a bio-techne brand

RNAscope® 2.5 LS Reagent Kit – BROWN
User Manual for BDZ 11

For use with Leica Biosystems' BOND RX System

For Research Use Only. Not for diagnostic use.

Trademarks

RNAscope® is a registered trademark of Advanced Cell Diagnostics, Inc. Leica BOND RX is a registered trademark of Leica Biosystems. All other trademarks belong to their respective owners.

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

Advanced Cell Diagnostics, Inc. reserves the right to change its products and services at any time to incorporate technological developments. This manual is subject to change without notice.

Although this manual has been prepared with every precaution to ensure accuracy, Advanced Cell Diagnostics, Inc. assumes no liability for any errors, omissions, or for any damages resulting from the use of this information.

Copyright

Contents

1 Chapter 1. Product Information

Before using this product, read and understand the information in **Appendix C. Safety** on page Error! Bookmark not defined. 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) 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

Figure 1 on page 6 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 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 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 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) and Hematoxylin*	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 and Pretreat Samples** on page 10 for preparation of FFPE 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 best results.
- Do not let your sections dry out during the procedure.
- Use good laboratory practices and follow all necessary safety procedures. Refer to **Appendix D. Safety** on page Error! Bookmark not defined. for more information.

3 Chapter 3. Prepare and Pretreat Samples

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

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

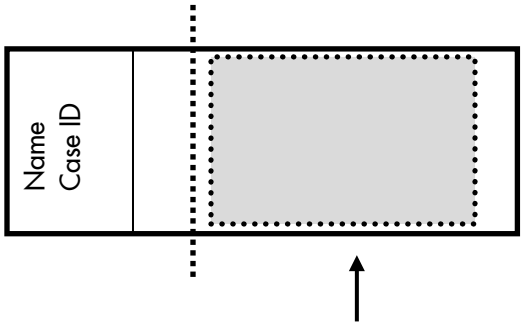
IMPORTANT! Use fresh reagents.

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 paraffin ribbon in a **40–45°C** water bath, and mount 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 3 months. Store sections with desiccants at room temperature.

4 Chapter 4. Set Up the BDZ 11 Software

IMPORTANT! We strongly recommend you run the RNAscope® 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.

Workflow

Note: A few of the procedures in this chapter need to be completed only once per reagent.

Register the reagents

↓

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

↓

Create a staining protocol (ONCE FOR EACH PROBE)

↓

Register the mock probe (ONE TIME ONLY)

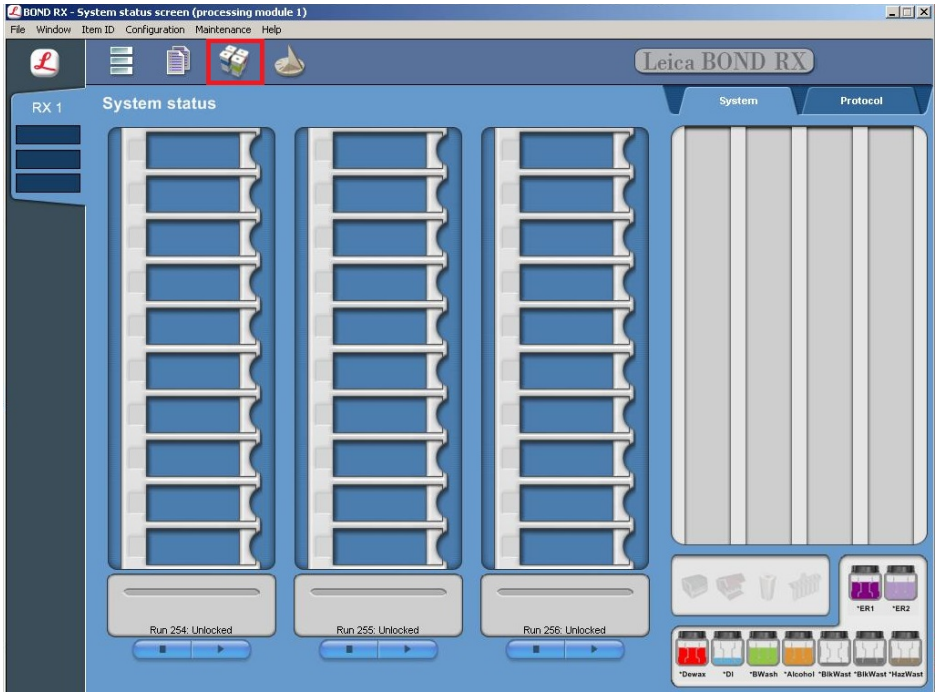
↓

Set up a study

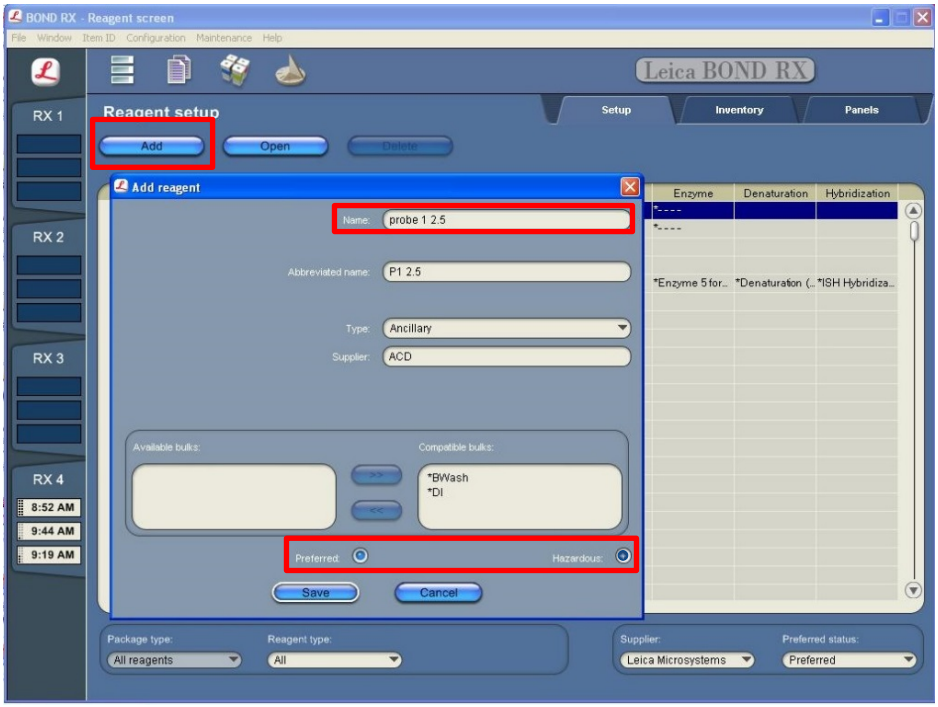
Register the reagents

This step is required as a "work around" to the existing BDZ 11 software to accommodate the RNAscope® 2.5 LS Brown assay. Your ACD Field Application Specialist (FAS) should implement this procedure. In summary, a probe is created as an ancillary reagent and added to the staining protocol.

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



2. Select **Add** to enter reagent information.
3. To create probe, enter a name (for example, **probe 1 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 **Preferred** and **Hazardous** (for probe reagent only) boxes.

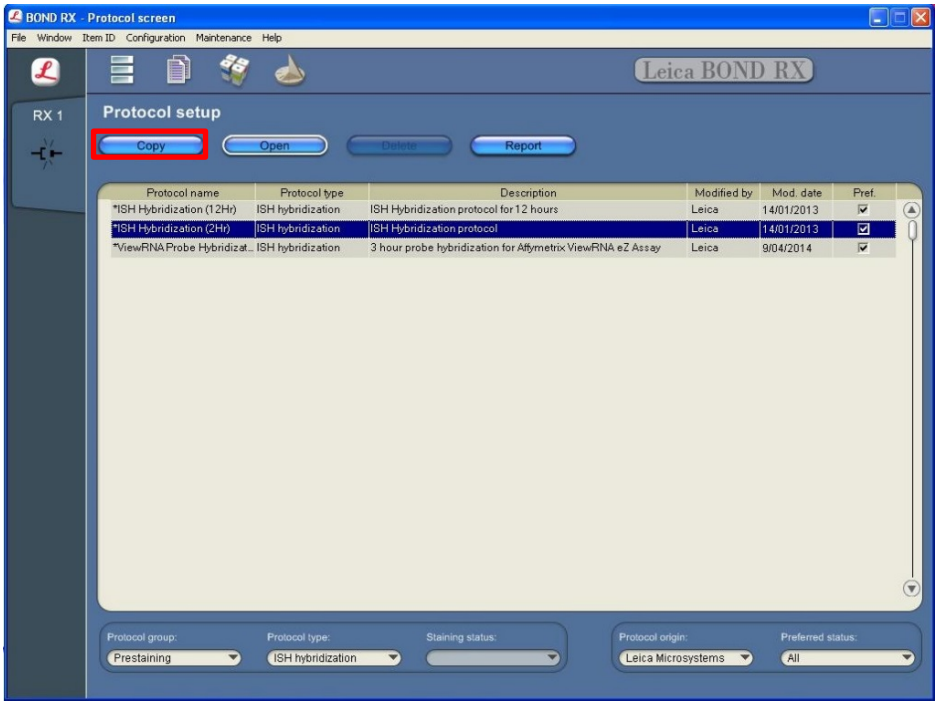
IMPORTANT! Only probe reagents are marked hazardous. RNAscope Amp reagents do not require that designation.

8. Select **Save**.
9. Perform these steps only once for each reagent and for each target probe.

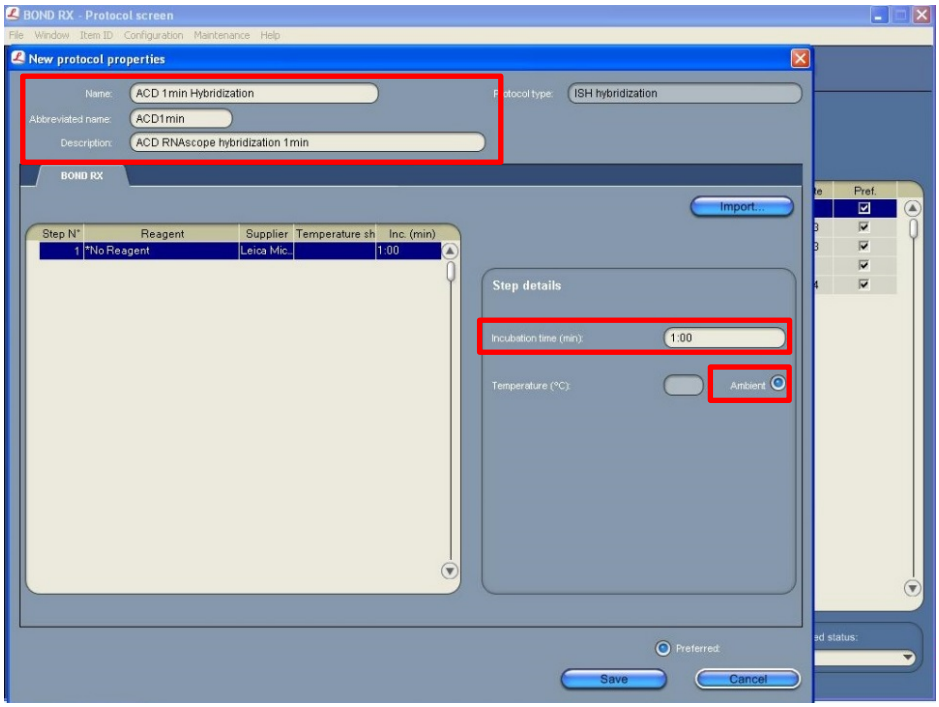
Create a one-minute probe hybridization protocol

A mock probe hybridization step must be created as part of the BDZ 11 software workaround for the RNAscope® 2.5 LS assay. You need to set this up only once. The following example copies the existing two hour hybridization protocol and changes the incubation time to one minute.

1. In the Protocol setup screen, select **ISH hybridization** under the Protocol type menu.
2. Highlight the ***ISH Hybridization (2Hr)** protocol. Select **Copy**.



3. Change the Name to **ACD 1min Hybridization**, the Abbreviated Name to **ACD1min**, and the Description to **ACD RNAscope hybridization 1min**.

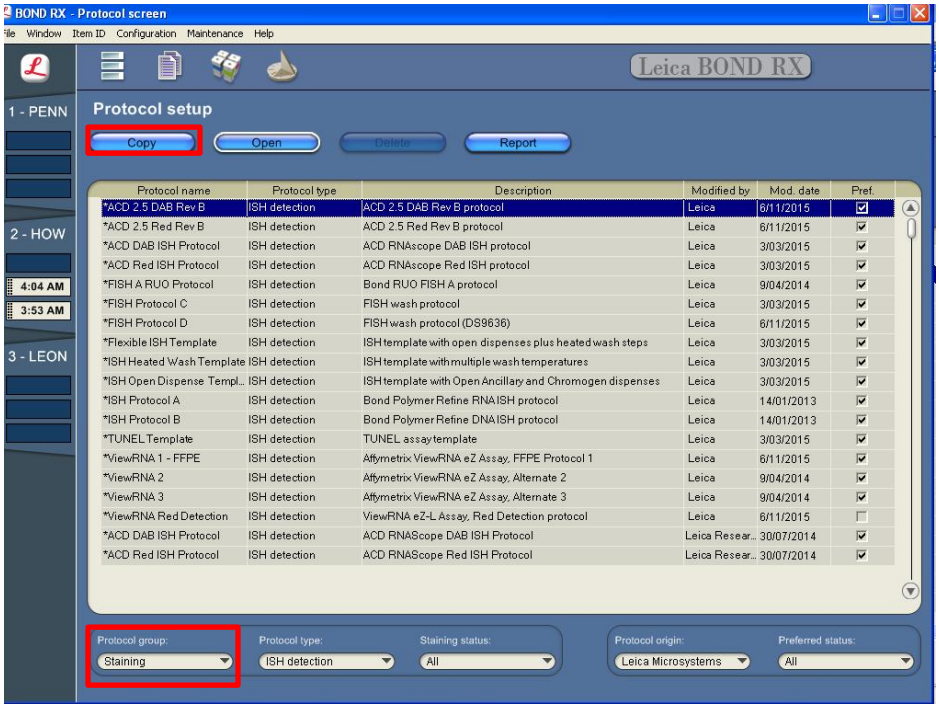


4. Highlight the ***No Reagent** step.
5. Change the incubation time to **1 MIN** and select **Ambient** as Temperature (°C).
6. Select **Save**.

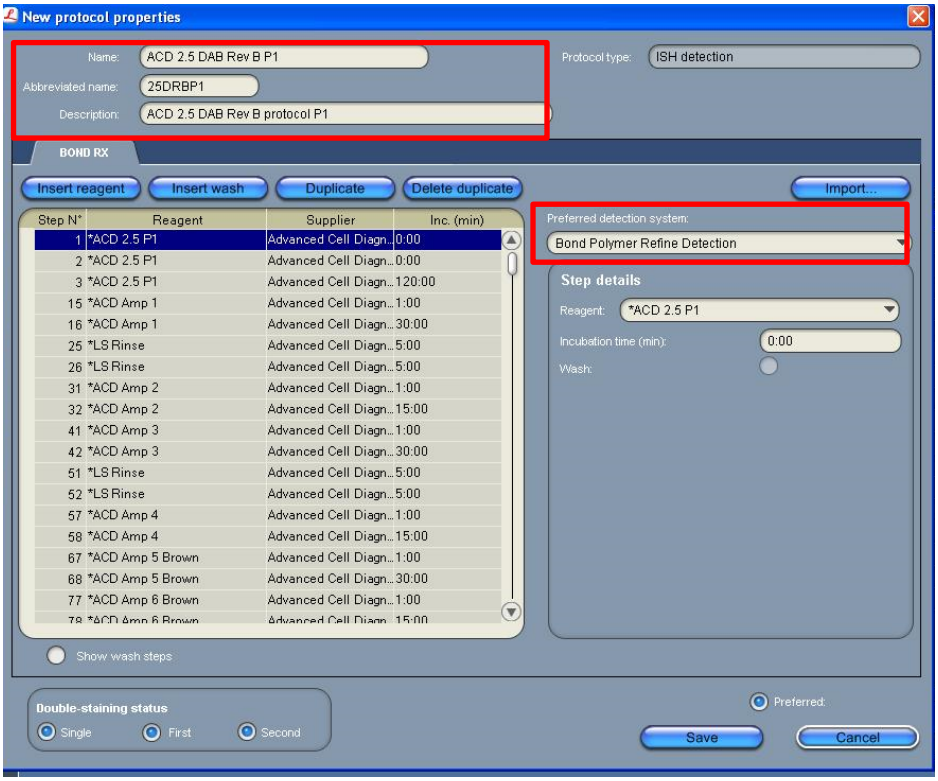
Create a staining protocol

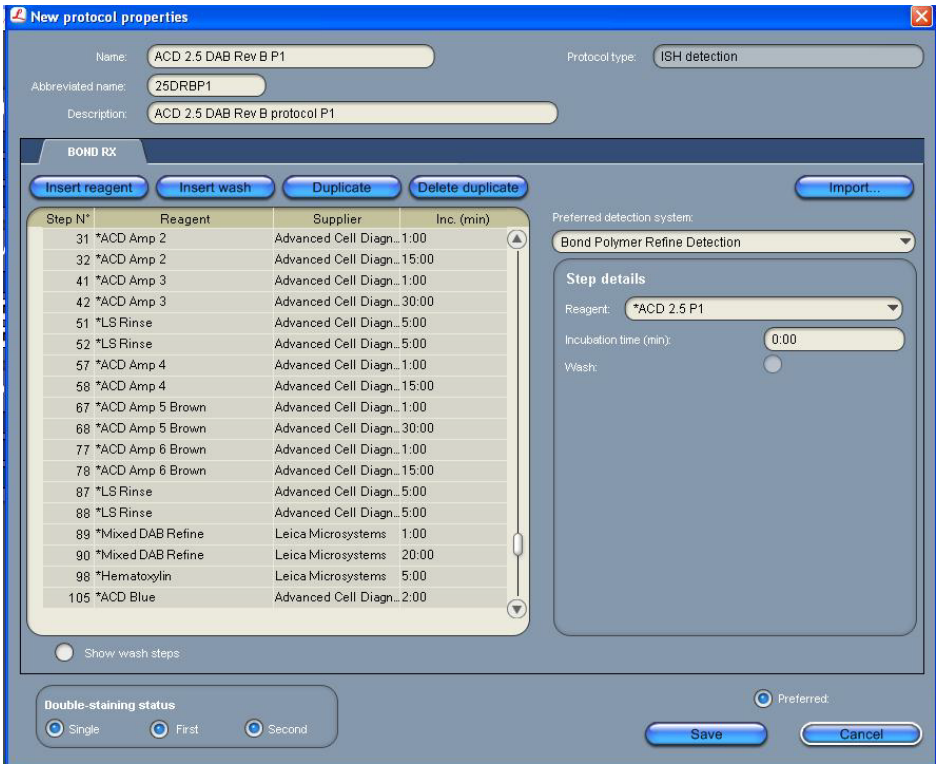
Due to the BDZ 11 software workaround for the RNAscope® 2.5 LS assay, unique staining protocols *must be created for each probe.*

1. In the Protocol setup screen, select **Staining** under 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 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.
4. Select **Bond Polymer Refine Detection** from the Preferred detection system menu.





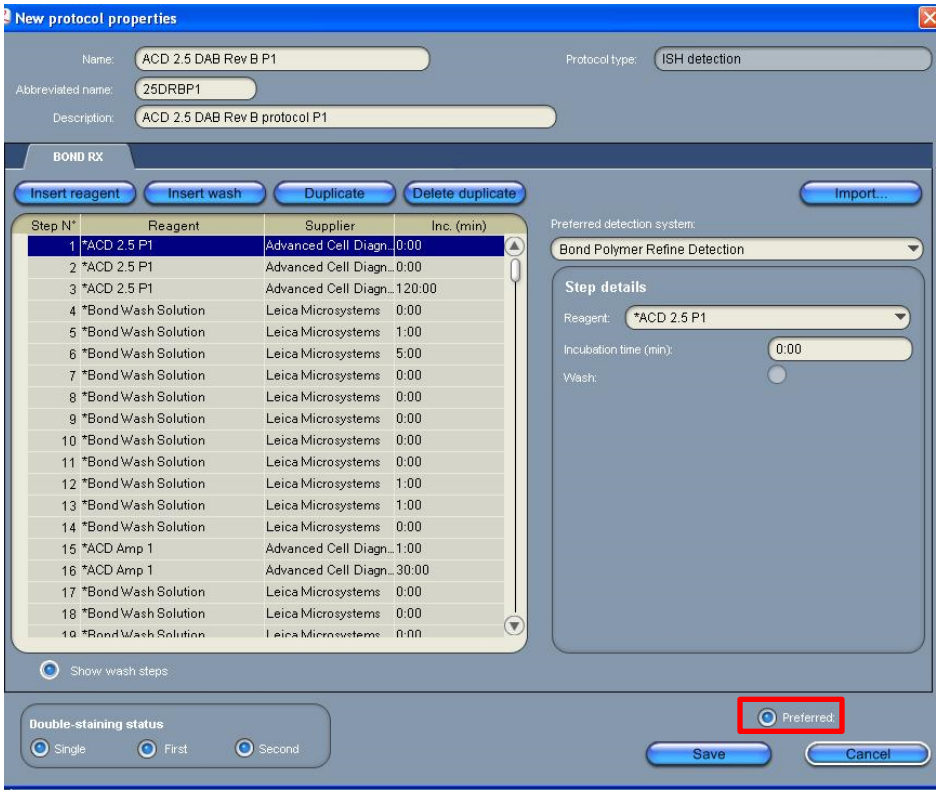
Note: The preceding two figures display all reagent steps.

5. Highlight and select each Reagent step to edit.

IMPORTANT! You can change the incubation times, but not the temperature for these steps.

6. Click **Show wash steps** (see the following figure) to view the washing steps found between each reagent. Insert BOND Washes to match each of the protocol steps shown in the table above.

7. Compare and confirm screen protocol with the full protocol listed in **Appendix A. BDZ 11 Protocol** on page 30.
8. Make sure that **Preferred** is selected (bottom right corner of window).



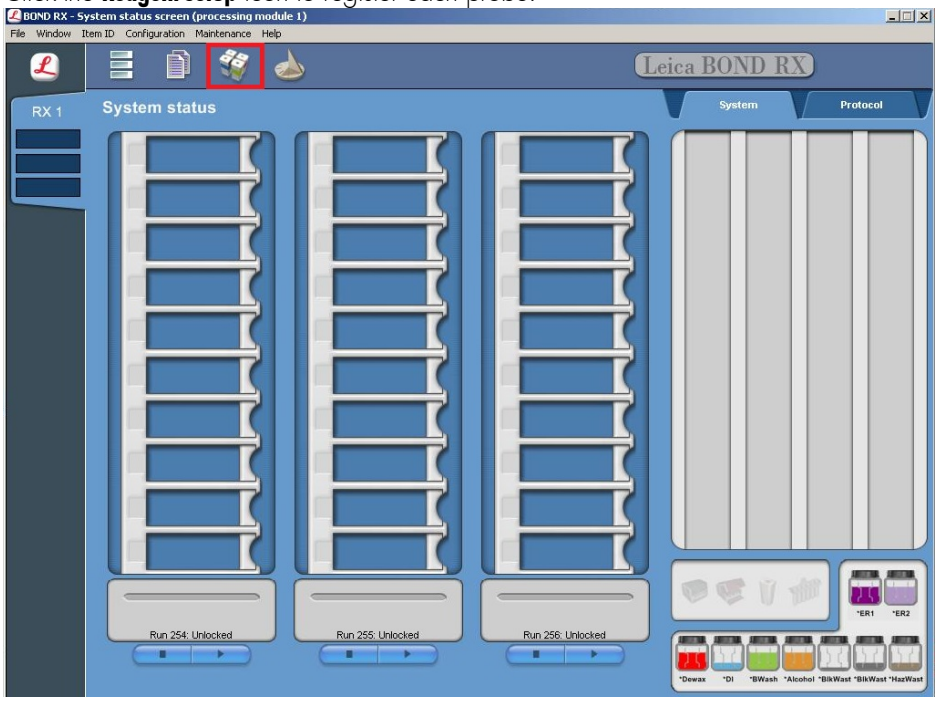
9. Select **Save**.
10. Click **Next** to proceed. Ignore pop-ups that say Step 3 exceeds the recommended time and that the protocol is not validated by Leica. If you see any other pop-ups please contact your FAS or technical support at ACD.
11. Create a new probe protocol.

Note: You must create a new protocol for each new probe you use.

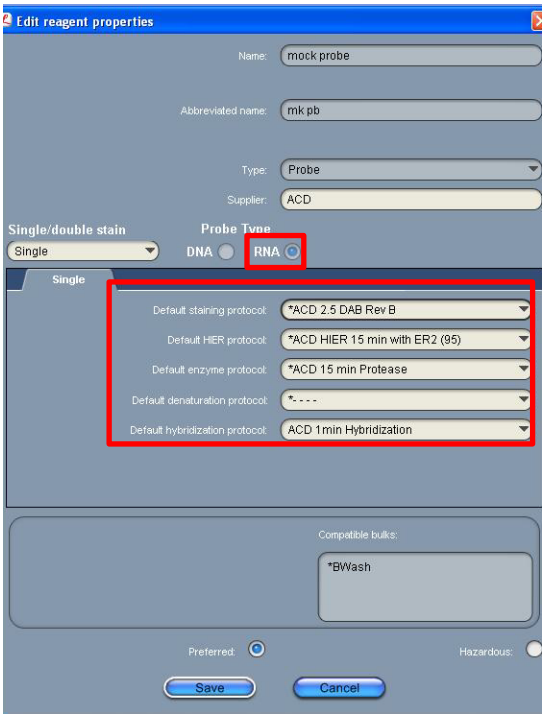
Register the mock probe

Create a mock probe in the reagent set up. You need to do this only once.

1. Click the **Reagent setup** icon to register each probe.



2. Select **Add**.
3. Enter the **mock probe** in the Name and Abbreviated name text boxes.
4. Check **RNA** for Probe Type Select **Probe** in the Type drop-down menu. Enter **ACD** in the Supplier text box.

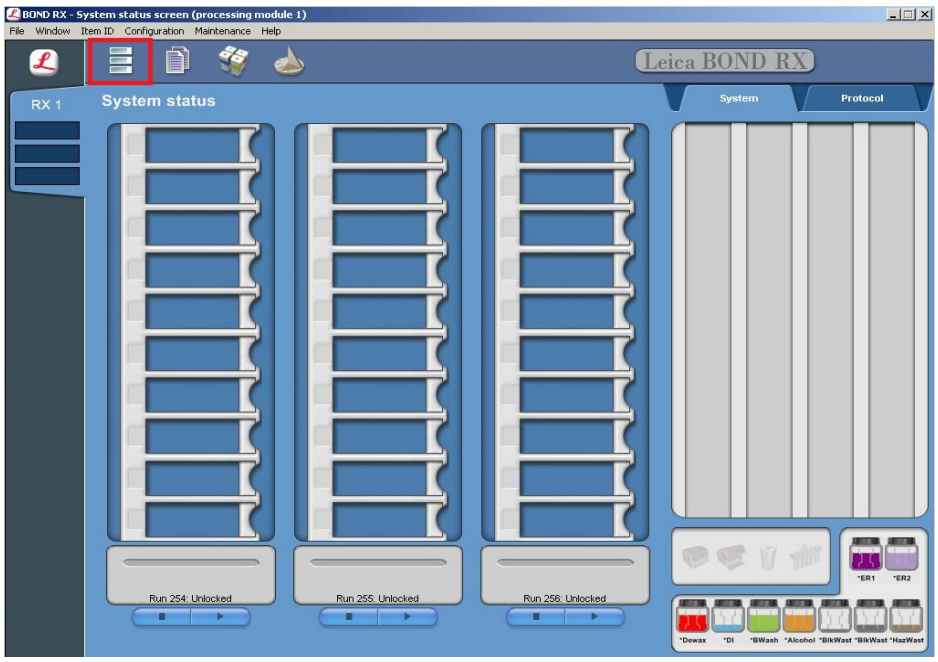


5. Select ***ACD 2.5 DAB Rev B** as the Default staining protocol.

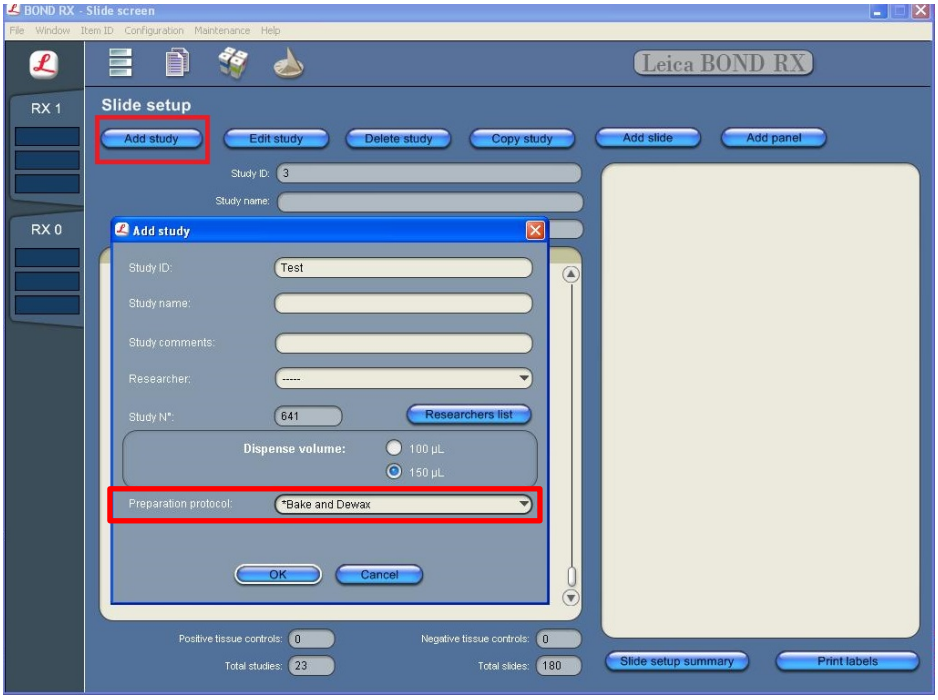
6. Select ***ACD HIER 15min with ER2 (95)*** as the Default HIER protocol.
7. Select ***ACD 15min Protease*** as the Default enzyme protocol.
8. Leave the Default denaturation protocol blank.
9. Select **ACD 1 min Hybridization** as the Default hybridization protocol.
10. Mock Probe is not a hazardous reagent.
11. Select **Save**.

Set up a study

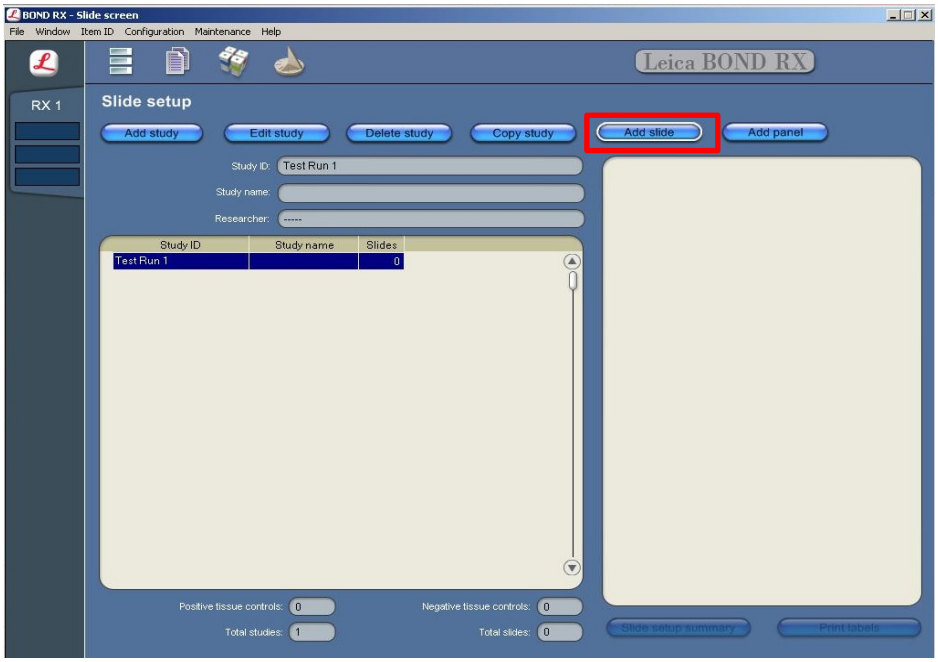
1. To build a study, select the **Slide setup** icon at the top of the screen.



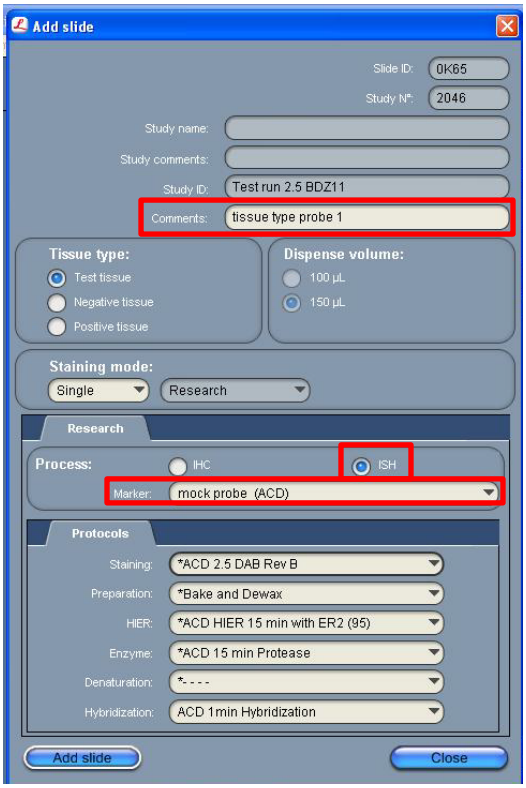
2. Select **Add study** and enter a name in the **Study ID** field (keep the Dispense volume default as shown at **150** µL).



3. Select ***Bake and Dewax** as the Preparation protocol.
4. Select **OK**.
5. Select **Add slide** to assign a protocol to each slide.

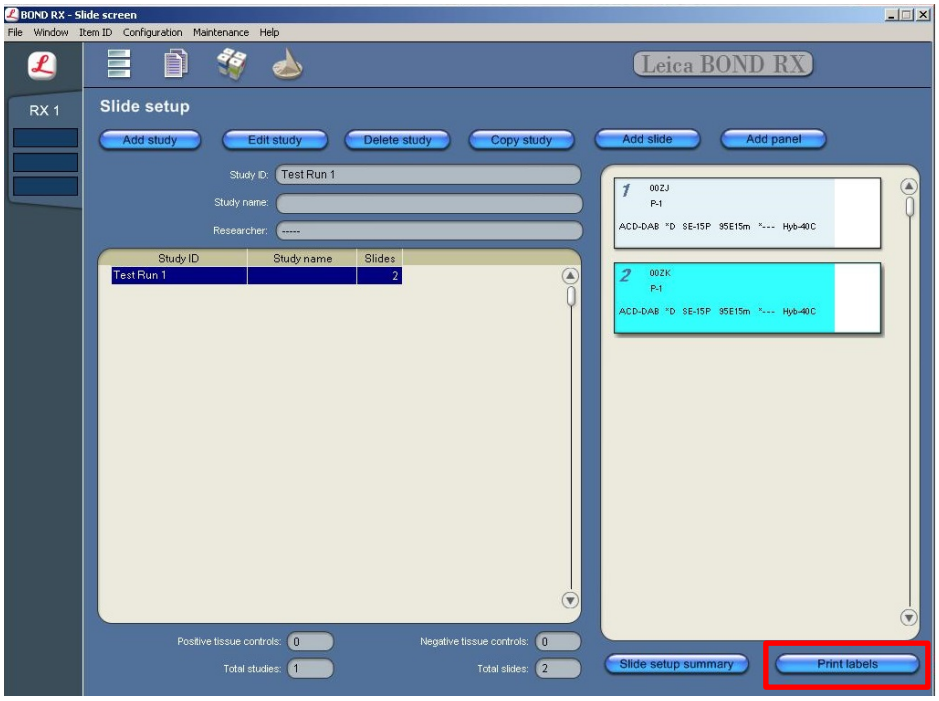


6. Enter the tissue type and probe name under the Comments field.



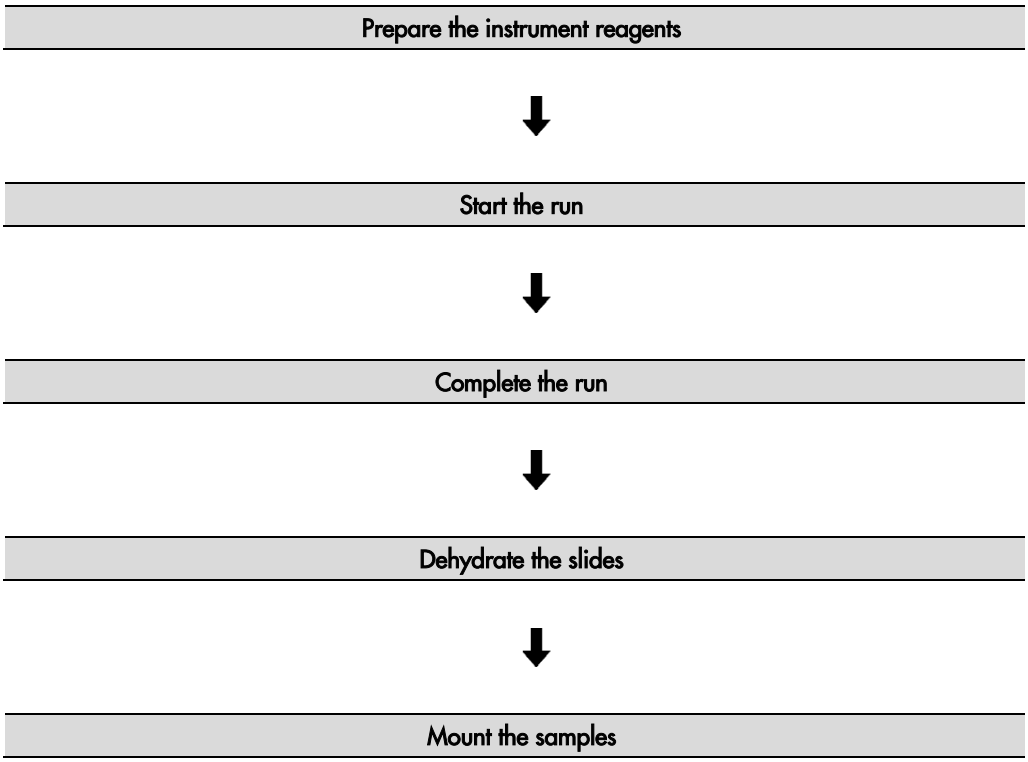
7. From the Research tab, select **ISH** under Process and **mock probe (ACD)** from the Marker drop down menu.
8. For RNAscope® 2.5 LS assays, under the **Protocols** tab:
   a. Make sure that each probe is associated with a different protocol (for example, ACD DAB RevB, P2), and select a protocol from the Staining drop down menu.
   b. For standard FFPE tissues, select the protocol ***Bake and Dewax** from the Preparation drop down menu.
   c. Select ***ACD HIER 15 min with ER2 (95)** as the HIER protocol.
   d. Select ***ACD 15 min Protease** for Enzyme.
   e. Select **ACD 1 min Hybridization** for Hybridization.

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



5 Chapter 5. Run the RNAscope® 2.5 LS Brown Assay

Workflow



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 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 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) plus Hematoxylin	• 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.

2. Use clean, dry covertiles for every run. Clean used covertiles with water, bleach, and ethanol following Lecia guidance. 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

1. Obtain one empty 30mL Open BOND container and label it "**Mock Probe**".
2. Carefully transfer all the RNAscope® LS reagents into empty 30 mL Open Bond containers.
3. Fill the **Mock Probe** container with Leica Biosystems' 1X BOND Wash.
4. Using the Barcode Scanner, scan the front barcode on the 30 mL Open Bond container. A window will appear.

NOTE: If using other open containers for probe delivery, please ensure that you do account for the dead volume required in each container with volumes suggested in the table below.

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

5. From the drop down menu, select the corresponding name of the reagent as shown in the following table under **Container Name**:

Reagents	Container Name
RNAscope® 2.5 LS Hydrogen Peroxide	*Open 0 Haz
RNAscope® 2.5 LS Protease III	*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 5 – BROWN	*ACD Amp 5 Brown
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: Leica BOND DAB plus Hematoxylin comes in a pre-filled Leica BOND RX container.

6. 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 refer to **Troubleshooting** on page 29.

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 6. Evaluate the Results** on page 28.

6 Chapter 6. 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:

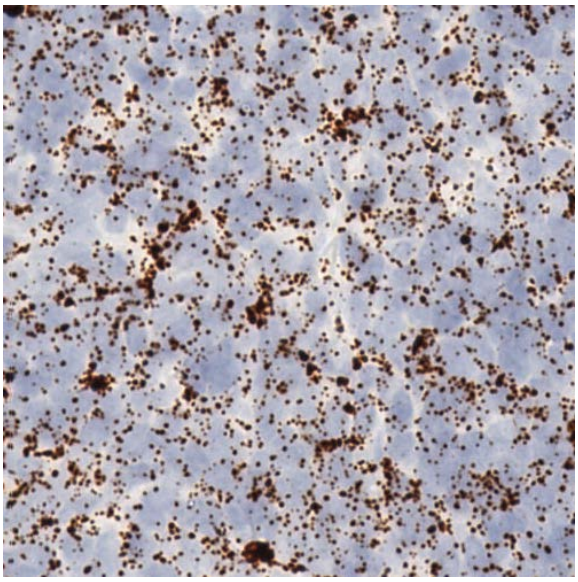
Staining Score	Microscope Objective Scoring*
0	No staining or less than 1 dot for every 10 cells (40X magnification)
1	1–3 dots/cell (visible at 20–40X magnification)
2	4–9 dots/cell. No or very few dot clusters (visible at 20–40X magnification)
3	10–15 dots/cell and /or< 10% positive cells have dot 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 FFPE lymph node 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 5 minutes and increase the Enzyme time in increments of 10 minutes (see **Appendix B. Edit the Protease Protocol** on page 37).
- 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 with the exception of 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. BDZ 11 Protocol

The following table displays the full software protocol.

Note: Heated bond washes 4–6 come from the bulk reagents and are heated by the instrument. You cannot delete these steps. You may delete other wash steps.

Step No.	Reagent	Step Type	Incubation Time	Temperature
1	Probe 1 2.5	Reagent	0 MIN	Ambient
2	Probe 1 2.5	Reagent	0 MIN	Ambient
3	Probe 1 2.5	Reagent	120 MIN	42°C
4	*Bond wash	Reagent	0 MIN	42°C
5	*Bond wash	Reagent	1 MIN	42°C
6	*Bond wash	Reagent	5 MIN	42°C
7	*Bond Wash Solution	Wash	0 MIN	Ambient
8	*Bond Wash Solution	Wash	0 MIN	Ambient
9	*Bond Wash Solution	Wash	0 MIN	Ambient
10	*Bond Wash Solution	Wash	0 MIN	Ambient
11	*Bond Wash Solution	Wash	0 MIN	Ambient
12	*Bond Wash Solution	Wash	1 MIN	Ambient
13	*Bond Wash Solution	Wash	1 MIN	Ambient
14	*Bond Wash Solution	Wash	0 MIN	Ambient
15	*ACD AMP 1	Reagent	1 MIN	42°C
16	*ACD AMP 1	Reagent	30 MIN	42°C
17	*Bond Wash Solution	Wash	0 MIN	Ambient
18	*Bond Wash Solution	Wash	0 MIN	Ambient
19	*Bond Wash Solution	Wash	0 MIN	Ambient
20	*Bond Wash Solution	Wash	3 MIN	Ambient
21	*Bond Wash Solution	Wash	3 MIN	Ambient
22	*Bond Wash Solution	Wash	0 MIN	Ambient
23	*Bond Wash Solution	Wash	0 MIN	Ambient
24	*Bond Wash Solution	Wash	0 MIN	Ambient
25	* LS Rinse	Reagent	5 MIN	Ambient
26	* LS Rinse	Reagent	5 MIN	Ambient
27	*Bond Wash Solution	Wash	0 MIN	Ambient
28	*Bond Wash Solution	Wash	0 MIN	Ambient
29	*Bond Wash Solution	Open wash	0 MIN	Ambient
30	*Bond Wash Solution	Wash	0 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature
31	*ACD AMP 2	Reagent	1 MIN	42°C
32	*ACD AMP 2	Reagent	15 MIN	42°C
33	*Bond Wash Solution	Wash	0 MIN	Ambient
34	*Bond Wash Solution	Wash	0 MIN	Ambient
35	*Bond Wash Solution	Wash	0 MIN	Ambient
36	*Bond Wash Solution	Wash	1 MIN	Ambient
37	*Bond Wash Solution	Wash	1 MIN	Ambient
38	*Bond Wash Solution	Wash	1 MIN	Ambient
39	*Bond Wash Solution	Wash	1 MIN	Ambient
40	*Bond Wash Solution	Wash	1 MIN	Ambient
41	*ACD AMP 3	Reagent	1 MIN	42°C
42	*ACD AMP 3	Reagent	30 MIN	42°C
43	*Bond Wash Solution	Wash	0 MIN	Ambient
44	*Bond Wash Solution	Wash	0 MIN	Ambient
45	*Bond Wash Solution	Wash	0 MIN	Ambient
46	*Bond Wash Solution	Wash	3 MIN	Ambient
47	*Bond Wash Solution	Wash	3 MIN	Ambient
48	*Bond Wash Solution	Wash	0 MIN	Ambient
49	*Bond Wash Solution	Wash	0 MIN	Ambient
50	*Bond Wash Solution	Wash	0 MIN	Ambient
51	*LS Rinse	Reagent	5 MIN	Ambient
52	*LS Rinse	Reagent	5 MIN	Ambient
53	*Bond Wash Solution	Wash	0 MIN	Ambient
54	*Bond Wash Solution	Wash	1 MIN	Ambient
55	*Bond Wash Solution	Open wash	1 MIN	Ambient
56	*Bond Wash Solution	Wash	1 MIN	Ambient
57	*ACD AMP 4	Reagent	1 MIN	42°C
58	*ACD AMP 4	Reagent	15 MIN	42°C
59	*Bond Wash Solution	Wash	0 MIN	Ambient
60	*Bond Wash Solution	Wash	0 MIN	Ambient
61	*Bond Wash Solution	Wash	0 MIN	Ambient
62	*Bond Wash Solution	Wash	1 MIN	Ambient
63	*Bond Wash Solution	Wash	1 MIN	Ambient
64	*Bond Wash Solution	Wash	1 MIN	Ambient
65	*Bond Wash Solution	Open Wash	1 MIN	Ambient
66	*Bond Wash Solution	Wash	1 MIN	Ambient
67	*ACD AMP 5 Brown	Reagent	1 MIN	Ambient
68	*ACD AMP 5 Brown	Reagent	30 MIN	Ambient
69	*Bond Wash Solution	Wash	0 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature
70	*Bond Wash Solution	Wash	0 MIN	Ambient
71	*Bond Wash Solution	Wash	0 MIN	Ambient
72	*Bond Wash Solution	Wash	1 MIN	Ambient
73	*Bond Wash Solution	Wash	1 MIN	Ambient
74	*Bond Wash Solution	Wash	1 MIN	Ambient
75	*Bond Wash Solution	Wash	1 MIN	Ambient
76	*Bond Wash Solution	Wash	1 MIN	Ambient
77	*ACD AMP 6 Brown	Reagent	1 MIN	Ambient
78	*ACD AMP 6 Brown	Reagent	15 MIN	Ambient
79	*Bond Wash Solution	Wash	0 MIN	Ambient
80	*Bond Wash Solution	Wash	0 MIN	Ambient
81	*Bond Wash Solution	Wash	0 MIN	Ambient
82	*Bond Wash Solution	Wash	1 MIN	Ambient
83	*Bond Wash Solution	Wash	1 MIN	Ambient
84	*Bond Wash Solution	Wash	1 MIN	Ambient
85	*Bond Wash Solution	Wash	1 MIN	Ambient
86	*Bond Wash Solution	Wash	1 MIN	Ambient
87	*LS Rinse	Reagent	5 MIN	Ambient
88	*LS Rinse	Reagent	5 MIN	Ambient
89	*Mixed DAB Refine	Reagent	1 MIN	Ambient
90	*Mixed DAB Refine	Reagent	20 MIN	Ambient
91	*De-ionized Water	Wash	0 MIN	Ambient
92	*De-ionized Water	Wash	0 MIN	Ambient
93	*De-ionized Water	Wash	0 MIN	Ambient
94	*De-ionized Water	Wash	0 MIN	Ambient
95	*De-ionized Water	Wash	0 MIN	Ambient
96	*De-ionized Water	Wash	0 MIN	Ambient
97	*De-ionized Water	Wash	0 MIN	Ambient
98	*Hematoxylin	Reagent	5 MIN	Ambient
99	*De-ionized Water	Wash	0 MIN	Ambient
100	*De-ionized Water	Wash	0 MIN	Ambient
101	*De-ionized Water	Wash	0 MIN	Ambient
102	*De-ionized Water	Wash	0 MIN	Ambient
103	*De-ionized Water	Wash	0 MIN	Ambient
104	*De-ionized Water	Wash	0 MIN	Ambient
105	*ACD Blue	Reagent	2 min	Ambient
106	*De-ionized Water	Wash	0 MIN	Ambient
107	*De-ionized Water	Wash	0 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature
108	*De-ionized Water	Wash	0 MIN	Ambient
109	*De-ionized Water	Wash	0 MIN	Ambient
110	*De-ionized Water	Wash	0 MIN	Ambient
111	*De-ionized Water	Wash	0 MIN	Ambient

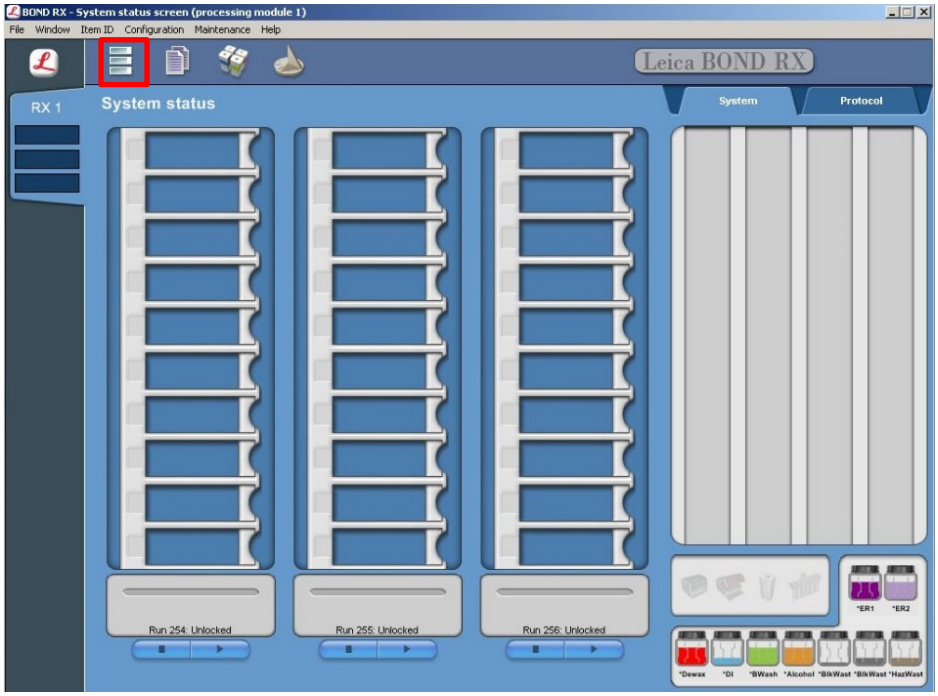
* Indicates reagent is hard–coded in software by Leica Biosystems.

B Appendix B. Edit the Epitope Retrieval Protocol

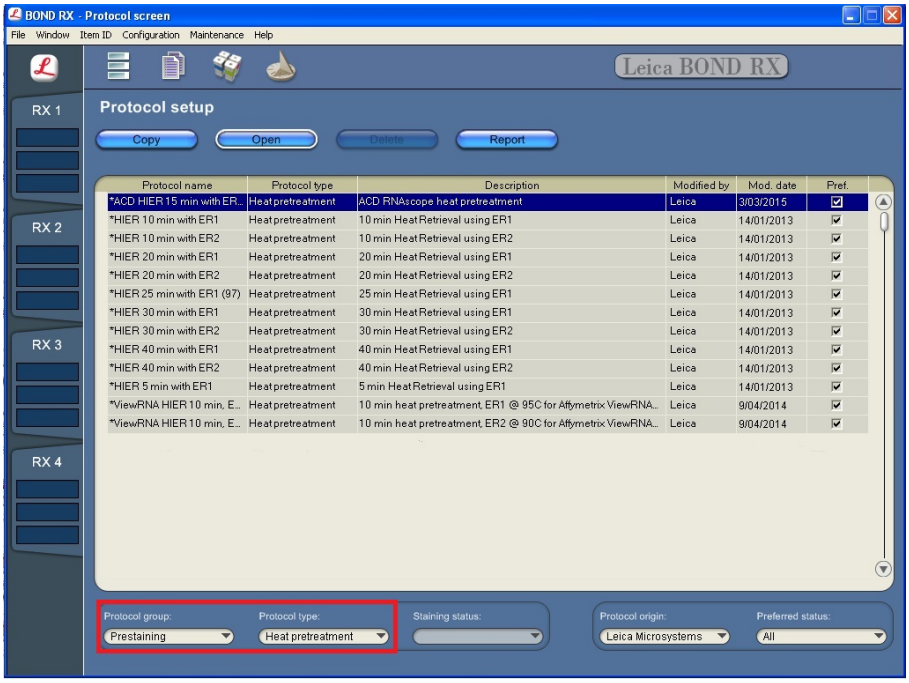
The following example shows how to edit the Epitope Retrieval procedure from within the software.

Create a prestaining protocol

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



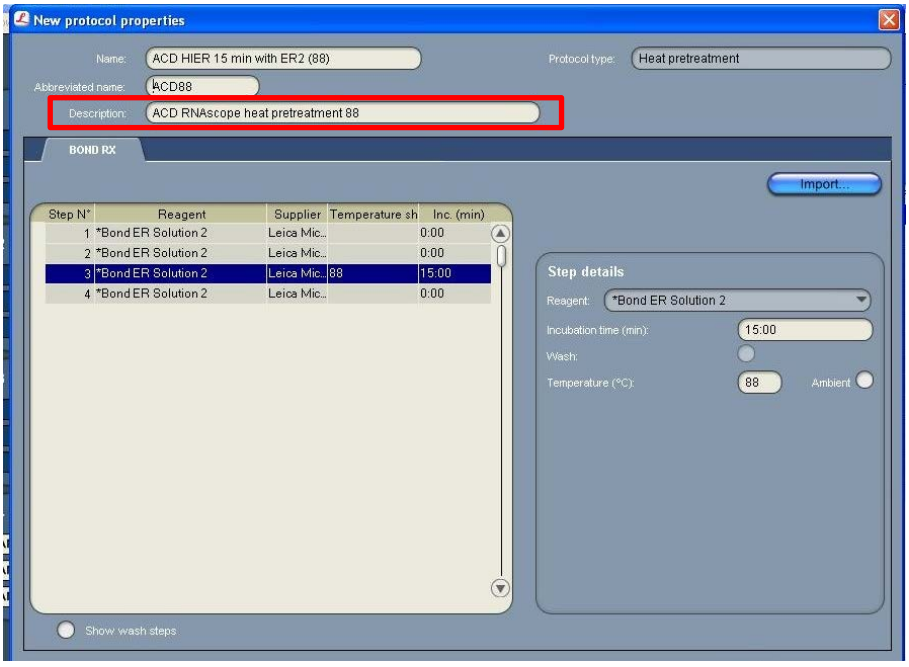
2. Select **Prestaining** under the Protocol group menu and **Heat pretreatment** under the Protocol type menu to access the heat pretreatment protocols.



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). Depending on the tissue type used, change the temperature and time as shown in the following table.

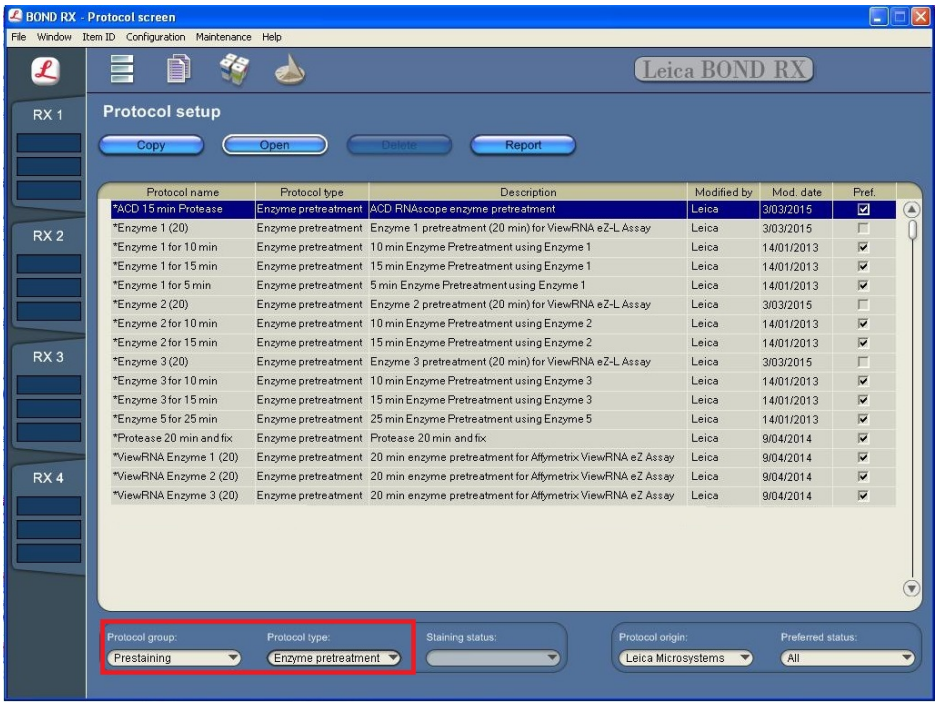
Tissue Type	ER2 Incubation Time	Temperature
Brain and spinal cord	15 MIN	95°C
Breast cancer	15 MIN	95°C
Cell pellet	15 MIN	88°C
Colon	15 MIN	95°C
GI tract	15 MIN	95°C
Head and neck cancer	15 MIN	95°C
Heart	15 MIN	95°C
Kidney	15 MIN	95°C
Liver	15 MIN	95°C
Lung	15 MIN	95°C
Lymphoma	15 MIN	95°C
Placenta	15 MIN	95°C
Prostate	15 MIN	95°C
Skin	15 MIN	95°C
Stomach	15 MIN	95°C
Thymus	15 MIN	88°C or 95°C
Tonsil	15 MIN	88°C or 95°C
Xenograft	15 MIN	88°C
Mouse tissue	15 MIN	88°C or 95°C

8. Select **Save** to create a protocol for ER2 pretreatment at 88°C.
9. If needed, repeat Steps 1 through 8 to create a new heating protocol (for example, ACD 25min ER2).

Appendix C. Edit the Protease Protocol

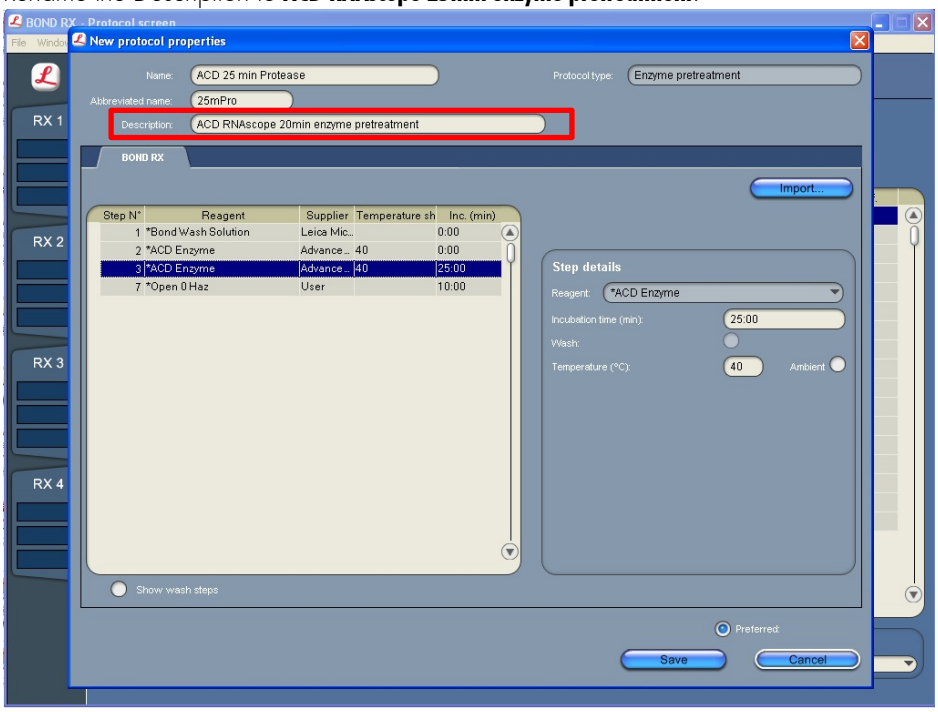
The following example shows how to edit the Protease procedure from within the software.

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 **25mPro**.

5. Rename the Description to **ACD RNAscope 25min enzyme pretreatment**.



6. Highlight the second ***ACD Enzyme** step. Keep the temperature at **40°C** and set the enzyme incubation time to **15 MIN** for the following:

Tissue Type
Brain and spinal cord
Breast cancer
Cell pellet
Colon
GI tract
Head and neck cancer
Heart
Kidney
Liver
Lung
Lymphoma
Placenta
Prostate
Skin
Stomach
Thymus
Tonsil
Xenograft

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

D Appendix D. Safety

Chemical safety

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

- Read and understand the Safety Data Sheets (SDSs) before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see **https://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: **https://www.cdc.gov/biosafety/**
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: **https://www.osha.gov/pls/oshaweb/owadisp.show_document?p_id=10051&p_table=STANDARDS**
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.
- Additional information about biohazard guidelines is available at: **https://www.cdc.gov/biosafety/**

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, Authorization and Restriction of Chemicals (REACH) can be found at: **https://echa.europa.eu/regulations/reach**

Documentation and Support

Obtaining SDSs

Safety Data Sheets (SDSs) are available at: **https:// 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: **https://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.
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 ACD website. If you have any questions, please contact Advanced Cell Diagnostics at **https://acdbio.com/about/contact**.

Headquarters
7707 Gateway Blvd Suite 200, Newark, CA 94545 Phone 1-510-576-8800 Toll Free 1-877-576-3636
For support, email **support.acd@biotechne.com**
www.acdbio.com

a bio-techne brand