

RNAscope[®] 2.5 LSx Reagent Kit – **BROWN** User Manual for BDZ 15/BXD 15

For use with Leica Biosystems' BOND RX System

Document Number UM-322700

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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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Chapter 1. Product Information



Before using this product, read and understand the information in **Appendix C. Safety** on page 40 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 LSx Reagent Kit for use with Leica Biosystems' BOND RX Research Advanced Staining System. RNAscope® 2.5 LSx Assays are compatible with a variety of sample types.

Product description

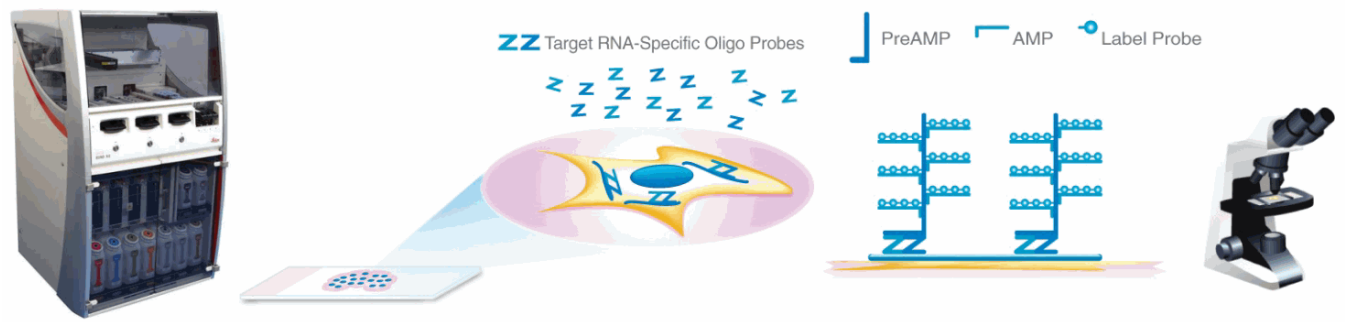
Background

The RNAscope® 2.5 LSx 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 LSx 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 LSx Assay procedure, which takes ~9–10 hours. You will first pretreat sections, and then hybridize RNA-specific probes to target RNA. The signal is then amplified using multiple steps, followed by hybridization to horseradish peroxidase (HRP)-labeled probes and detection using the 3,3'-diaminobenzidine (DAB) chromogenic substrate. Each single RNA transcript will appear as a distinct dot of chromogen precipitate that is visible using a common brightfield 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.	Hybridize probe to 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 LSx Assay requires the RNAscope® 2.5 LS Probes and the RNAscope® 2.5 LSx 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 <https://acdbio.com/products> to find a gene-specific Target Probe or appropriate Control Probes.

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

Target Probes					
<input checked="" type="checkbox"/>	Reagent	Cat. No.	Content	Quantity	Storage
	RNAscope® 2.5 LS Target Probe – [species]– [gene]	Various	Probe targeting specific RNA	18 mL x 1 bottle	2–8°C
Control Probes					
<input checked="" type="checkbox"/>	Reagent	Cat. No.	Content	Quantity	Storage
	RNAscope® 2.5 LS Positive Control Probe – [species]– PPIB	Various	Probe targeting common housekeeping gene	18 mL x 1 bottle	2–8°C
	RNAscope® 2.5 LS Negative Control Probe – DapB	312038	Probe targeting bacterial gene dapB	18 mL x 1 bottle	2–8°C

RNAscope® 2.5 LSx Reagents

The RNAscope® 2.5 LSx Reagent Kit – BROWN (Cat. No. 322700) contains the reagents required to run the RNAscope® 2.5 LSx 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 arrive in labeled Leica BOND compatible containers in two trays. Use the two trays in parallel as a single kit. The first tray has two tray barcodes and a label on the handle, and contains the LS Rinse, DAB chromogen, and counterstaining reagents. Register the reagents on this tray as a set using the tray barcodes. Do not remove or change the position of the reagents in the tray. Removing or changing the position of the containers will result in run failure.

The second tray contains the pretreatment and signal amplification reagents. The reagents on this tray need to be individually registered.

Note: You can use the empty spots on the trays for BOND Open containers, such as those containing custom probes.

RNAscope® 2.5 LSx Reagent Kit – BROWN (Cat. No. 322700)				
<input checked="" type="checkbox"/>	Reagent	Tray position	Quantity	Storage
	RNAscope® 2.5 LSx Rinse	Tray 1, positions 1–2	27 mL x 2 containers	2–8°C
	RNAscope® 2.5 LSx Hematoxylin	Tray 1, position 3	9 mL x 1 container	2–8°C
	RNAscope® 2.5 LSx DAB Part 1	Tray 1, position 4	1.2 mL x 1 container	2–8°C
	RNAscope® 2.5 LSx DAB Part B	Tray 1, position 5	22 mL x 1 container	2–8°C
	RNAscope® 2.5 LSx Bluing	Tray 1, position 6	9 mL x 1 container	2–8°C
	RNAscope® 2.5 LSx AMP 1 DAB	Tray 2, position 1	18 mL x 1 container	2–8°C
	RNAscope® 2.5 LSx AMP 2 DAB	Tray 2, position 2	18 mL x 1 container	2–8°C
	RNAscope® 2.5 LSx AMP 3 DAB	Tray 2, position 3	18 mL x 1 container	2–8°C
	RNAscope® 2.5 LSx AMP 4 DAB	Tray 2, position 4	18 mL x 1 container	2–8°C
	RNAscope® 2.5 LSx AMP 5 DAB	Tray 2, position 5	18 mL x 1 container	2–8°C
	RNAscope® 2.5 LSx AMP 6 DAB	Tray 2, position 6	18 mL x 1 container	2–8°C
	RNAscope® 2.5 LS H ₂ O ₂	Tray 2, position 7	9 mL x 1 container	2–8°C
	RNAscope® 2.5 LSx Protease	Tray 2, position 8	12 mL x 1 container	2–8°C

IMPORTANT! Use only the RNAscope® 2.5 LS Probes. Do not substitute the reagent components of the RNAscope® 2.5 LSx Reagent Kit with those of other RNAscope® Reagent Kits, including RNAscope® 2.0 or 2.5 LS Reagent Kits even if the components have the same name. Do not refill prefilled containers as the barcodes correlate to the volumes in the container. Do not mix trays between different RNAscope® 2.5 LSx Kits or other RNAscope® Reagent Kits.

Required materials from Leica BOND RX

The RNAscope® 2.5 LSx Assay requires specific materials and equipment available *only* from Leica Biosystems.

<input checked="" type="checkbox"/>	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

<input checked="" type="checkbox"/>	Component	Cat. No.	Storage
	BOND Epitope Retrieval Solution 2-1L (RTU)	AR9640	2–8°C
	BOND Dewax Solution – 1L (RTU)	AR9222	2–26°C
	BOND Wash Solution 10X Concentrate – 1L	AR9590	2–8°C
	BOND Aspirating Probe Cleaning System	CS9100	2–8°C
	BOND Mixing Stations	S21.1971	Room temp (20–25°C)

Equipment

<input checked="" type="checkbox"/>	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.

<input checked="" type="checkbox"/>	Description	Supplier	Cat. No.
	SuperFrost® Plus Slides (required)	Fisher Scientific	12-550-15
	95% Ethanol (EtOH)	American Master Tech Scientific/MLS*	ALREACS
	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	LWSRA24
	Tissue-Tek® Staining Dishes	American Master Tech Scientific/MLS	LWT4457EA
	Tissue-Tek® Clearing Agent Dishes, xylene resistant	American Master Tech Scientific/MLS	LWT4456EA
	Cover Glass 24 x 50 mm	Fisher Scientific/MLS	12-545-F
	Distilled water	MLS	—
	Fume hood	MLS	—

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

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Chapter 2. Before You Begin

Prior to running the RNAscope® 2.5 LSx 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@acdbio.com.
- Regularly maintain and clean your automated staining instrument.
- Always run positive and negative control probes on your sample to assess sample RNA quality and optimal permeabilization.
- Do not substitute required materials. The assay has been validated with these materials only.
- Follow the protocol exactly for 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 E. Safety** page 40 for more information.

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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

Fix the sample

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

1. Immediately following dissection, fix 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

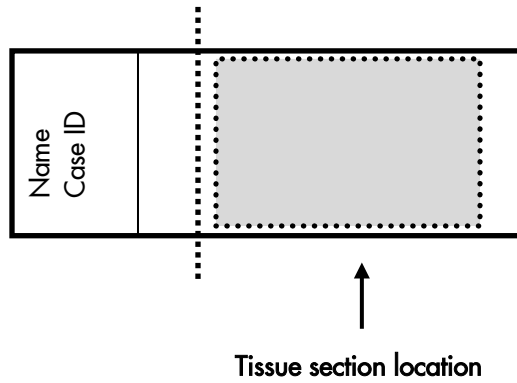
IMPORTANT! Use fresh reagents.

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), we recommend storing the slides at 2–8°C with desiccation.

4. Trim paraffin blocks as needed, and cut embedded tissue into 5 +/- 1 µm sections using a microtome.

- Place paraffin ribbon in a **40–45°C** water bath, and mount sections on **SUPERFROST® PLUS SLIDES**. Place tissue as shown for optimal staining:



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

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

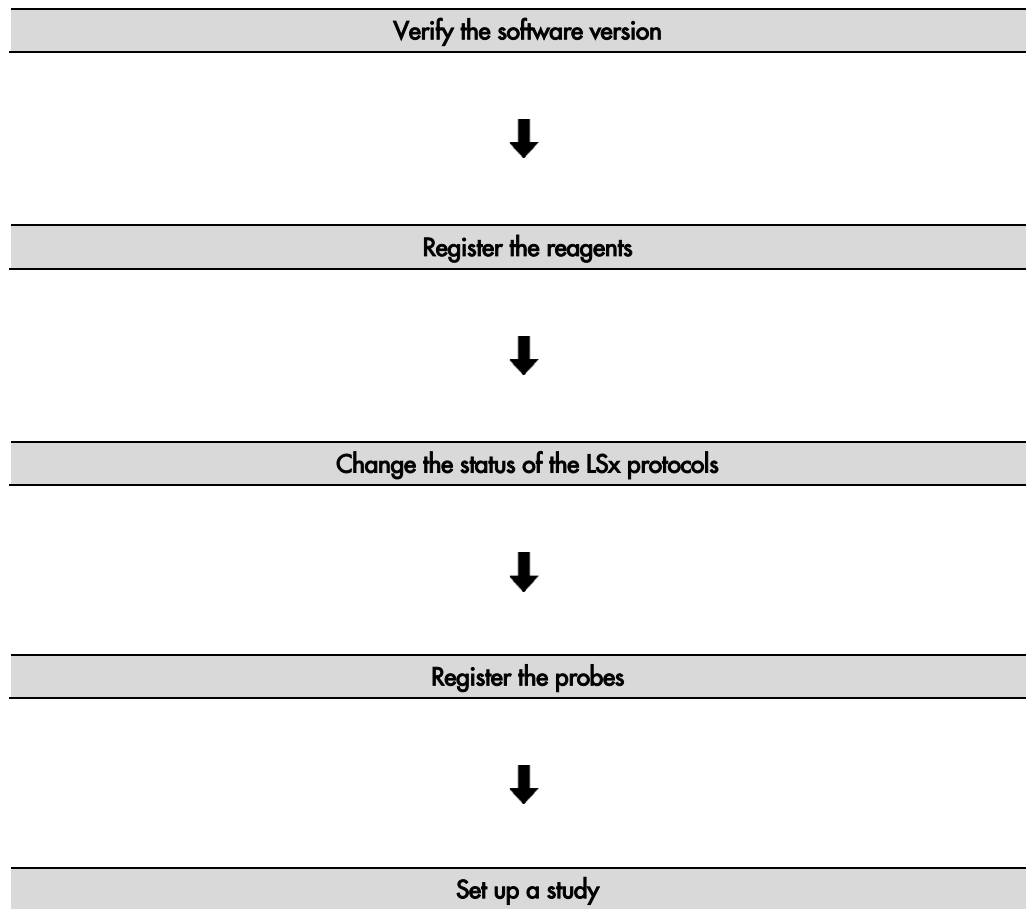
OPTIONAL STOPPING POINT. Use sectioned tissue within three months. Store sections with dessicants at room temperature.

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Chapter 4. Set Up the BDZ 15/BXD 15 Software

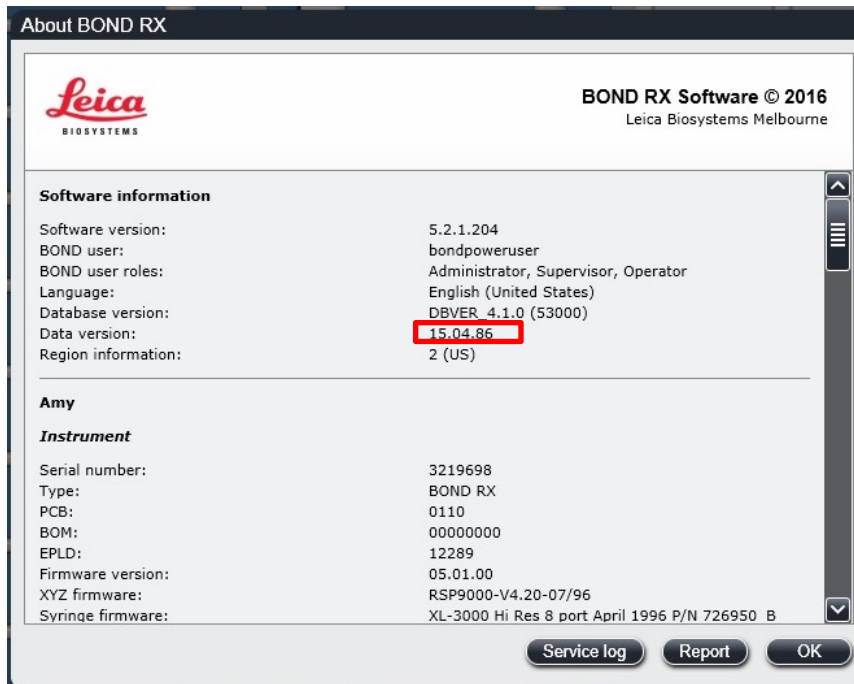
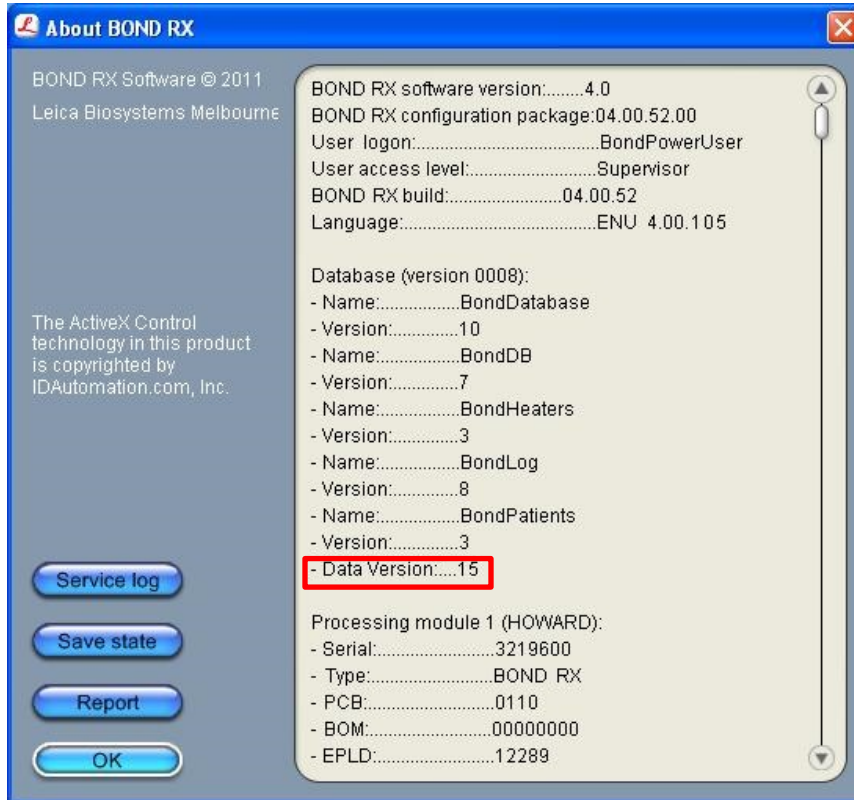
IMPORTANT! We strongly recommend you run the RNAscope® Control Slides (Cat. No. 310045 or Cat. No. 310023) using the RNAscope® 2.5 LS positive and negative control probes along with your samples in every run.

Workflow



Verify the software version

Prior to running the RNAscope® 2.5 LSx Brown Assay, you must update the BDZ/BXD software to database version 1.5. Please contact a Leica field service scientist for help with installing the software update.



Register the LSx reagents

You must register the two trays contained in the RNAscope® 2.5 LSx Brown Kit prior to use. The first tray contains the LS rinse, DAB chromogen, and counterstaining reagents, and has the label “RNAscope 2.5 LSx Reagent Kit- BROWN” on the handle. The second tray contains the pretreatment and amplification reagents.

1. Register the reagents on the first tray by scanning the two tray barcodes sequentially with the Barcode Scanner.
2. After reviewing the scanned information, select **OK**.

Note: You do not need to register the reagents on the tray individually. The tray barcodes contain all of the required information.

Pstrn	UPI	Reagent
1	09998454	*RNAscope 2.5 LSx Rinse
2	09998442	*RNAscope 2.5 LSx Rinse
3	09998466	*RNAscope 2.5 LSx Hematax...
4	09998586	*RNAscope 2.5 LSx DAB Part...
5	09998478	*RNAscope 2.5 LSx DAB Part...
6	09998490	*RNAscope 2.5 LSxBluing

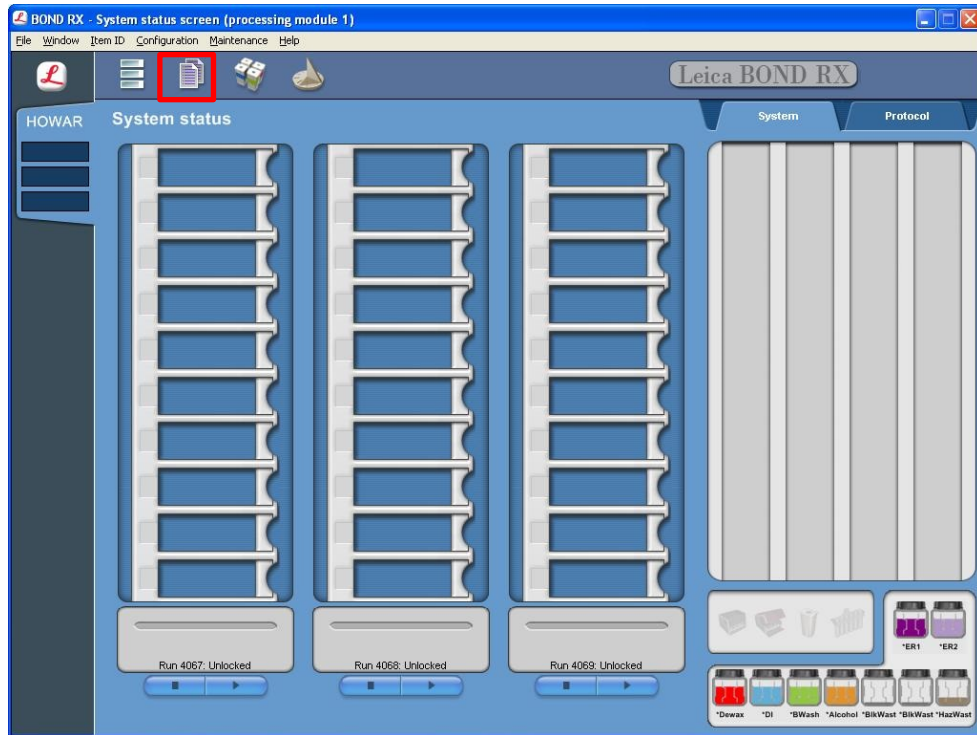
3. Register each component separately on the second tray by scanning the barcode on each reagent container with the Barcode Scanner.
4. Select **OK**.
5. Repeat steps 3–4 for all of the reagents on the second tray.

Change the status of the LSx protocols

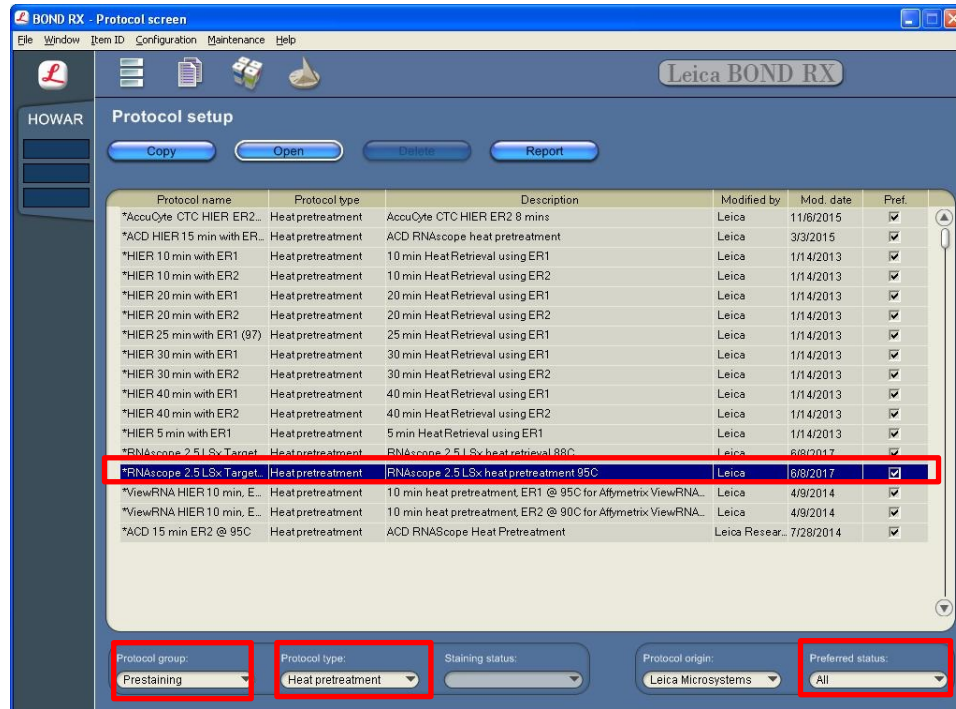
The BDZ/BDX 1.5 software update includes specific RNAscope® 2.5 LSx Brown pretreatment (HIER/protease), hybridization, and staining protocols specifically designed for the RNAscope® 2.5 LSx Brown Kit. Prior to registering the probes or setting up a study, you must change the status of the LSx protocols to **Preferred**.

Change the status of the LSx heat pretreatment protocols

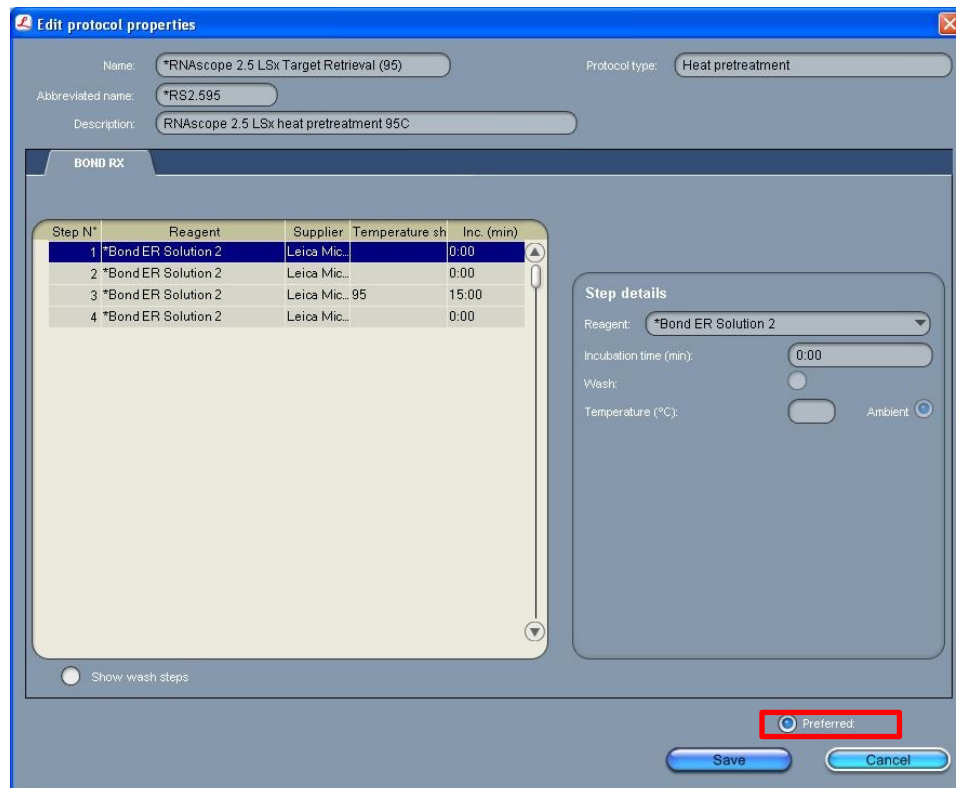
1. Select the **Protocol Setup** icon at the top of the screen.



- On the bottom of the screen, select **Prestaining** under the Protocol group menu, **Heat pretreatment** under the Protocol type menu, and **All** under the Preferred status menu.
- Open the ***RNAscope 2.5 LSx Target Retrieval (95)** protocol.



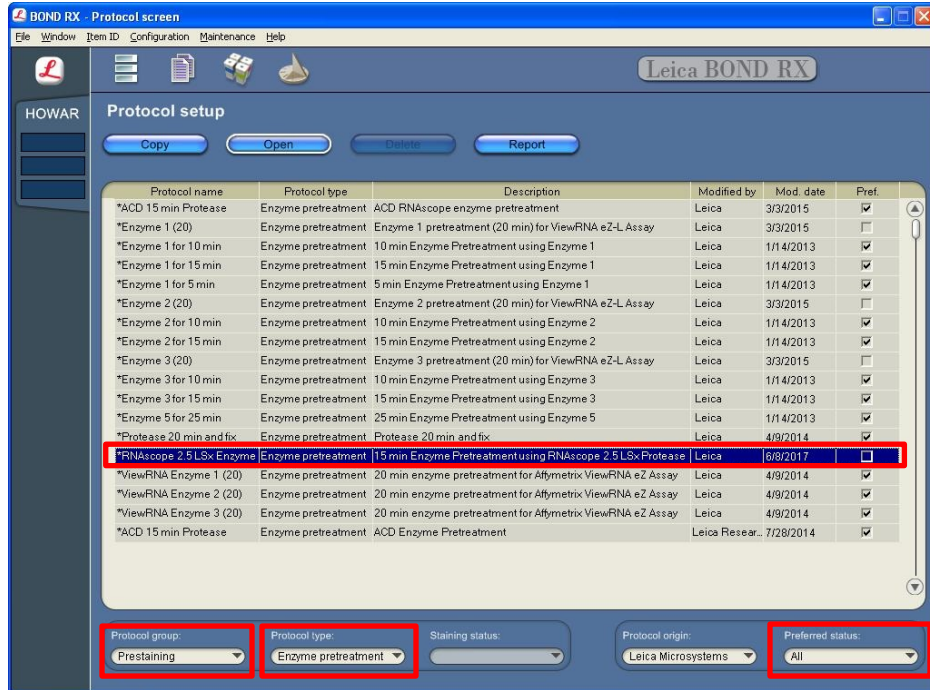
- On the bottom of the screen, select **Preferred**.
- Select **Save**.



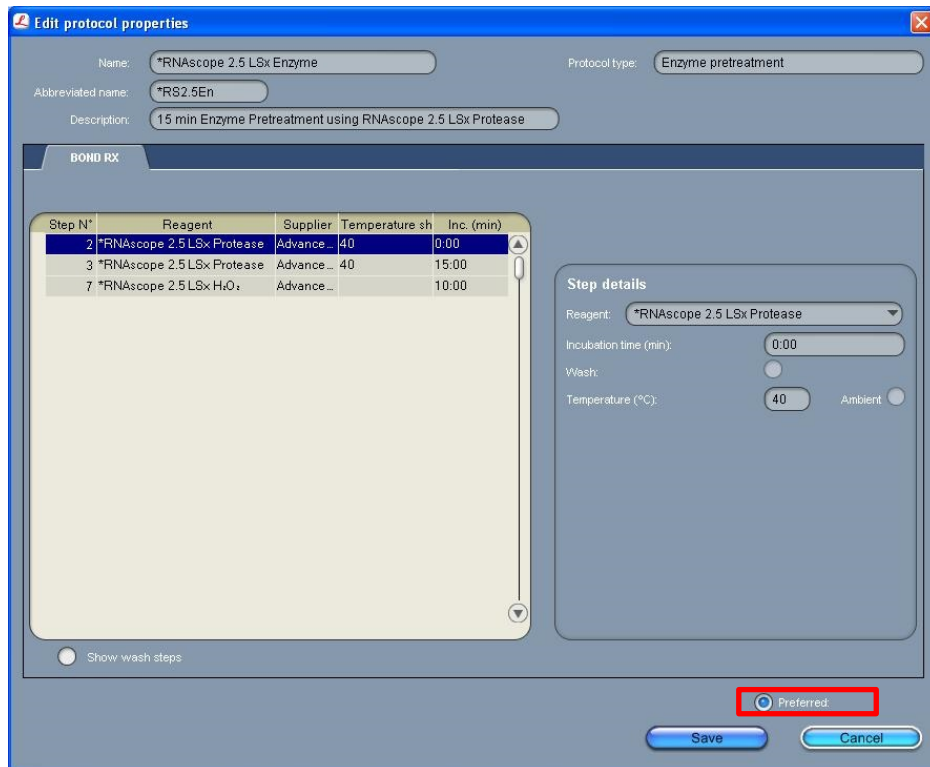
- Repeat steps 2–5 for the ***RNAscope 2.5 LSx Target Retrieval (88)** protocol.

Change the status of the LSx enzyme protocol

1. Select the **Protocol Setup** icon at the top of the screen.
2. On the bottom of the screen, select **Prestaining** under the Protocol group menu, **Enzyme pretreatment** under the Protocol type menu, and **All** under the Preferred status menu.
3. Open the ***RNAscope 2.5 LSx Enzyme** protocol.



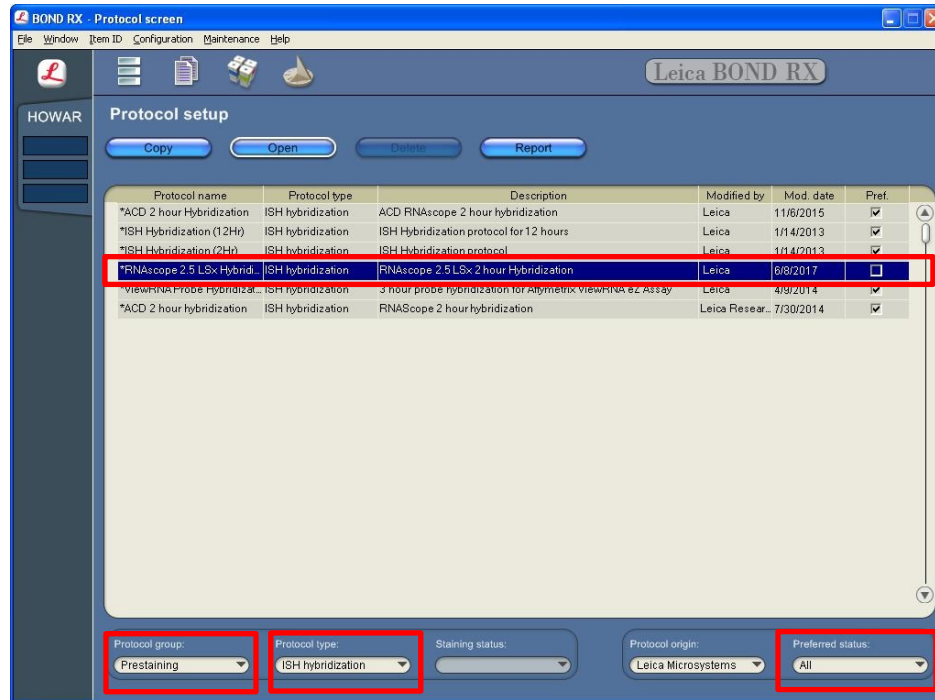
4. On the bottom of the screen, select **Preferred**.



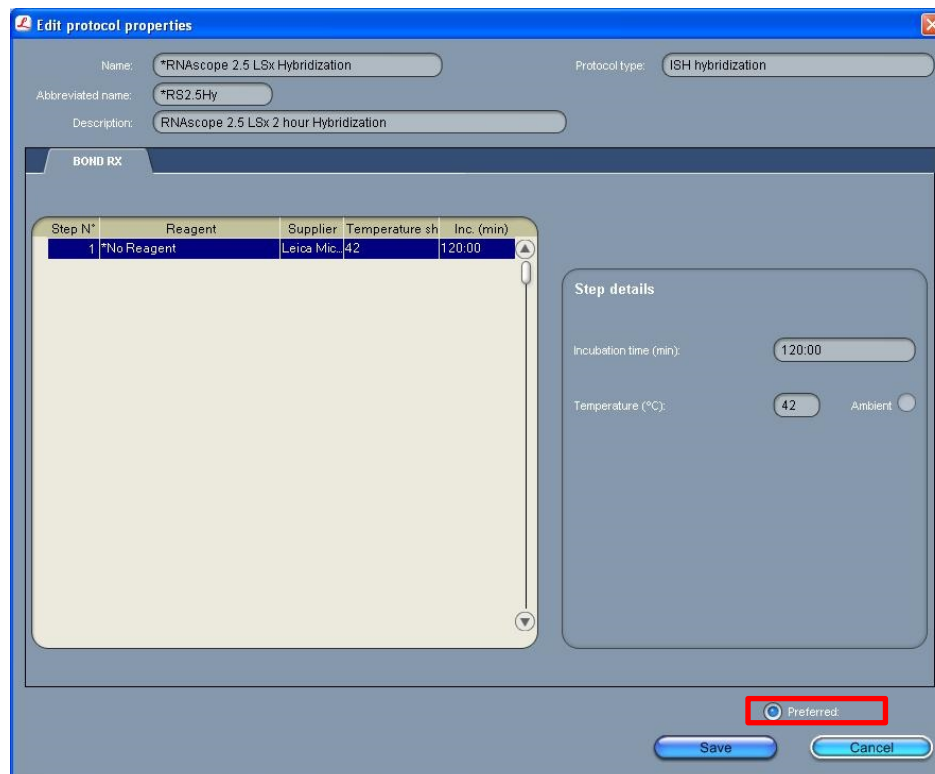
5. Select **Save**.

Change the status of the LSx hybridization protocol

1. Select the **Protocol Setup** icon at the top of the screen
2. On the bottom of the screen, select **Prestaining** under the Protocol group menu, **ISH hybridization** under the Protocol type menu, and **All** under the Preferred status menu.
3. Open the ***RNAscope 2.5 LSx Hybridization** protocol



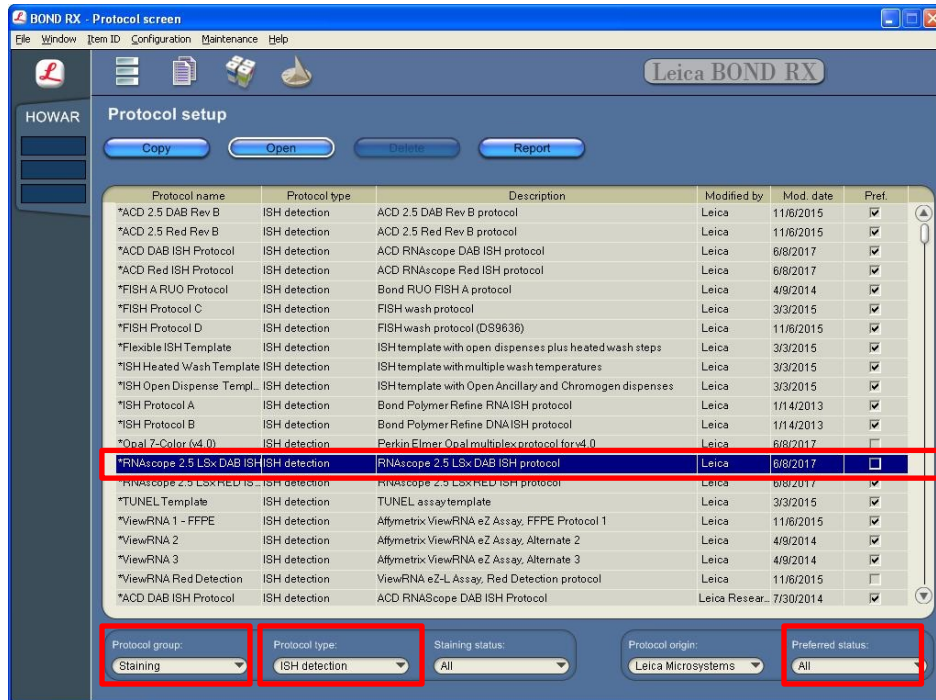
4. On the bottom of the screen, select **Preferred**.



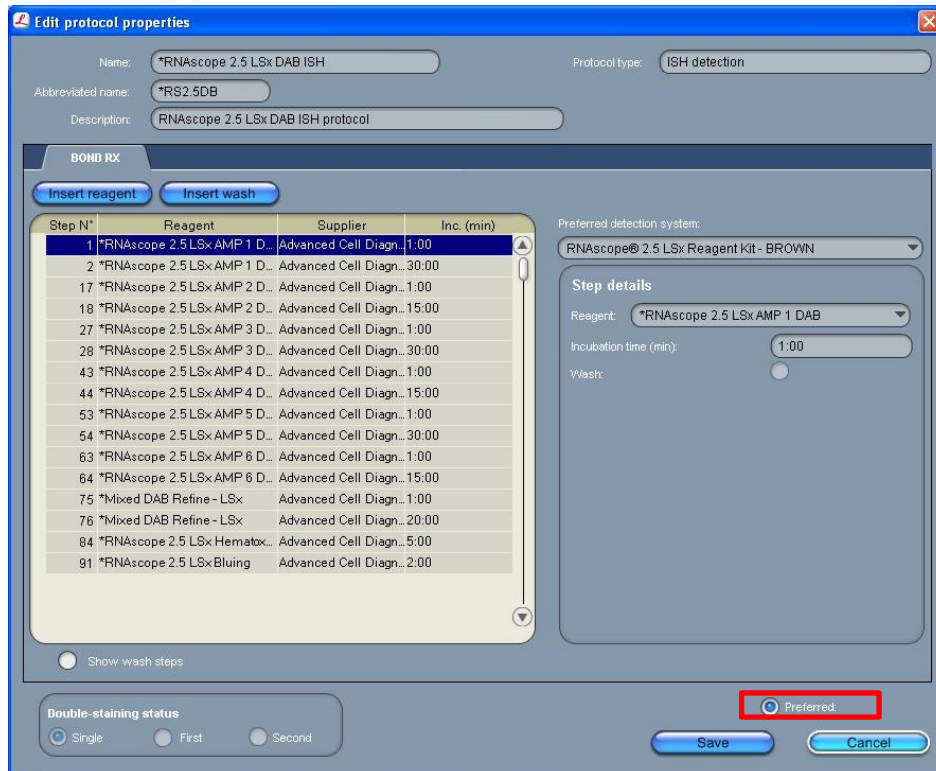
5. Select **Save**.

Change the status of LSx Brown Staining Protocol

1. Select the **Protocol Setup** icon at the top of the screen.
2. On the bottom of the screen, select **Staining** under the Protocol group menu, **ISH detection** under the Protocol type menu, and **All** under the Preferred status menu.
3. Open the ***RNAscope 2.5 LSx DAB ISH** protocol.



4. On the bottom of the screen, select **Preferred**.



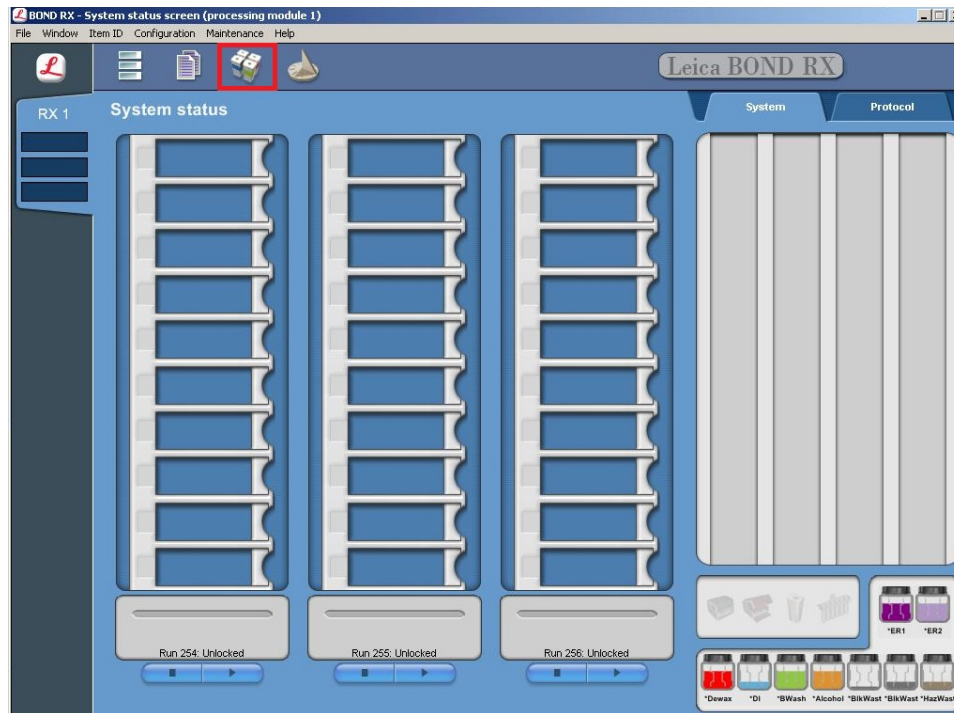
5. Select **Save**.

Register probes

Database version BDZ 11 required work around steps to accommodate the RNAscope® 2.5 LS Brown Assay. These steps included the probe as an ancillary reagent to the staining protocol, and created a mock probe hybridization step. For database version BDZ 15/BXD15, each probe is registered as a “probe” that is used as a marker for the ISH procedure. The staining protocol no longer contains the probe and starts from the AMP 1 step. A mock probe is no longer required to set up the assay.

Your ACD Field Application Specialist (FAS) will assist you with this procedure.

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



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

- To create a probe, enter a name (for example, **probe 1**) in the Name text box.

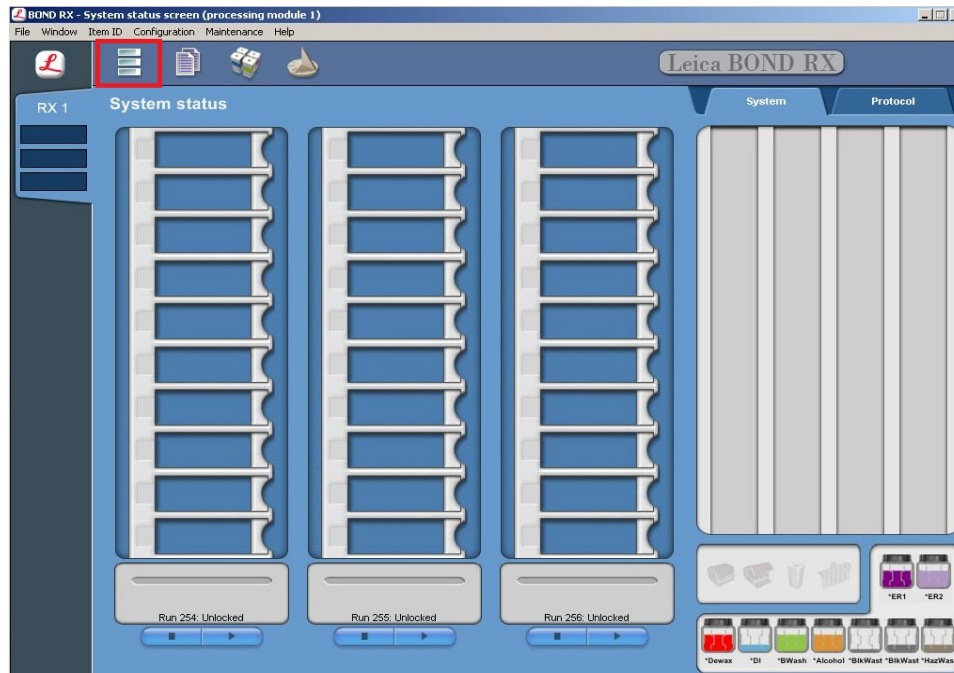
Note: Probe names must be different from the names used for **Ancillary** probes created for the 2.5 LS workaround protocols used in software version BDZ 11.

- Enter **Probe 1** (for example) in the Abbreviated name text box.
- Select **Probe** in the Type drop down menu for software version 4.0. Select **Probe RNA** in the Type drop down menu for software version 5.2.
- Enter **ACD** in the Supplier text box.
- For software version 4.0, select **RNA** for Probe Type.
- Select the default protocols for the probe you are registering. These are the most commonly used protocols.
- Select **Hazardous**.
- Select **Save**.

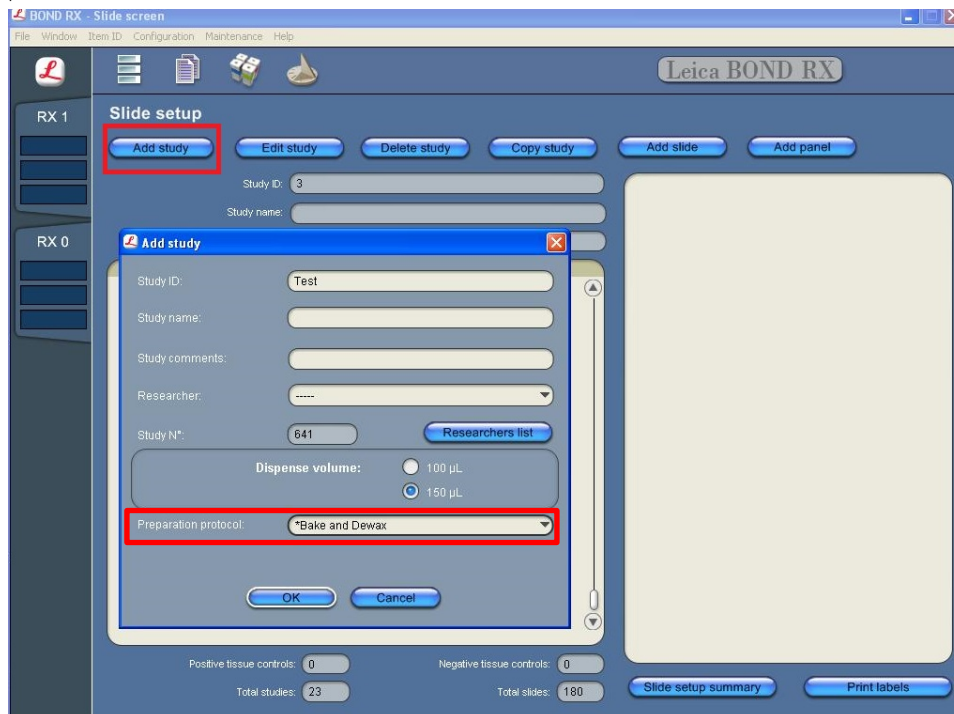
Note: Perform these steps only once for each target probe.

Set up a study

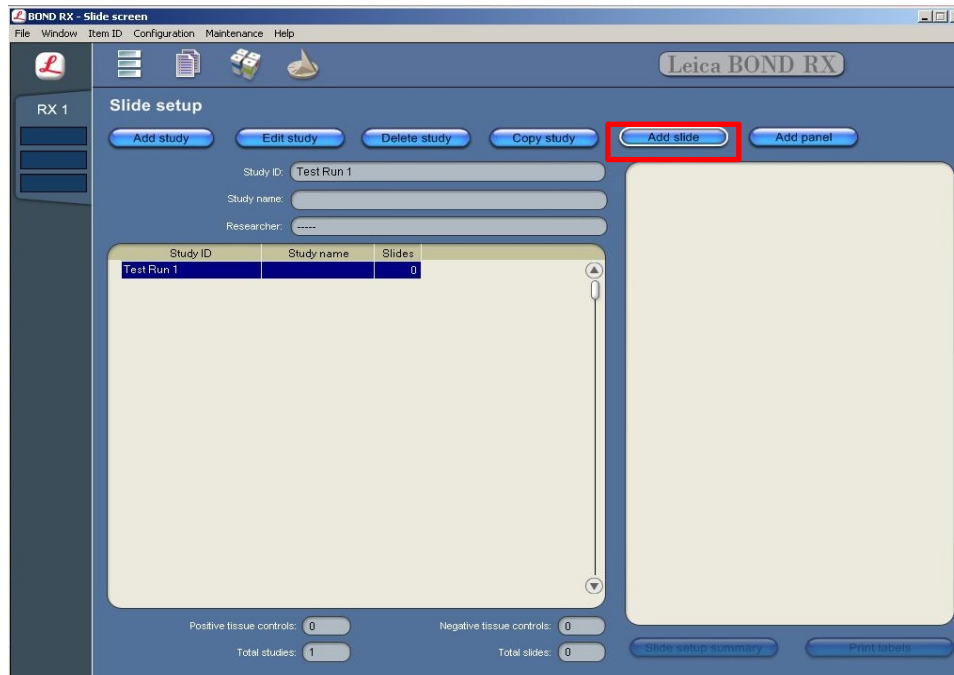
1. To build a study, select the **Slide setup** icon at the top of the screen.



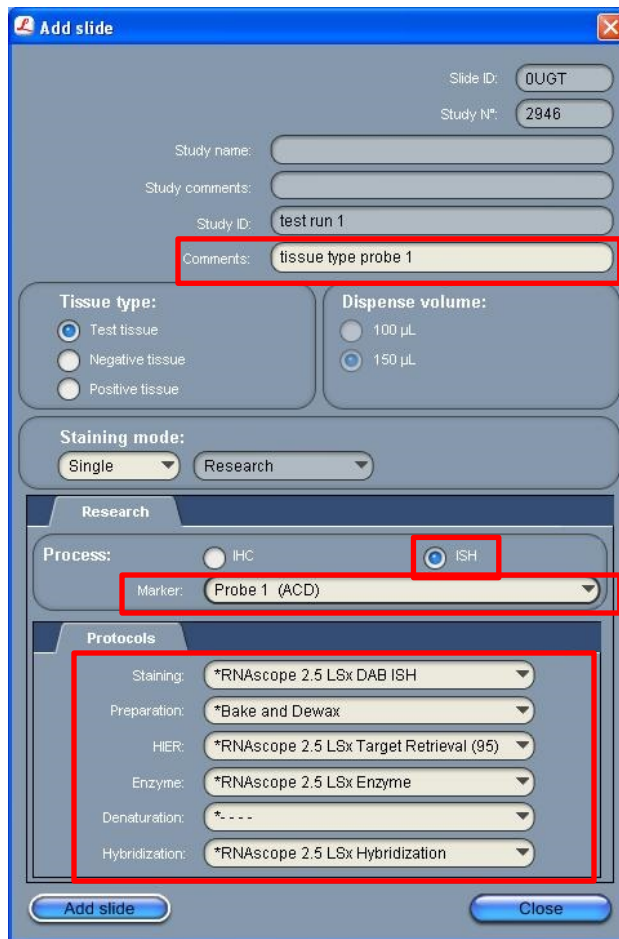
2. Select **Add study** and enter a name in the **Study ID** field (keep the Dispense volume default as shown at 150 μ L).



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

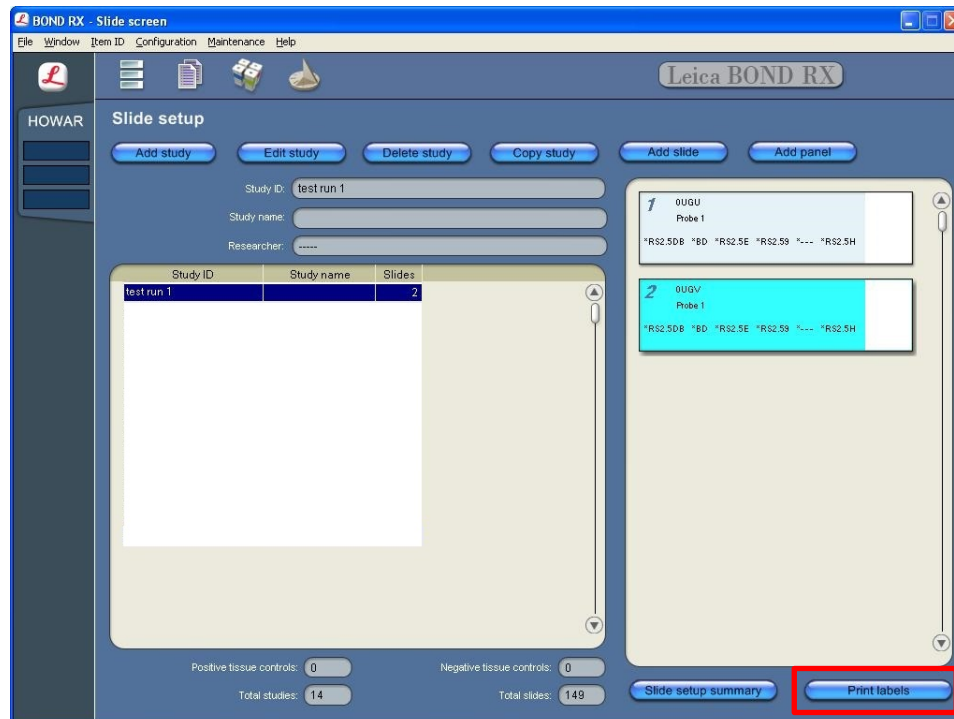


6. Enter the tissue type and/or probe name under the Comments field.



7. In the Research tab, select **ISH** under Process, and the registered probe name from the Marker drop down menu.

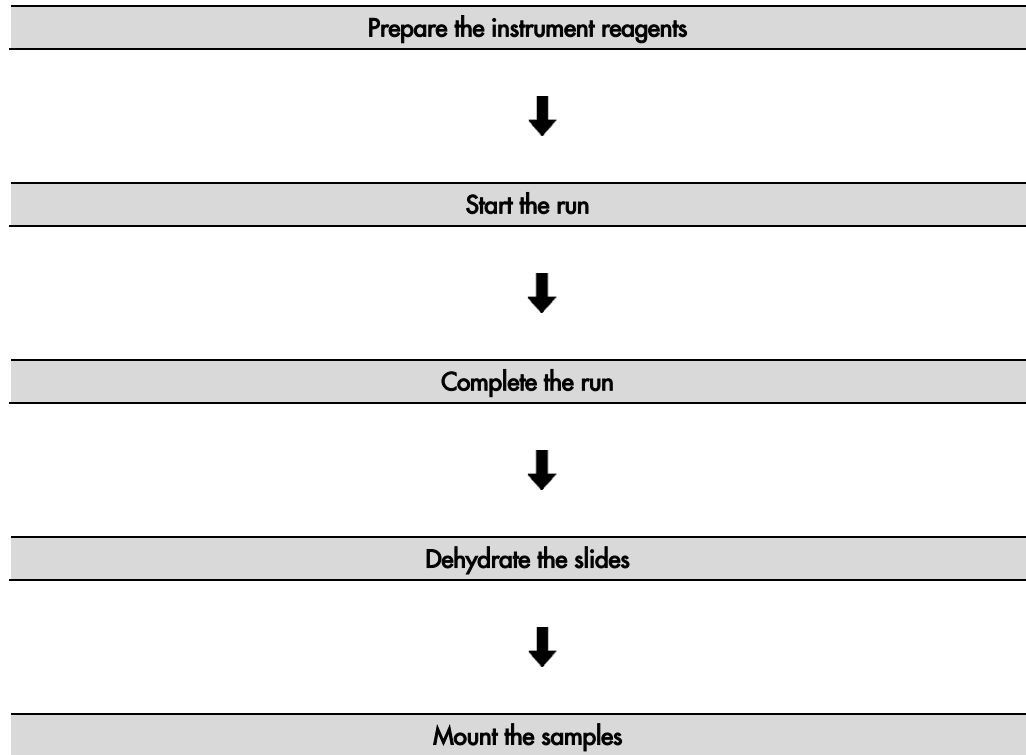
8. For RNAscope® 2.5 LSx assays, under the **Protocols** tab:
 - a. Select **RNAscope 2.5 LSx DAB ISH** protocol from the Staining drop down menu.
 - b. For standard FFPE tissues, select the protocol ***Bake and Dewax** from the Preparation drop down menu.
 - c. Select ***RNAscope 2.5 LSx Target Retrieval (95)** as the standard HIER protocol, or **RNAscope 2.5 LSx Target Retrieval (88)** if using mild conditions.
 - d. Select ***RNAscope 2.5 LSx Enzyme** for Enzyme.
 - e. Select ***RNAscope 2.5 LSx 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.



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Chapter 5. Run the RNAscope® 2.5 LS Assay

Workflow



Materials required

Materials provided by Advanced Cell Diagnostics	Materials provided by Leica Biosystems	Materials provided by User
<ul style="list-style-type: none"> • RNAscope® 2.5 LS Target Probe • RNAscope® 2.5 LS Positive Control Probe • RNAscope® 2.5 LS Negative Control Probe • RNAscope® 2.5 LSx Rinse • RNAscope® 2.5 LSx Hematoxylin • RNAscope® 2.5 LSx DAB Part 1 • RNAscope® 2.5 LSx DAB Part B • RNAscope® 2.5 LSx Bluing • RNAscope® 2.5 LSx AMP 1 DAB • RNAscope® 2.5 LSx AMP 2 DAB • RNAscope® 2.5 LSx AMP 3 DAB • RNAscope® 2.5 LSx AMP 4 DAB • RNAscope® 2.5 LSx AMP 5 DAB • RNAscope® 2.5 LSx AMP 6 DAB • RNAscope® 2.5 LS H2O2 • RNAscope® 2.5 LSx Protease 	<ul style="list-style-type: none"> • Leica Biosystems' BOND RX System • BOND 30 mL Open containers • BOND Universal Covertiles • BOND Aspirating Probe Cleaning System • BOND Mixing Stations <p>LS Bulk Reagents</p> <ul style="list-style-type: none"> • BOND Wash Solution, 10X • BOND Dewax Solution • BOND Epitope Retrieval Solution 1 • BOND Epitope Retrieval Solution 2 Reagents 	<ul style="list-style-type: none"> • Distilled water • Conical tube, 50 mL • 95% Ethanol (EtOH) • Drying oven

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. Air dry before reuse.
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. Use the Barcode Scanner to scan both tray barcodes on the labeled tray. A window will appear on the instrument containing the name and information of the detection system, including the configurations of all the reagents on the tray.
2. Select **OK**.
3. For the reagents in the second tray, use the Barcode Scanner to scan the barcode on the front of each container. A window will appear on the instrument containing the name and information for each reagent.
4. Select **OK**.
5. Empty each target probe into a new 30mL Open container.
6. Use the Barcode Scanner to scan the barcode on the front of the 30mL Open container.

7. From the drop down menu, select the corresponding name of the probe.
8. Enter the probe lot number and the expiration date in their respective fields. Select **OK**.

IMPORTANT! Do not introduce bubbles into the solutions by shaking the containers. If bubbles are present, leave the containers out at room temperature until the bubbles dissipate.

Start the run

1. Attach the barcodes 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 the placement and seating of the covertile.
2. Place the tray in the Leica BOND RX™ and press the button to load the tray onto the machine.
3. After scanning the slides, select the **PLAY** (triangular) button on the screen located under the start tray to start the run. You may also right-click on the 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.

Note: You may also use an automated coverslipper in the following sections.

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 a staining dish containing 95% 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 slide rack into a clearing agent dish containing xylene for **1 MIN** with occasional agitation.
5. Move the Tissue-Tek® Slide rack into a second clearing agent dish containing xylene for **1 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	4–10 dots/cell. No or very few dot clusters (visible at 20–40X magnification)
3	>10 dots/cell. Less than 10% positive cells have dot clusters (visible at 20X magnification)
4	>10 dots/cell. More than 10% positive cells have dot clusters (visible at 20X magnification)

* Discount cells with artificially high nuclear background staining.

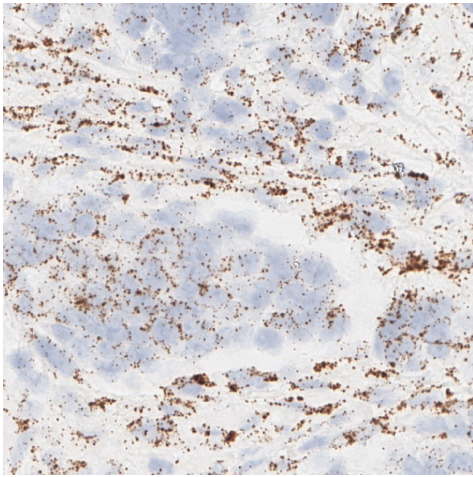
Quantitative image analysis

RNAscope® Spot Studio Software is designed for pathologists with no prior training in image analysis. This intuitive software allows users to get statistical results with complete information of cell-count/region and number of spots/cell. Simply load any image, select a region of interest, define settings, and run the analysis, followed by a quality control review before results are exported.

Control example

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

Figure 2. RNAscope® 2.5 LSx Assay detection of PPIB mRNA in colon FFPE tissue



Troubleshooting

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

- If using over-fixed tissues, increase the Epitope Retrieval 2 (ER2) in increments of five minutes and increase the Enzyme time in increments of five minutes (see **Appendix B. Edit the Protease Protocol** on page 35).
- Do not attempt to refill the pre-filled containers. The 2.5 LSx-BROWN Reagent Kit contains all necessary reagents including chromogen to run the RNAscope® assay, except for probes. The volumes in the pre-filled containers match the barcodes.
- The BOND Polymer Refine Detection reagent is no longer required.
- Pre-filled containers are locked in the trays. Changing the positions of the containers will cause the kit to malfunction.
- 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 are optimized to run the RNAscope® assay on the instrument.

For troubleshooting information, please contact technical support at support@acdbio.com.

3. Change the protocol name, the abbreviated name, and the description to reflect any changes made to the protocol.

New protocol properties

Name: RNAscope 2.5 LSx DAB ISH no bluing
Abbreviated name: LSxBnoBI
Description: RNAscope 2.5 LSx DAB ISH protocol no bluing
Protocol type: ISH detection

BOND RX

Insert reagent Insert wash Duplicate Delete duplicate Import...

Step N*	Reagent	Supplier	Inc. (min)
1	*RNAscope 2.5 LSx AMP 1 D...	Advanced Cell Diagn.	1:00
2	*RNAscope 2.5 LSx AMP 1 D...	Advanced Cell Diagn.	30:00
3	*Bond Wash Solution	Leica Microsystems	0:00
4	*Bond Wash Solution	Leica Microsystems	0:00
5	*Bond Wash Solution	Leica Microsystems	0:00
6	*Bond Wash Solution	Leica Microsystems	3:00
7	*Bond Wash Solution	Leica Microsystems	3:00
8	*Bond Wash Solution	Leica Microsystems	0:00
9	*Bond Wash Solution	Leica Microsystems	0:00
10	*Bond Wash Solution	Leica Microsystems	0:00
11	*RNAscope 2.5 LSx Rinse	Advanced Cell Diagn.	5:00
12	*RNAscope 2.5 LSx Rinse	Advanced Cell Diagn.	5:00
13	*Bond Wash Solution	Leica Microsystems	0:00
14	*Bond Wash Solution	Leica Microsystems	0:00
15	*Bond Wash Solution	Leica Microsystems	0:00
16	*Bond Wash Solution	Leica Microsystems	0:00
17	*RNAscope 2.5 LSx AMP 2 D...	Advanced Cell Diagn.	1:00
18	*RNAscope 2.5 LSx AMP 2 D...	Advanced Cell Diagn.	15:00
19	*Bond Wash Solution	Leica Microsystems	0:00

Preferred detection system:
RNAscope® 2.5 LSx Reagent Kit - BROWN

Step details

Reagent: *RNAscope 2.5 LSx AMP 1 DAB
Incubation time (min): 1:00
Wash:

Show wash steps

Double-staining status
 Single First Second

Preferred

Save Cancel

4. Highlight and select the Reagent step to edit it.
5. If using software version 4.0, you can change the incubation times, but not the temperature of the steps. If using software version 5.2, you can change both the time and temperature for each step.
6. Click **Show wash steps** to view the washing steps found between each reagent. Make sure that **Preferred** is selected (bottom right corner of the window).
7. Select **Save**.
8. Click **Next** to proceed. Ignore any pop ups that may appear on the screen.

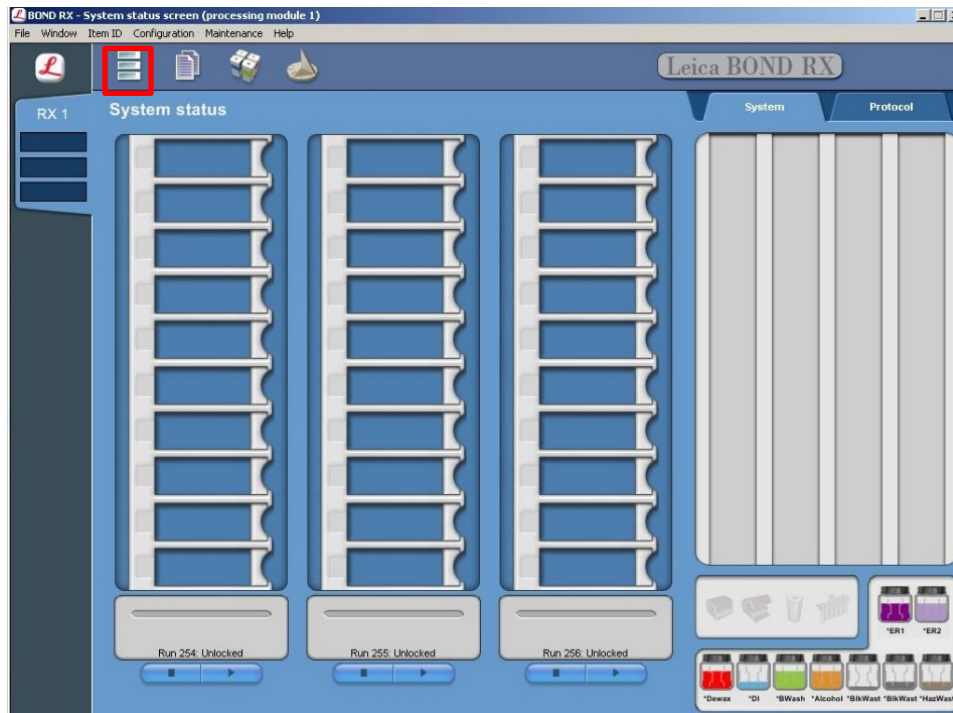
B

Appendix B. Edit the Epitope Retrieval Protocol

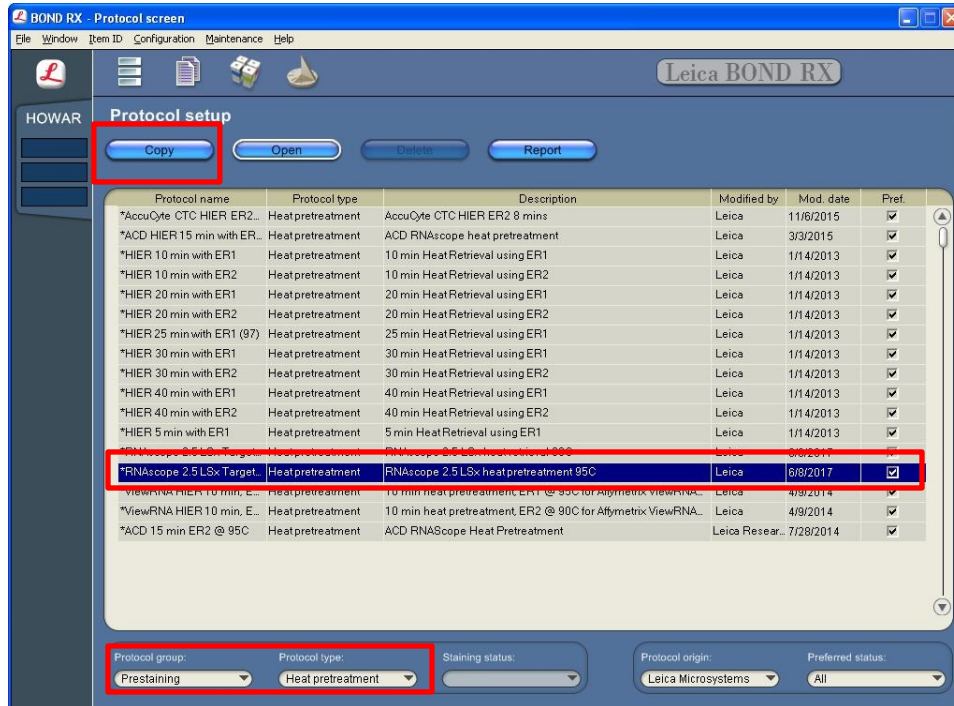
The following example shows how to edit the Epitope Retrieval procedure from within the software.

Create a prestaining protocol

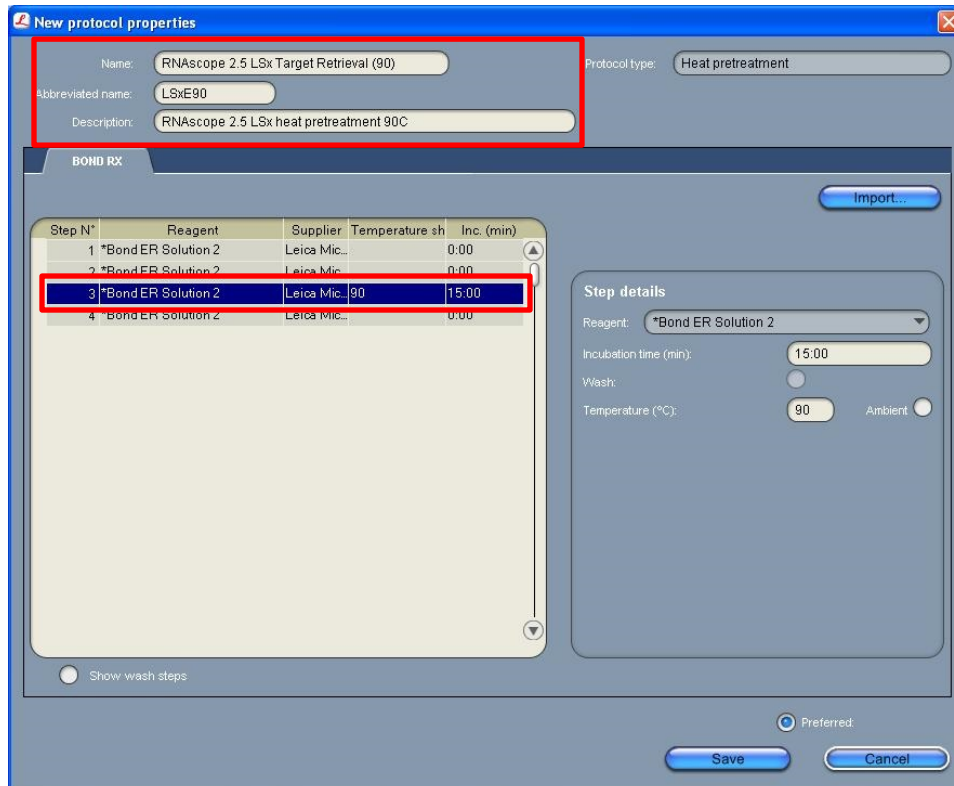
1. Open the Leica BOND software and click on the **Protocol setup** icon as shown.



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



- Highlight the ***RNAscope 2.5 LSx Target Retrieval (95)** protocol. Select **Copy**.
- Rename the protocol as **RNAscope 2.5 LSx Target Retrieval (90)**.
- Change the Abbreviated name to **LSxE90**.
- Change the Description to **RNAscope 2.5 LSx heat pretreatment 90C**.



7. Highlight the third ***Bond ER Solution 2** step. 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

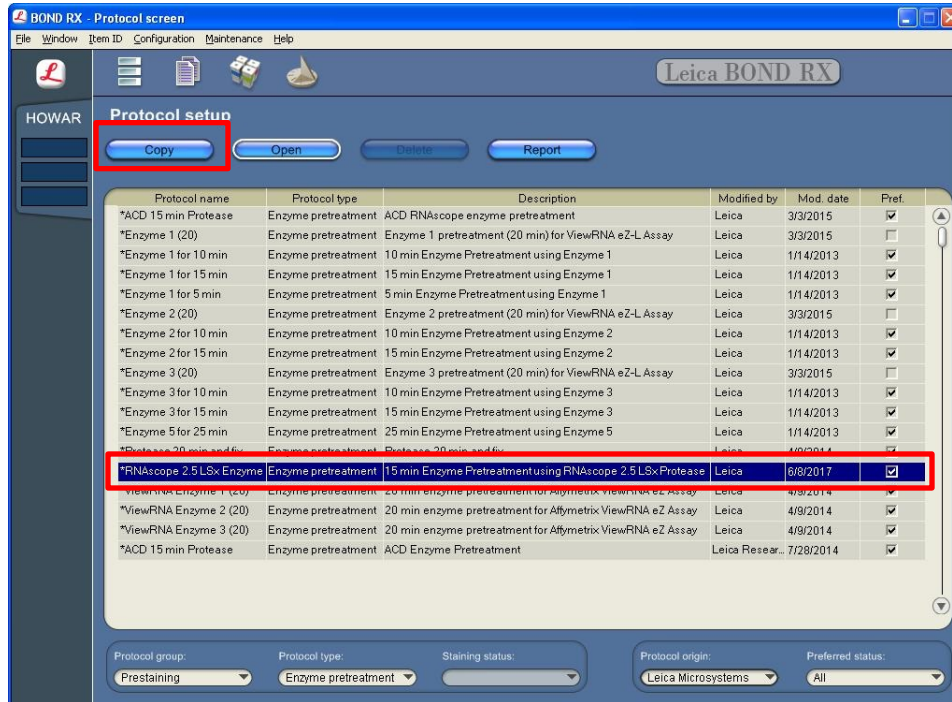
8. Make sure that **Preferred** is selected (bottom right corner of window).
9. Select **Save**.
10. If needed, repeat Steps 1–8 to create a new heating protocol (for example, RNAscope 2.5 LSx Target Retrieval 25min).



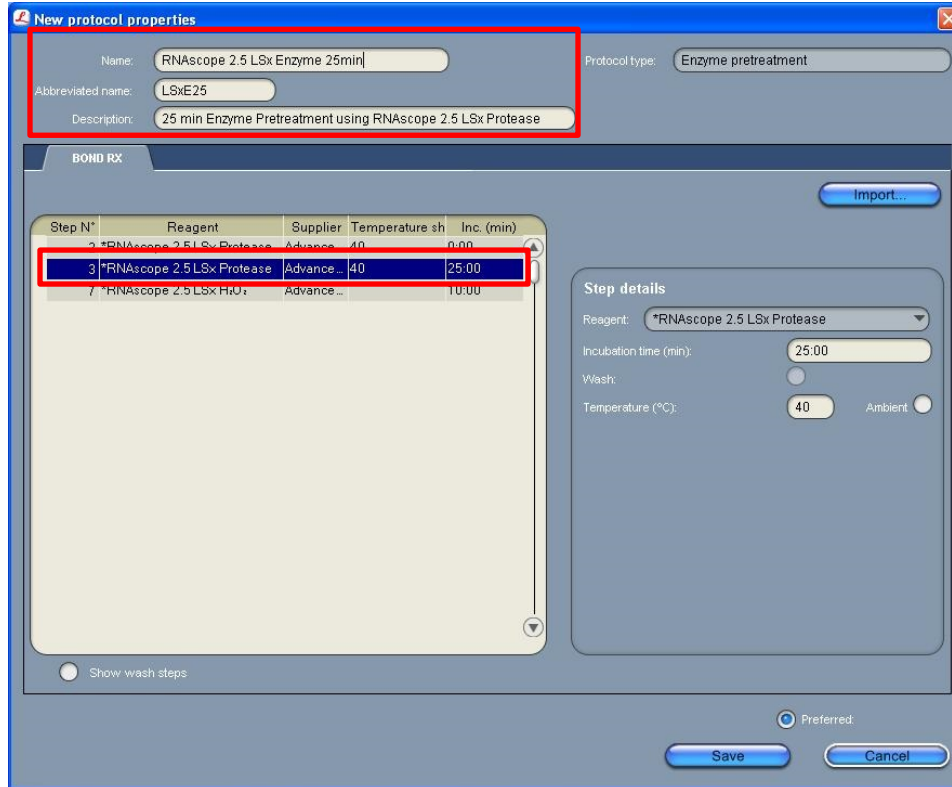
Appendix C. Edit the Protease Protocol

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 ***RNAscope 2.5 LSx Enzyme** protocol. Select **Copy**.



- Rename the protocol to **RNAscope 2.5 LSx Enzyme 25min**.
- Rename the Abbreviated name to **LSxE25**.
- Change the Description to **25 min Enzyme Pretreatment using RNAscope 2.5 LSx Protease**.



- Highlight the second ***RNAscope 2.5 LSx Protease** step. Keep the temperature at **40°C**, and set the enzyme incubation time to **25 MIN**.
- Make sure that **Preferred** is selected (bottom right corner of window).
- Select **Save**.
- If needed, repeat Steps 1–7 to create a new protease protocol for different sample types (for example, RNAscope 2.5 LSx Protease 10min or RNAscope 2.5 LSx Protease 1.5min at ambient temperature).
- For the following tissue types, we recommend protease conditions of **15 MIN** at **40°C**:

Tissue Type (continued)
Brain and spinal cord
Breast cancer
Cell pellet
Colon
GI tract
Head and neck cancer
Heart
Kidney
Liver
Lung
Lymphoma
Placenta

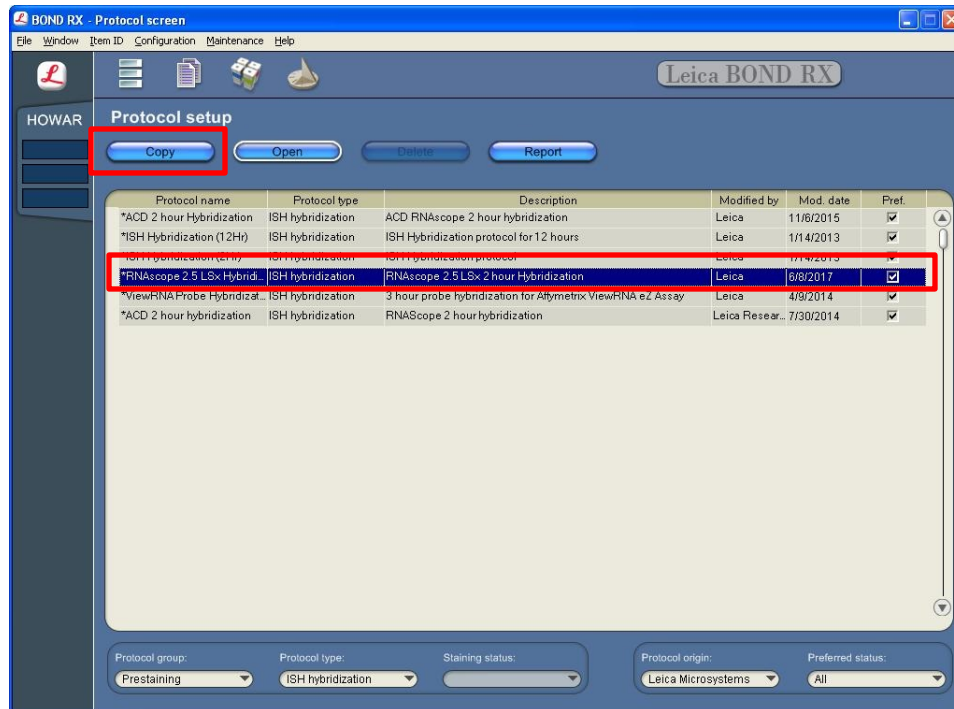
Tissue Type (continued)
Prostate
Skin
Stomach
Thymus
Tonsil
Xenograft



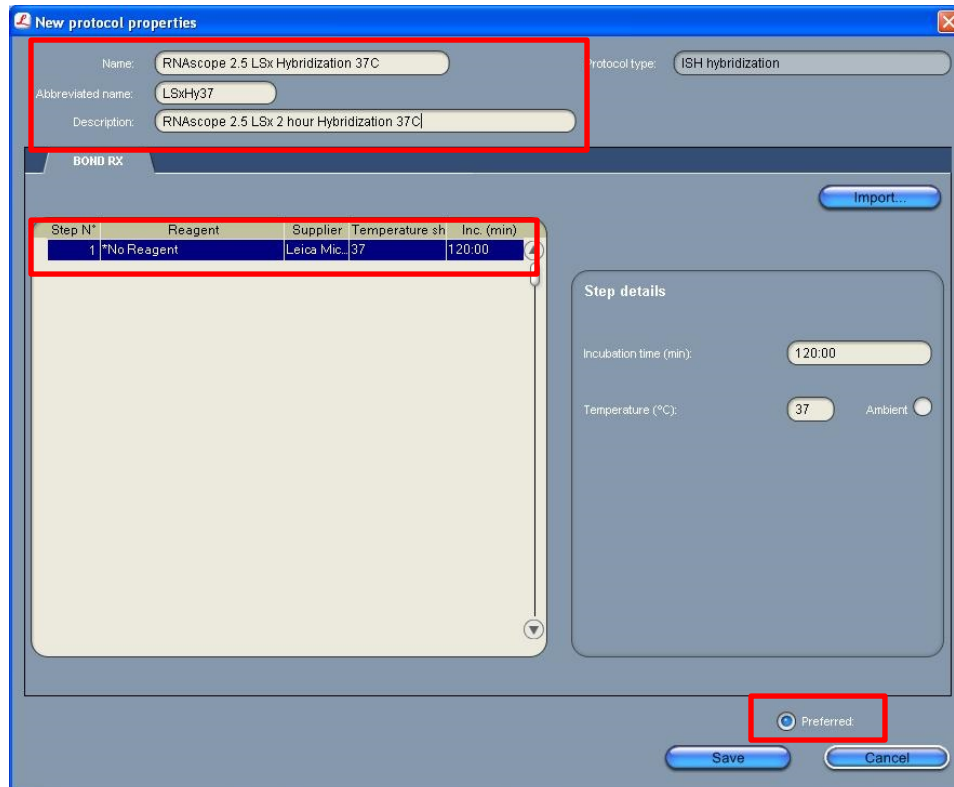
Appendix D. Edit the Hybridization Protocol

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

1. Select **ISH Hybridization** under the Protocol type menu (bottom left).
2. Highlight the ***RNAscope 2.5 LSx Hybridization** protocol. Select **Copy**.



3. Rename the protocol to **RNAscope 2.5 LSx Hybridization 37C**.
4. Rename the Abbreviated name to **LSxHy37**.
5. Change the description to **RNAscope 2.5 LSx 2 hour Hybridization 37C**.
6. Highlight the ***No Reagent** step. Keep the time at 120.00 min and change the temperature to 37°C.
7. Make sure that **Preferred** is selected (bottom right corner of window).
8. Select **Save**.
9. If needed, repeat Steps 1– 7 to create a new hybridization protocol (for example, 2.5 LSx Hybridization 4hr).





Appendix E. Safety

Chemical safety



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

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

Biological hazard safety



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

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: www.cdc.gov/biosafety
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§ 1910.1030), found at: www.access.gpo.gov/nara/cfr/waisidx_01/%2029cfr1910a_01.html
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.
- Additional information about biohazard guidelines is available at: www.cdc.gov

In the EU:

- Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition, found at:
http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/
- Information about the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) can be found at: **<http://echa.europa.eu/regulations/reach>**

Documentation and Support

Obtaining SDSs

Safety Data Sheets (SDSs) are available at: <https://acdbio.com/technical-support/product-documents>. 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.

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Newark, CA 94545

Toll Free: 1-877-576-3636

Direct: 1-510-576-8800

Fax: 1-510-576-8801

Information: info@acdbio.com

Orders: orders@acdbio.com

Support Email: support@acdbio.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

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For support, email support@acdbio.com

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