amp 4
RNAscope 2.5 LS AMP 5 – BROWN	*ACD Amp 5 Brown	*ACD Amp 5 Brown
RNAscope 2.5 LS AMP Pro	ACD Amp Pro	ACD Amp Pro
RNAscope 2.5 LS AMP 6 – BROWN	*ACD Amp 6 Brown	*ACD Amp 6 Brown
RNAscope 2.5 LS Rinse	*LS Rinse	*LS Rinse
Hematoxylin	N/A -- in BOND Polymer Refine Detection	N/A -- in BOND Polymer Refine Detection
RNAscope 2.5 LS Bluing Reagent	*ACD Blue	*ACD Blue
RNAscope 2.5 LS Target Probe	Appropriate Probe RNA name	Appropriate Ancillary probe name
1X BOND Wash	N/A	Mock Probe

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

Note: Leica BOND Polymer Refine Detection comes in a pre-filled Leica BOND RX container.

7. Enter the RNAscope 2.5 LS Reagent Kit lot number and the expiration date in their respective fields. Select **OK**.

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.

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 Leica 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. Wash the slides by lifting the slide rack up and down several times.

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 **Chapter 8. Evaluate the Results**.

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 DAB 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:

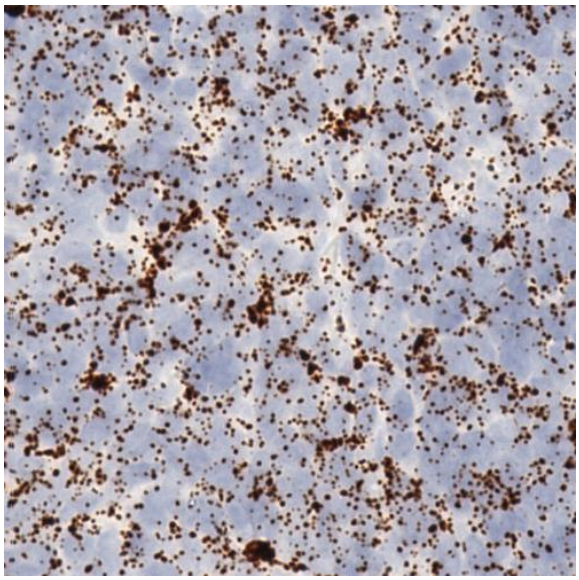
Staining Score	Microscope Objective Scoring*
0	No staining or less than 1 dot for every 10 cells (40X magnification)
1	1–3 dots/cell (visible at 20–40X magnification)
2	4–9 dots/cell. No or very few dot clusters (visible at 20–40X magnification)
3	10–15 dots/cell and/or < 10% positive cells have dot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

- 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).
- Use the above process for over-fixed tissues.
- The RNAscope 2.5 LS BROWN and LS RED assays utilize 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 and bluing incubation times. 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
- Samples prepared differently than the sample preparation protocol found in **Chapter 3**.
- For specific guidance on other sample preparations contact ACD Support at **support.acd@bio-techne.com**

Tissue-specific pretreatment conditions

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

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

Species	Tissue Type	Pathology	Pretreatment Condition
Human	Head	Cancer	Standard
	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 cells, fixed with 10% Formaldehyde /PBS/ACD Control	—	Mild

	Brain	Normal	Standard

	Xenograft tissue	—	Mild

*Tissue Microarray

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

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

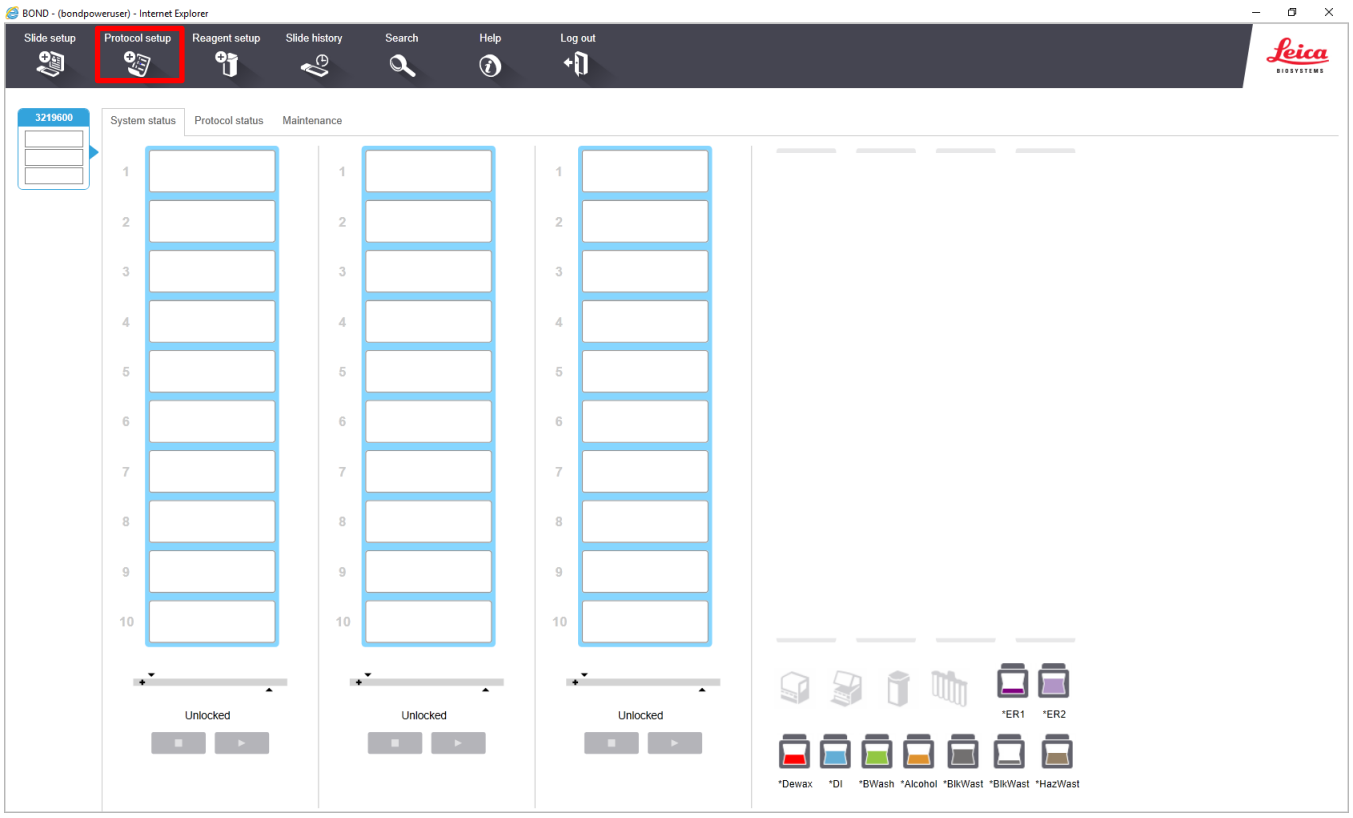
	Jejunum	Normal	Standard
	Colon	Normal	Standard
	Adrenal Gland	Normal	N.A

B Appendix B. Edit the Epitope Retrieval Protocol

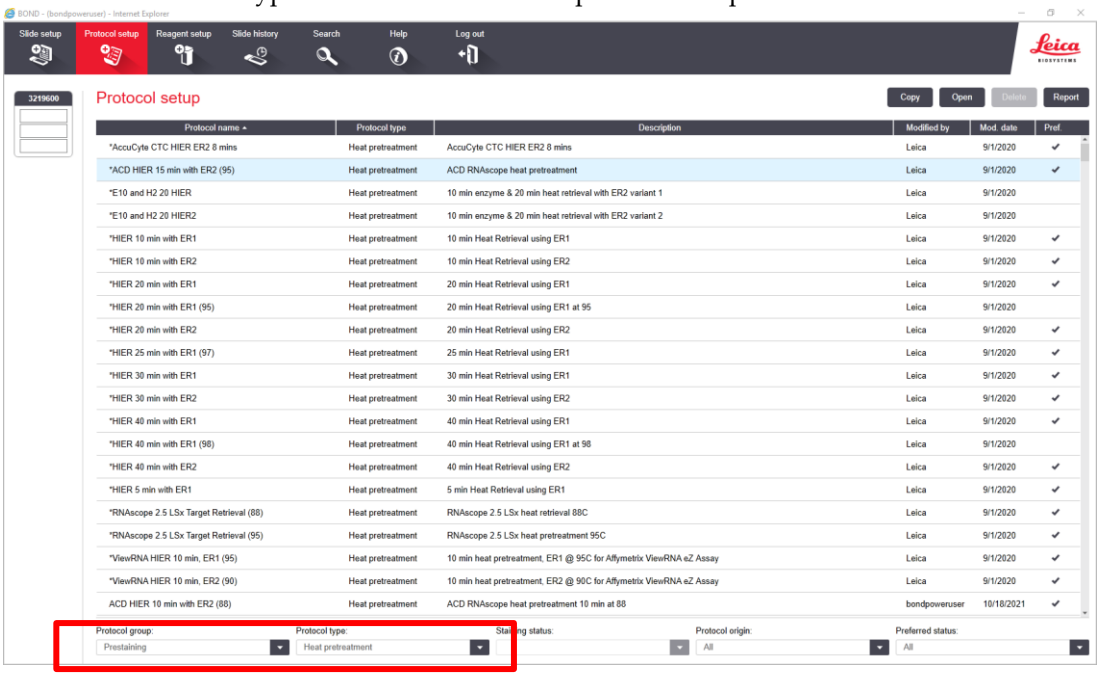
The following example shows how to edit the Epitope Retrieval procedure from within the software.

Create a prestaining protocol

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



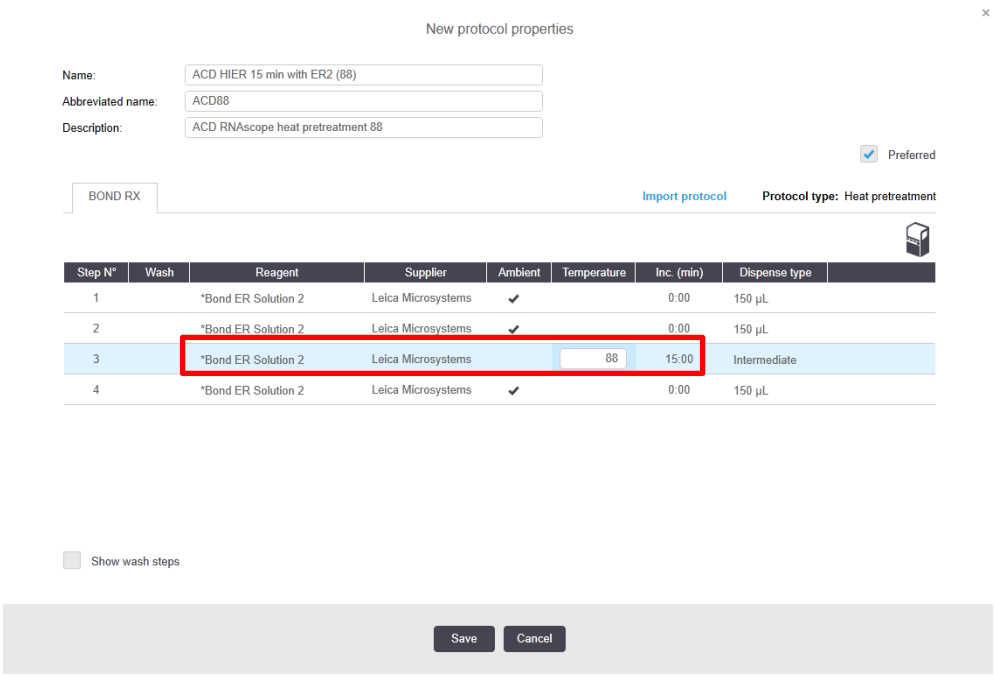
2. Select **Prestaining** under the Protocol group menu and **Heat pretreatment** under the Protocol type menu to access the heat pretreatment protocols.



3. Highlight the ***ACD HIER 15 min with ER2 (95)** protocol**.** 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**.

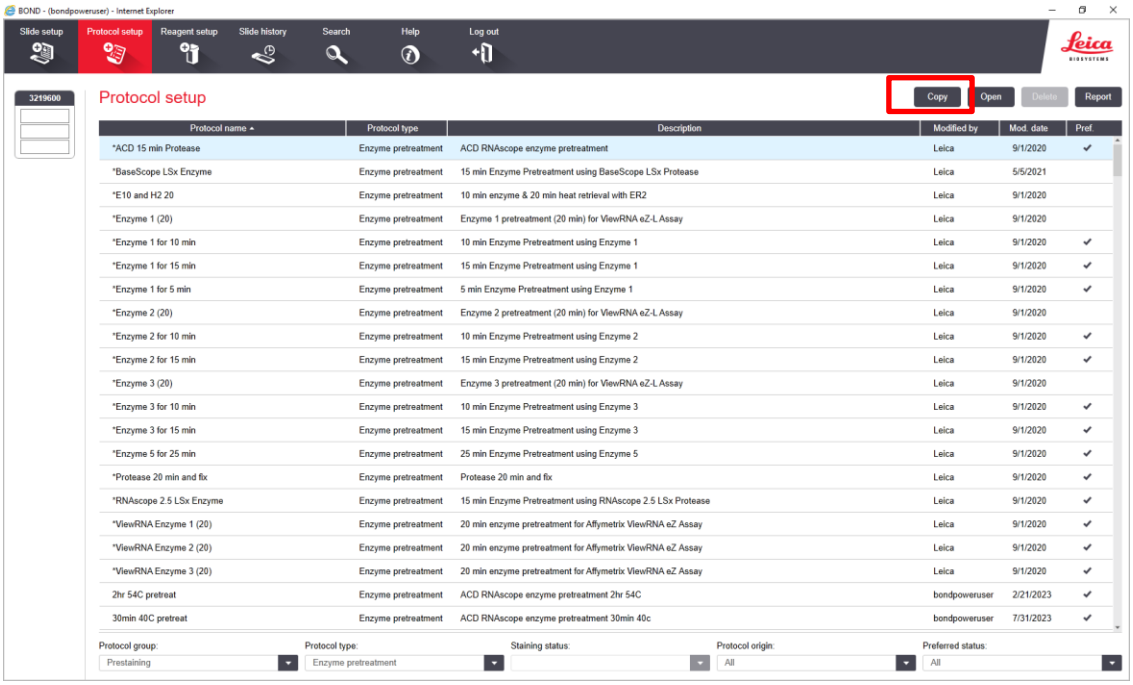


8. For RNAscope, ER 2 temperature varies between **95°C** and **88°C** depending on the tissue type used. Please see Appendix A for a list of tissues.
9. Select **Save** to create a protocol for ER2 pretreatment at **88°C**.
10. If needed, repeat Steps 1–8 to create new heating protocols for different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

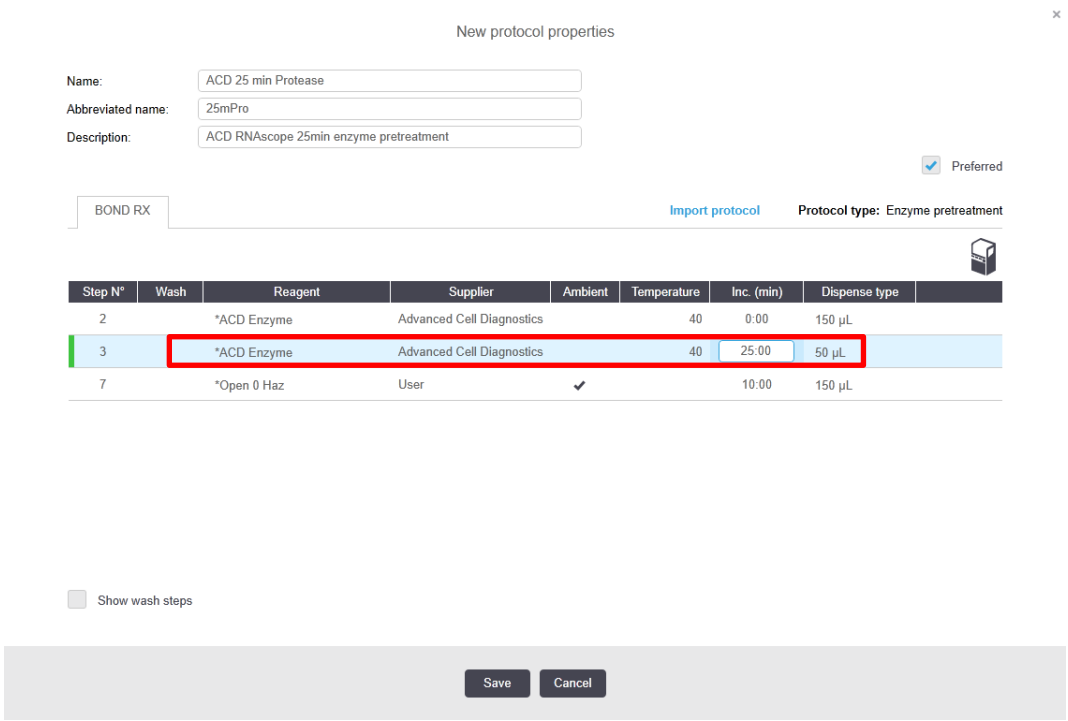
C Appendix C. Edit the Protease Protocol

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



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

Appendix D. How to use LS PretreatPro

Prepare the instrument reagents

In this workflow, two new reagents are used in place of traditional reagents.

Traditional protease workflow	New protease free workflow
LS Protease III	LS PretreatPro
ACD Amp5 - Brown	LS Amp Pro

1. Place RNAscope 2.5 LS PretreatPro into a BOND Open container and register it as ***ACD Enzyme.**

Note: Visually identify the containers as LS PretreatPro to avoid unintended use.

2. Register RNAscope 2.5 LS AMP Pro as a new reagent (for example, ACD Amp Pro) in the BOND database. See **Register the reagents** in Chapter 5.
3. Place the ACD Amp Pro into a BOND Open container and register it as reagent created.

Note: Place only one *ACD Enzyme reagent on the instrument at a time.

4. When registering containers select the corresponding name of the reagent from the drop-down menu as showing in the following table under **Container Name**:

Reagents	Container Name
RNAscope 2.5 LS Hydrogen Peroxide	*Open 0 Haz
RNAscope 2.5 LS PretreatPro	*ACD Enzyme
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 2.5 LS AMP Pro	ACD 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	Variable (probe 1 2.5)
1X BOND Wash	Mock Probe

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

Note: When paired with correlated Enzyme treatment protocol, container names *Open 0 Haz and *RNAscope 2.5 LSx Hydrogen Peroxide are equivalent and *ACD Enzyme and **RNAscope 2.5 LSx Enzyme are equivalent.

5. Enter the RNAscope 2.5 LS Reagent Kit lot number and the expiration date in their respective fields. Select **OK**.

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.

Create the protocol changes needed

1. Filter **Protocol Group** by **Prestaining**, and filter for **Protocol type** by **Enzyme Pretreatment**. Find existing protocol ***ACD 15min Protease** that includes the two reagents *ACD Enzyme and *Open 0 Haz.
2. Copy and create a new protocol:
   a. Change the *ACD Enzyme incubation time to **15 MIN** per step for two steps and the temperature to **40°C**.
   b. Keep ***Open 0 Haz** incubation time at **10 MIN** and temperature at **Ambient**. Save the protocol with a new name such as **ACD 15min PretreatPro**.
3. Refer to **Appendix C** for detailed instructions on editing an Enzyme treatment protocol.
4. Filter **Protocol Group** by **Staining**.
5. Copy the standard LS 2.5 DAB staining protocol used and replace the Amp 5 reagents steps with the newly created ACD Amp Pro reagent using the same incubations conditions.
6. Run all the other parts of the assay as usual and change only these two reagents.
7. The final Slide setup for an FFPE sample should look like the following considering proper HIER condition choice.
   a. Staining: Choose an edited staining protocol with ACD Amp Pro incorporation depending on your 5.2 or 6.0 workflow
   b. Preparation: Select ***Bake and Dewax**.
   c. HIER: Choose ***ACD HIER 15 min with ER2 (95)**.
   d. Enzyme: Choose the protocol created in step 3.
   e. Probe Application: Select ***RNAscope 2.5 LSx Probe Application.**
   f. Denaturation: Select ***….**
   g. Hybridization: Select **ACD 1min Hybridization OR *RNAscope 2.5 LSx Hybridization** depending on your 5.2 or 6.0 workflow
   h. Probe Removal: Select ***RNAscope 2.5 LSx Probe Removal**.
   i. Select **Add slide**.
8. After adding all the slides to the study, select **Close** to return to the Slide setup screen.
9. Proceed to **Chapter 7. Run the RNAscope 2.5 LS Assay**.

   Optimization Note: For all protease-free workflows using LS PretreatPro and AMP Pro involving a low RNA expressor and/or a dense tissue like liver or spleen, we recommend

boosting the ISH signal by increasing the antigen retrieval strength [duration, temperature].

E Appendix E. Slide Setup for Additional Tissue Types

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

Note: Choose appropriate staining and hybridization related protocols depending on whether you are using the mock probe workflow (**Chapter 5**) or the standard probe workflow (**Chapter 6**).

Fixed-frozen tissues

As described in **Chapter 4**, these tissues need a gentle target retrieval step.

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

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.

Fresh-frozen tissues

As described in Chapter 4, these 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 for the chemistry and workflow you are using.
   b. Preparation: Choose ***----**.
   c. HIER: Choose ***----**.
   d. Enzyme: Select **ACD 30min RT with LS Protease IV**†.
   e. Probe Application: Select ***RNAscope 2.5 LSx Probe Application**.
   f. Denaturation: Select ***….**
   g. Hybridization: Choose the appropriate protocol for the chemistry and workflow you are using: **ACD 1 min Hybridization** or ***RNAscope 2.5 LSx Hybridization**.
   h. Probe Removal: Select ***RNAscope 2.5 LSx Probe Removal**.

   †See **Appendix C**. Edit the Protease Protocol to edit the protease protocol.

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

F Appendix F. 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 at: **http://www.acdbio.com/technical-support/user-manuals**. For the SDSs of chemicals not distributed by Advanced Cell Diagnostics, contact the chemical manufacturer.

Obtaining support

For the latest services and support information, go to: **http://www.acdbio.com/technical-support/support-overview**

At the website, you can:

- Access telephone and fax numbers to contact Technical Support and Sales facilities.
- 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.
- Find out information about customer training events.

Contact information

Advanced Cell Diagnostics, Inc.
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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 ACD website at **http://www.acdbio.com/store/terms**. If you have any questions, please contact Advanced Cell Diagnostics at **http:/www.acdbio.com/about/contact.**