USER MANUAL

Fully Automated RNAscope® Assay

RNAscope® LS Reagent Kit

For use with Leica Biosystems' BOND RX System

BROWN

Document Number 321038

For Molecular Biology Applications (MBA), not intended for diagnosis. Refer to appropriate regulations.

Rev. Date 20141112

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Citing RNAscope® LS in Publications

When describing a procedure for publication using this product, please refer to it as the RNAscope® LS 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 A. Safety** on page 35 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® LS Reagent Kit for use with Leica Biosystems' BOND RX Research Advanced Staining System. RNAscope® LS Assays are compatible with a variety of sample types.

Product description

Background

The RNAscope® 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® LS Assay allows users to automate the highly sensitive RNAscope® Assay using Leica Biosystems' BOND RX System.

Overview

The RNAscope® LS Assay procedure is illustrated in Figure 1 on page 6 and 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 hybridization to 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.	Probes are hybridized 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® LS Assay requires the RNAscope® LS Probes and the RNAscope® LS Reagents, available from Advanced Cell Diagnostics.

RNAscope® LS Probes

The RNAscope® 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. 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 six months from the shipment date when stored as indicated in the following table:

Target Probes					
☑	**Reagent**	**Cat. No.**	**Content**	**Quantity**	**Storage**
	RNAscope® LS — Target Probe – *[species]*– *[gene]*	Various	Probe targeting specific RNA	11 mL x 1 bottle	4°C
Control Probes					
☑	Reagent	Cat. No.	Content	Quantity	Storage
	RNAscope® LS — Positive Control Probe – *[species]*– PPIB	Various	Probe targeting common housekeeping gene	11 mL x 1 bottle	4°C
	RNAscope® LS — Negative Control Probe – DapB	312037	Probe targeting bacterial gene dapB	11 mL x 1 bottle	4°C

RNAscope® Reagents

The RNAscope® LS Reagent Kit, BROWN (Cat. No. 321100) contains all the reagents needed to run the RNAscope® 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 have a shelf life of six months from the shipment date when stored as indicated in the following table:

RNAscope® LS Reagent Kit–BROWN (Cat. No. 321100)			
☑	Reagent	Quantity	Storage
	LS Pretreat1 – H_2O_2	10 mL x 1 bottle	4°C
	LS Pretreat 3 – Protease	21 mL x 1 bottle	4°C
	LS Amp 1	21 mL x 1 bottle	4°C
	LS Amp 2	21 mL x 1 bottle	4°C
	LS Amp 3	21 mL x 1 bottle	4°C
	LS Amp 4	21 mL x 1 bottle	4°C
	LS Amp 5 BROWN	21 mL x 1 bottle	4°C
	LS Amp 6 BROWN	21 mL x 1 bottle	4°C
	LS 10X Wash Buffer	5 mL x 1 bottle	4°C

IMPORTANT! Do not substitute the reagent components of the RNAscope® Reagent Kit with those of other RNAscope® Reagent Kits, even those having the same name.

Required materials from Leica BOND RX

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

☑	Component	Cat. No.	Storage
	BOND Open Containers 30 mL	Op309700	Room temperature (20–25°C)
	BOND Universal Covertiles 100 pack	S21.2001	Room temperature (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 temperature (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
	100% ethanol (EtOH)	American Master Tech Scientific/MLS*	ALREAGAL
	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 Dish (4 required)	American Master Tech Scientific/MLS	LWT4457EA
	Tissue-Tek® Clearing Agent Dish, xylene resistant (2 required)	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.

2 Chapter 2. Before You Begin

Prior to running the RNAscope® 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® 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 11 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. 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 A. Safety** on page 35 for more information.

3 Chapter 3. Prepare and Pretreat Samples

Formalin-fixed, paraffin-embedded (FFPE) sample preparation and pretreatment are described in the following protocols.

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
- 100% EtOH
- Xylene
- Microtome
- Water bath
- SuperFrost® Plus slides

Fix the sample

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® Assay.

Dehydrate, embed, and cut the sample

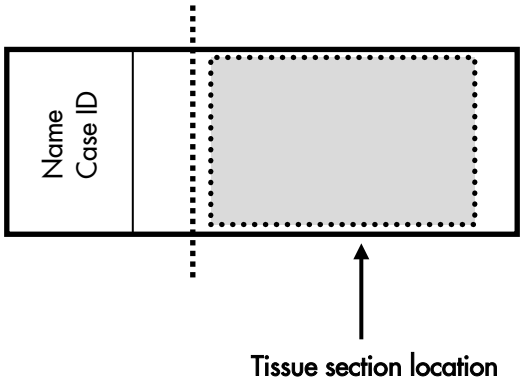
IMPORTANT! Use fresh reagents.

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 **RT** for years.

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:



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

OPTIONAL STOPPING POINT. Use sectioned tissue within 3 months. Store sections with dessicants at **RT**.

4 Chapter 4. Fully Automated RNAscope® LS Assay

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

Workflow

Prepare the materials

⬇

Set up the instrument

⬇

Run the RNAscope® Assay ~9 hours

Prepare the materials

Materials can be prepared ahead of time, unless otherwise stated.

Materials required

Materials provided by Advanced Cell Diagnostics	Materials provided by Leica Biosystems	Materials provided by user
• RNAscope® LS Target Probe • RNAscope® LS Positive Control Probe • RNAscope® LS Negative Control Probe • LS Pretreat 1 • LS Pretreat 3 • LS Amp 1 • LS Amp 2 • LS Amp 3 • LS Amp 4 • LS Amp 5 BROWN • LS Amp 6 BROWN • LS 10X Wash Buffer	**Stainer** • Leica Biosystems' BOND RX System **Bulk Reagents** • BOND Wash Solution 10X • BOND Dewax Solution • BOND Epitope Retrieval Solution 1 • BOND Epitope Retrieval Solution 2 **Reagents** • BOND PolymerRefine Detection (DAB) plus Hematoxylin	• Distilled water • Conical tube 50 mL • Drying oven

Prepare the reagents

- Warm up LS Amp 1, LS Amp 3, LS 10X Wash Buffer, and all LS target probes in a **40°C** oven for **30 MIN** before the run.

  Note: Loss of signal will occur if precipitates do not dissolve.

- Prepare two conical tubes of 30 mL of LS 1X Wash Buffer by adding 27 mL distilled water and 3 mL of LS 10X Wash Buffer to each tube. Mix well by inverting the tubes slowly at least five times. Do not shake the tube.

Prepare the instrument

- Fill the large containers located in the bottom of the instrument with the Leica BOND RX bulk reagents. Dilute BOND Wash Solution 1:10.

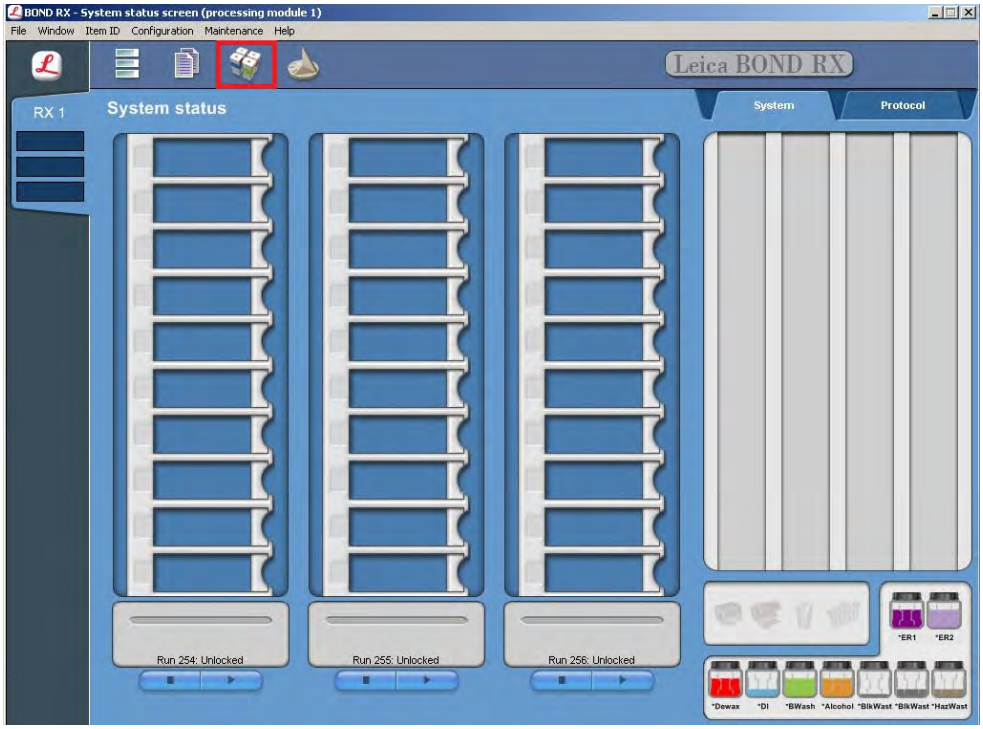
  Note: Insufficient bulk reagent volumes may lead to run failure.

  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.

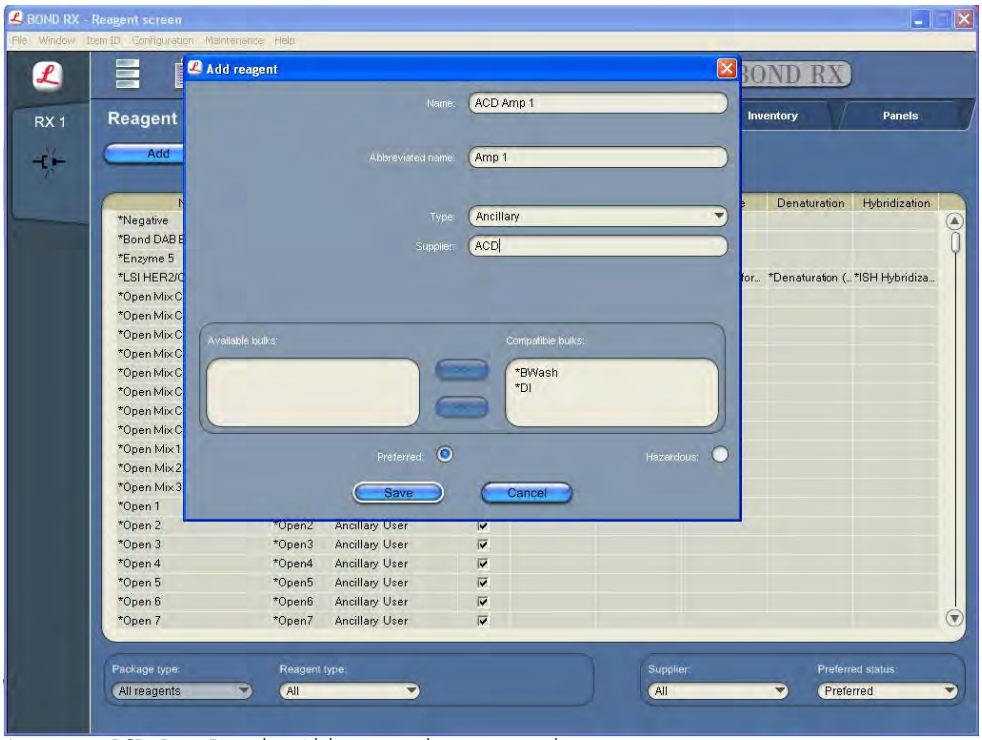
- Use clean, dry covertiles for every run. Clean used covertiles with water, bleach, and ethanol Air dry before reuse.
- Ensure waste bulk containers are emptied before starting a run. Discard waste according to all local, state/provincial, and/or national regulations.

Register the reagents

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



2. Select **Add** to enter reagent information.
3. Enter **ACD Amp 1** in the Name text box.



4. Enter **ACD Amp 1** in the Abbreviated name text box.
5. Select **Ancillary** in the Type drop-down menu.
6. Enter **ACD** in the Supplier text box.
7. Select **Save**.
8. Repeat steps 2–7 for Amp 2 – Amp 6 Brown and 1X LS Wash Buffer.

Note: Do not add reagents for LS Pretreat 1 and LS Pretreat 3. They will be directly scanned and registered as ***Open 0 Haz** and ***Enzyme 1**, respectively. See **Prepare instrument reagents**.

Prepare instrument reagents

Fill the Leica BOND RX containers with the appropriate reagents from the RNAscope® LS Reagent Kit according to the following table:

Reagents	Container Name
LS Pretreat 1	*Open 0 Haz
LS Pretreat 3	*Enzyme 1
LS Amp 1	ACD Amp 1
LS Amp 2	ACD Amp 2
LS Amp 3	ACD Amp 3
LS Amp 4	ACD Amp 4
LS Amp 5	ACD Amp 5 Brown
LS Amp 6	ACD Amp 6 Brown
LS 1X Wash Buffer	ACD Wash Buffer
LS Target Probe	Variable

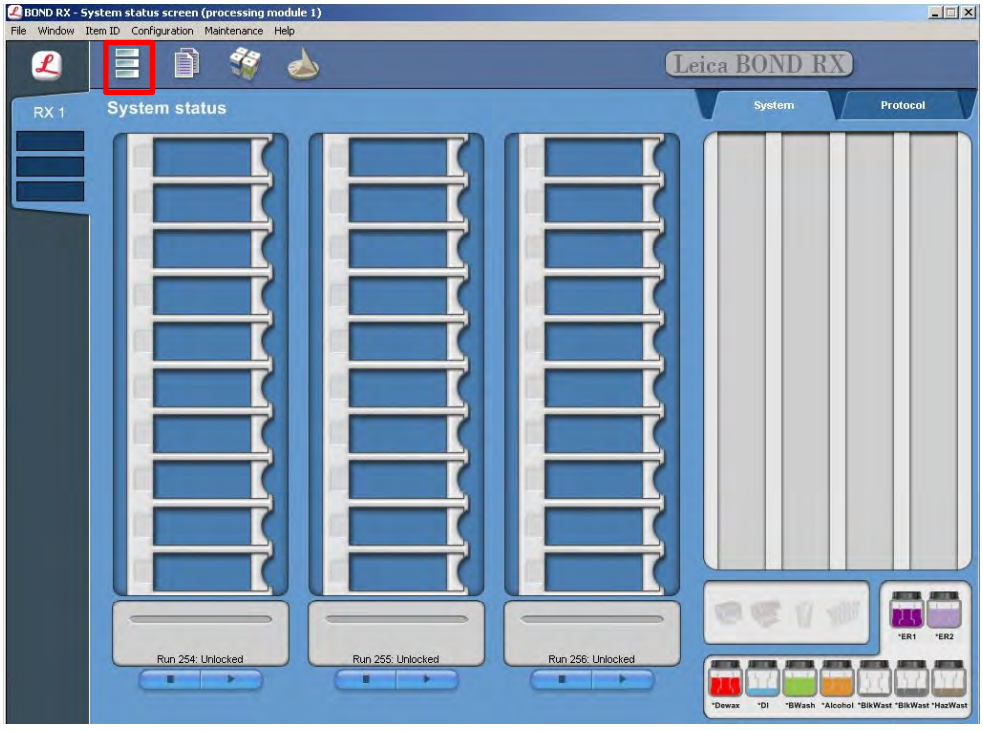
Note: Leica BOND DAB and Hematoxylin come in pre-filled Leica BOND RX containers.

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.

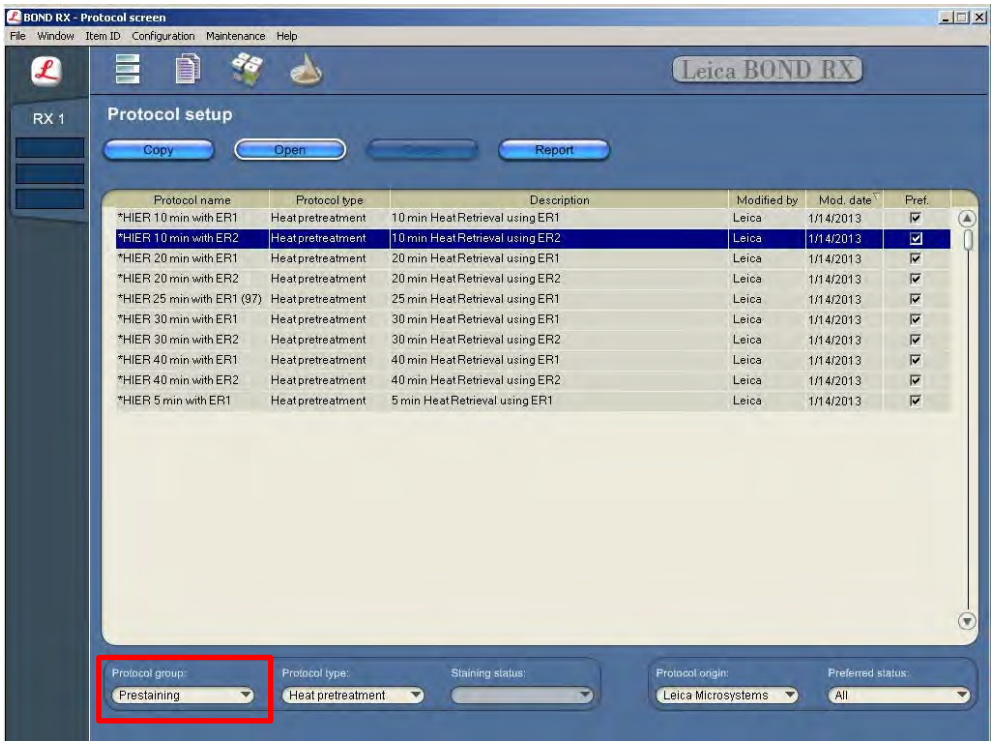
Set up the instrument

Create a prestaining protocol

1. Open the instrument software (version BDZ 6.0 or higher) and click on the **Protocol setup** icon as shown.

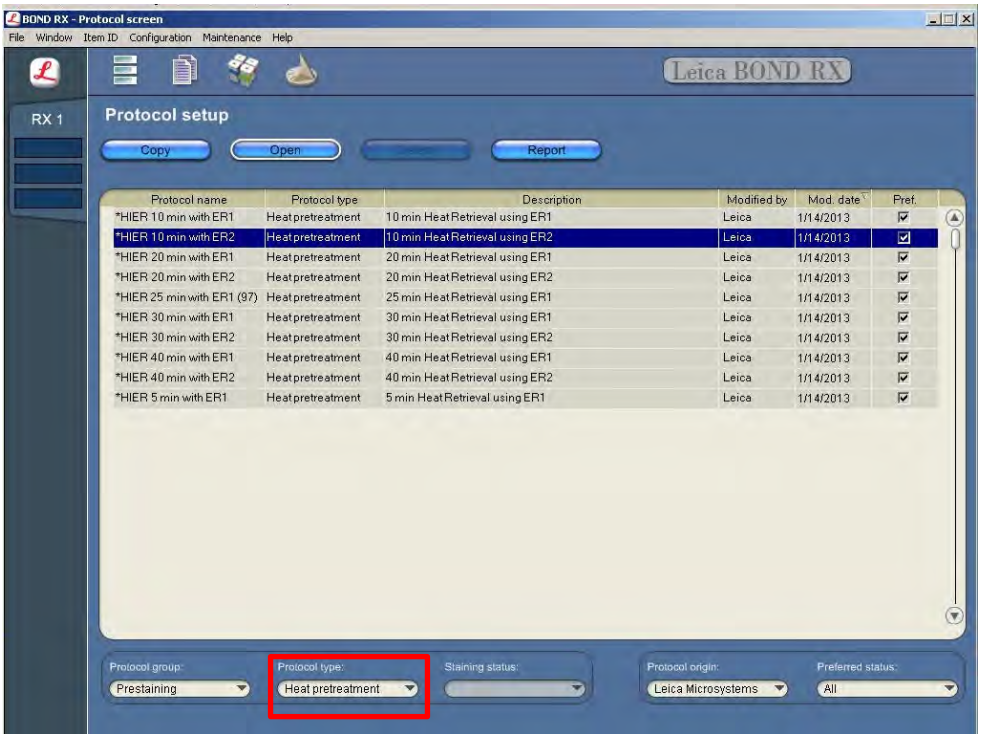


2. Select **Prestaining** under the Protocol group menu located in the bottom left corner of the screen to access the Enzyme Pretreatment, Heat pretreatment, and ISH Hybridization protocols.

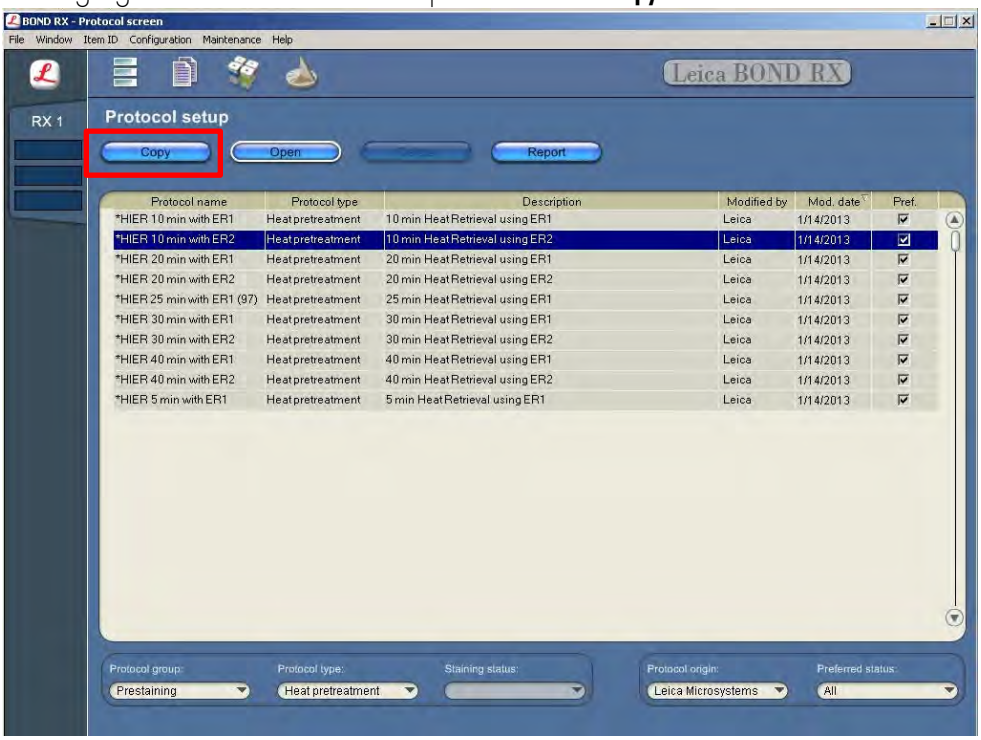


Antigen Retrieval

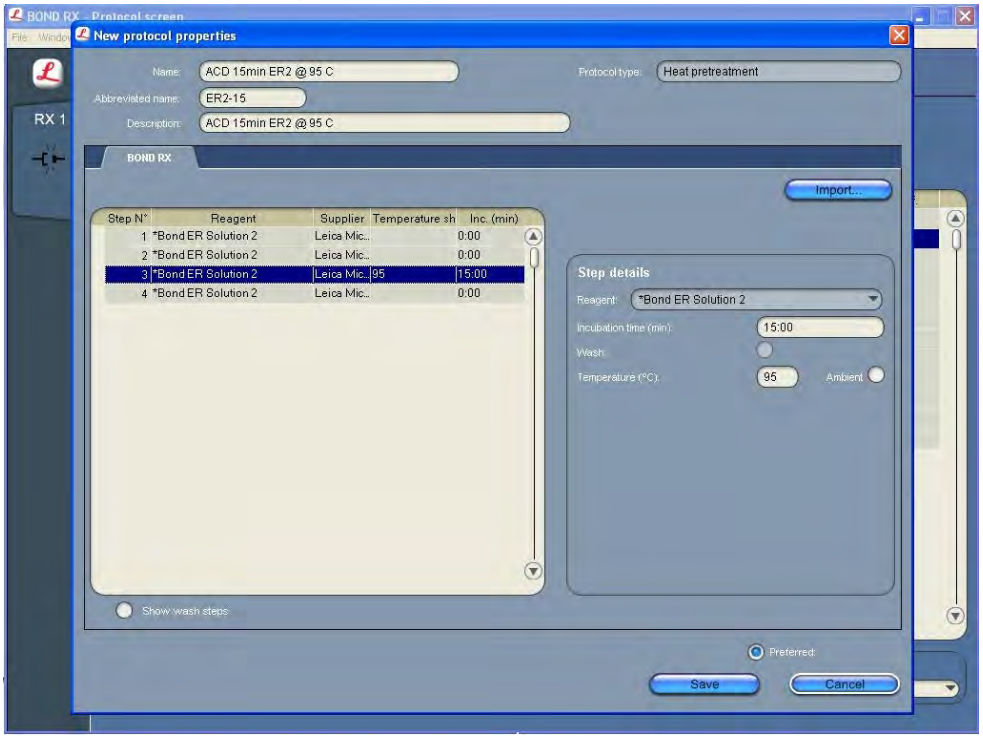
1. Under the Protocol type menu select **Heat pretreatment**.



2. Highlight the ***HIER 10 min with ER2** protocol. Select **Copy**.



3. Rename the protocol (e.g. **ACD 15 min ER2 @ 95°C**). Change the Abbreviated name (e.g. **ER2-15)** and the Description (e.g. **ACD 15 min ER2 @ 95°C**).



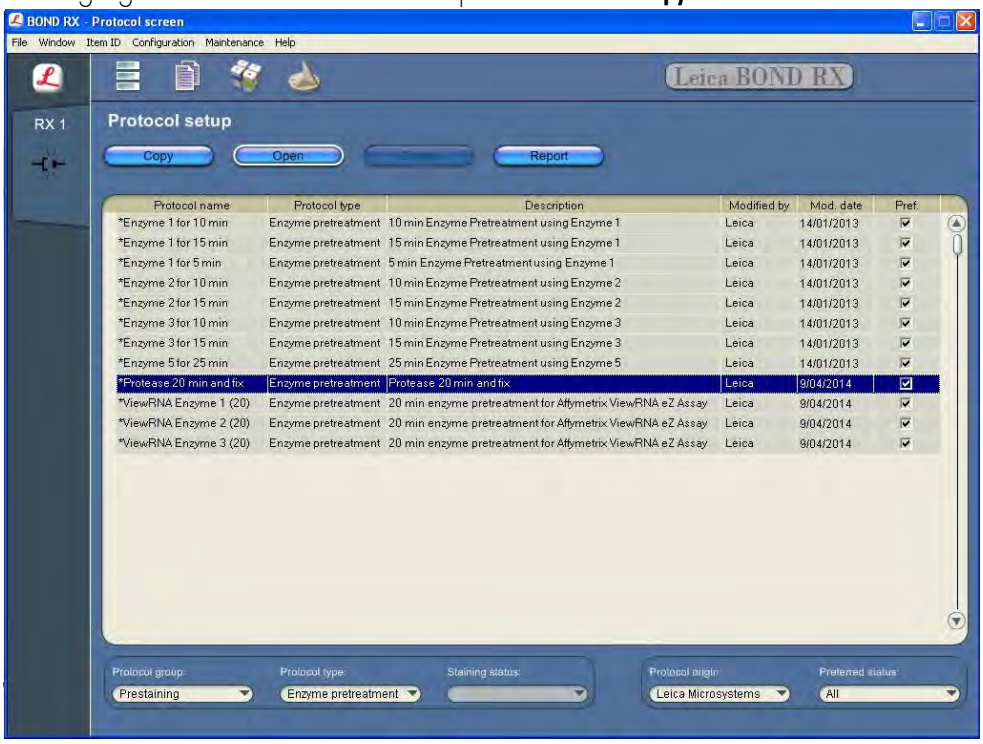
4. Highlight the third ***Bond ER Solution 2** step. Change the temperature to **95°C** and incubation time according to the following table:

Tissue Type	ER2 Incubation Time	Temperature
Brain and spinal cord	15 MIN	95°C
Breast cancer	15 MIN	95°C
Cell pellet	15 MIN	95°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	20 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	95°C
Tonsil	15 MIN	95°C
Xenograft	15 MIN	95°C

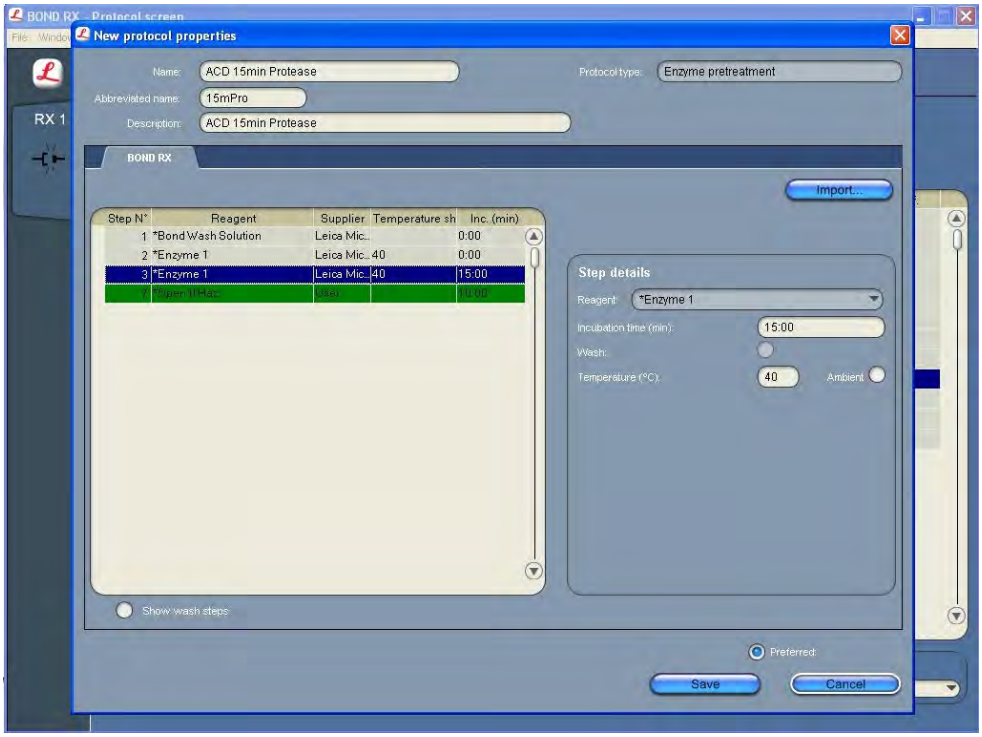
5. Select **Save**.

Protease and H_2O_2 Treatment

1. Under the Protocol type menu select **Enzyme Pretreatment**.
2. Highlight the ***Protease 20 min and fix** protocol. Select **Copy**.



3. Rename the protocol (e.g. **ACD 15min Protease**). Change the Abbreviated name (e.g. **15mPro)** and the Description (e.g. **ACD 15min Protease**).



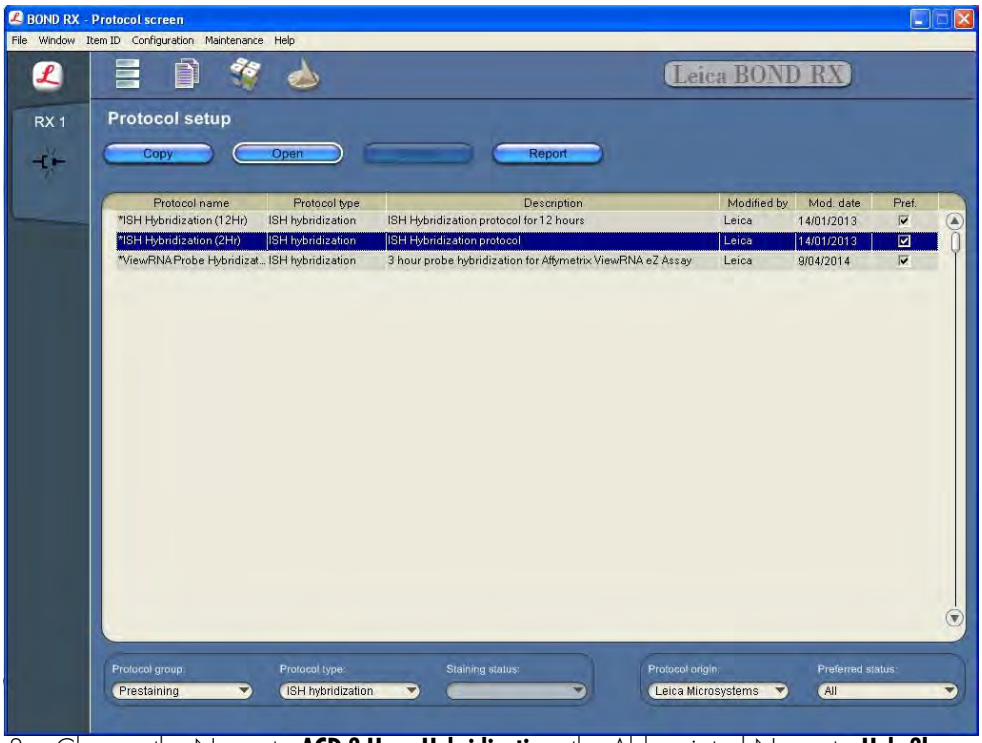
4. Highlight the second ***Enzyme 1** step. Change the temperature to **40°C** and incubation time according to the following table:

Tissue Type	Enzyme 1 Incubation Time	Temperature
Brain and spinal cord	15 MIN	40°C
Breast cancer	15 MIN	40°C
Cell pellet	15 MIN	40°C
Colon	15 MIN	40°C
GI tract	15 MIN	40°C
Head and neck cancer	15 MIN	40°C
Heart	15 MIN	40°C
Kidney	15 MIN	40°C
Liver	25 MIN	40°C
Lung	15 MIN	40°C
Lymphoma	15 MIN	40°C
Placenta	15 MIN	40°C
Prostate	15 MIN	40°C
Skin	15 MIN	40°C
Stomach	15 MIN	40°C
Thymus	15 MIN	40°C
Tonsil	15 MIN	40°C
Xenograft	15 MIN	40°C

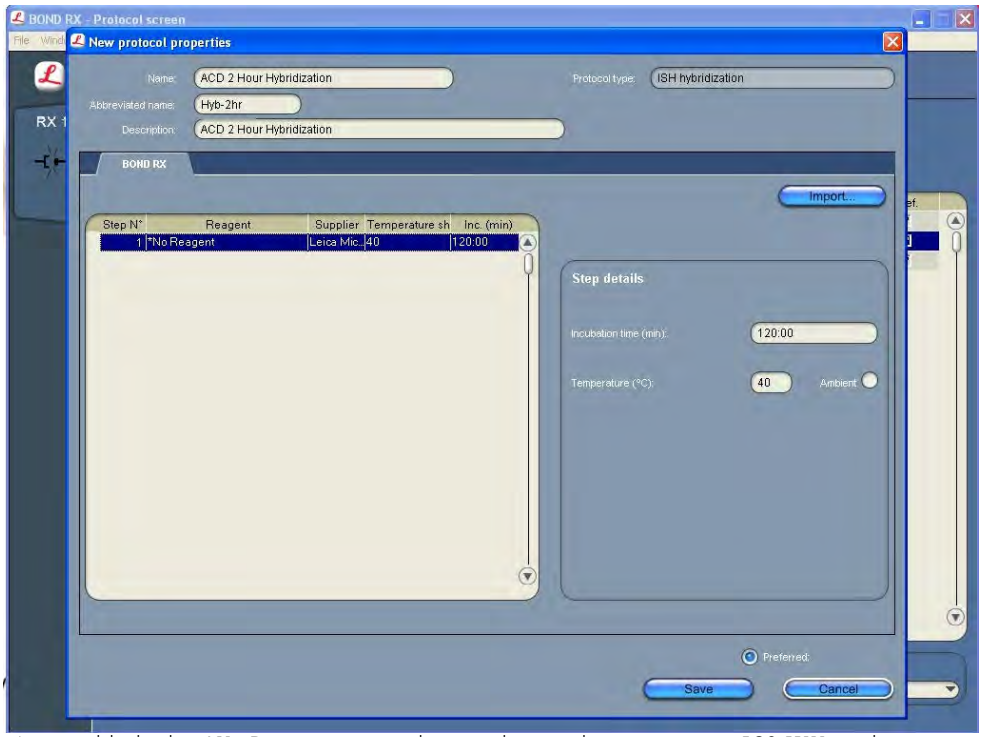
5. Highlight the ***Open 0 Haz** step. Change the incubation time to **10 MIN**.
6. Select **Save**.

Probe Hybridization

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 2 Hour Hybridization**, the Abbreviated Name to **Hyb-2hr**, and the Description to **ACD 2 Hour Hybridization**.



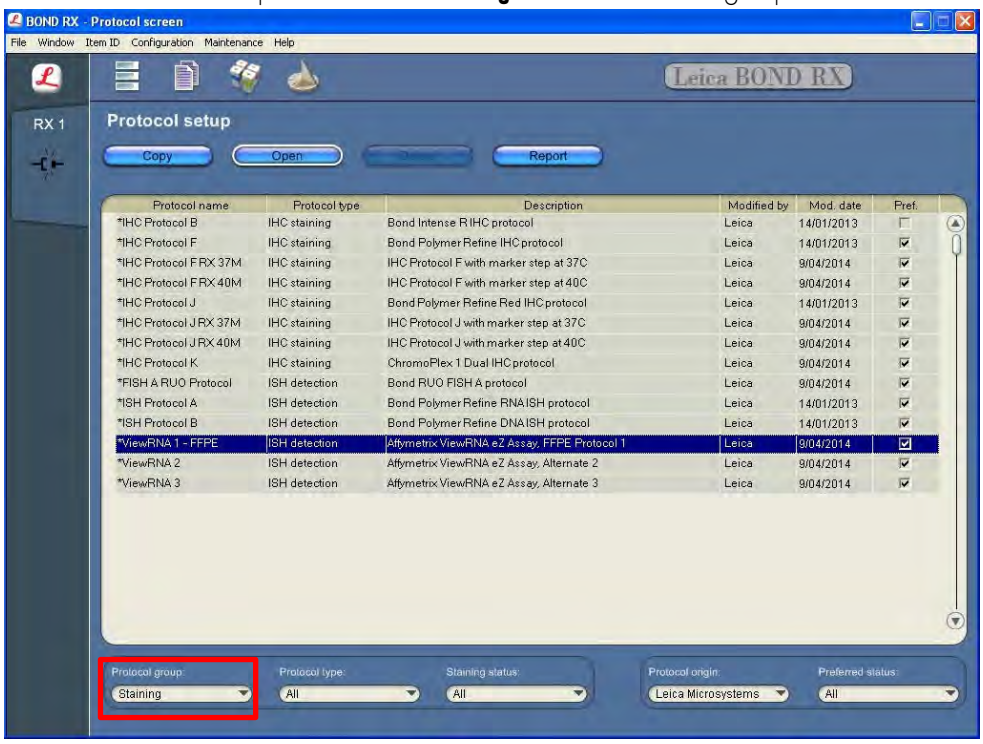
4. Highlight the ***No Reagent** step. Change the incubation time to **120 MIN** and temperature to

40°C.

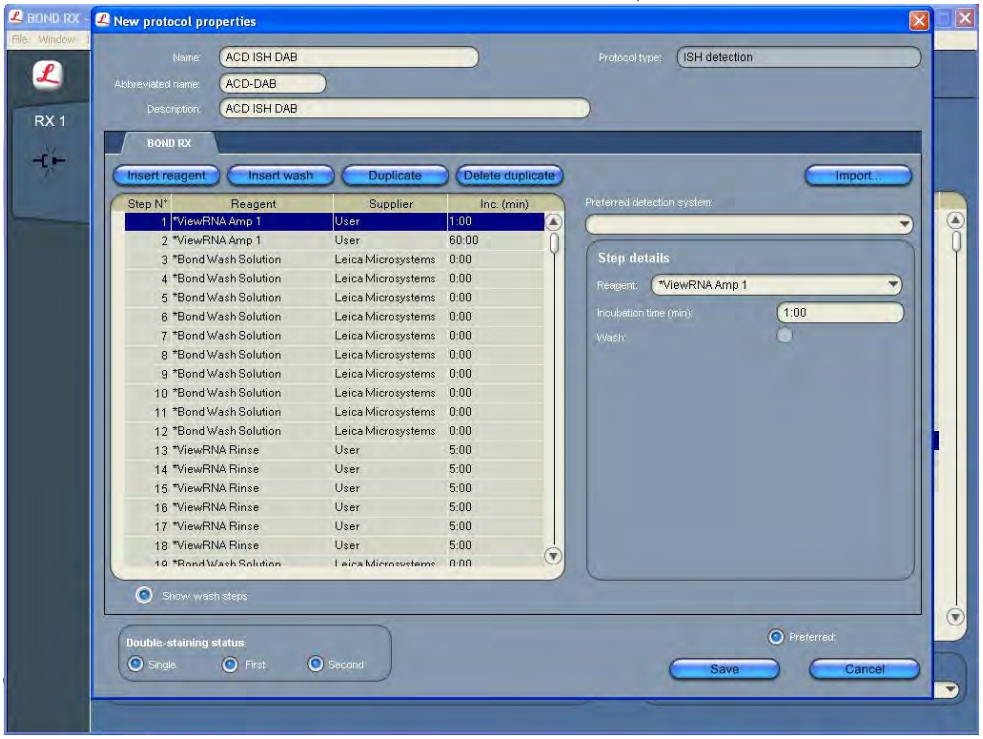
5. Select **Save**.

Create a staining protocol

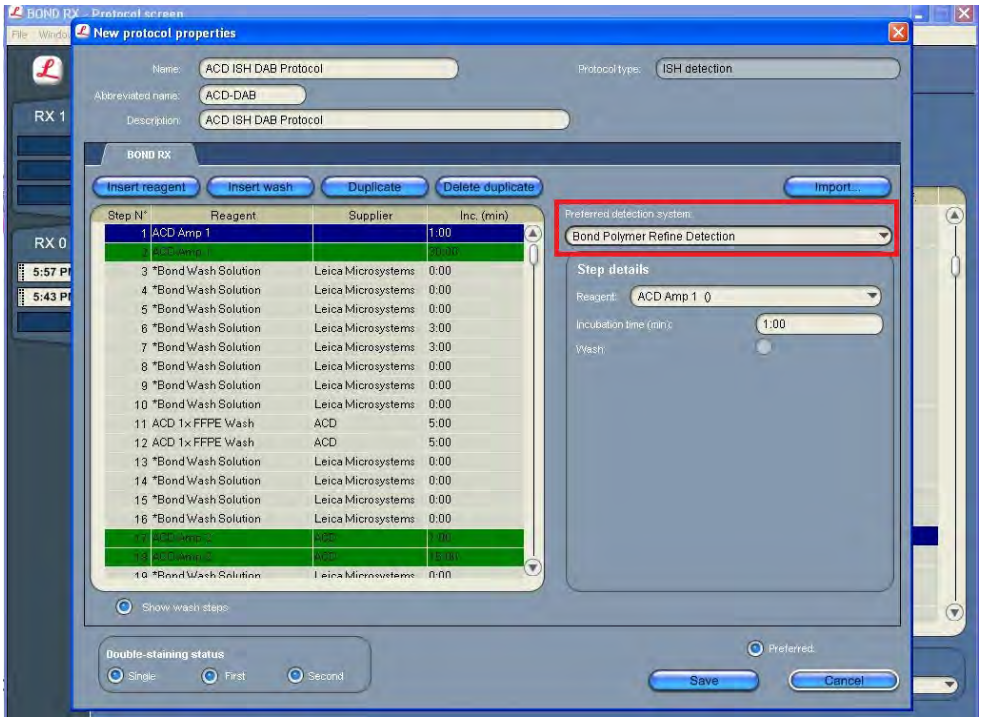
1. In the Protocol setup screen select **Staining** under the Protocol group menu.



2. Highlight the ***ViewRNA 1- FFPE** protocol. Select **Copy**.
3. Change the name to **ACD ISH DAB Procol** in the Name text box, **ACD-DAB** in the Abbreviated name text box, and **ACD ISH DAB Protocol** in the Description text box.



4. Select **Bond Polymer Refine Detection** under the Preferred detection system menu.



5. Highlight and select on each Reagent step to edit each step. Set up the protocol steps (highlighted rows) according to the following table:

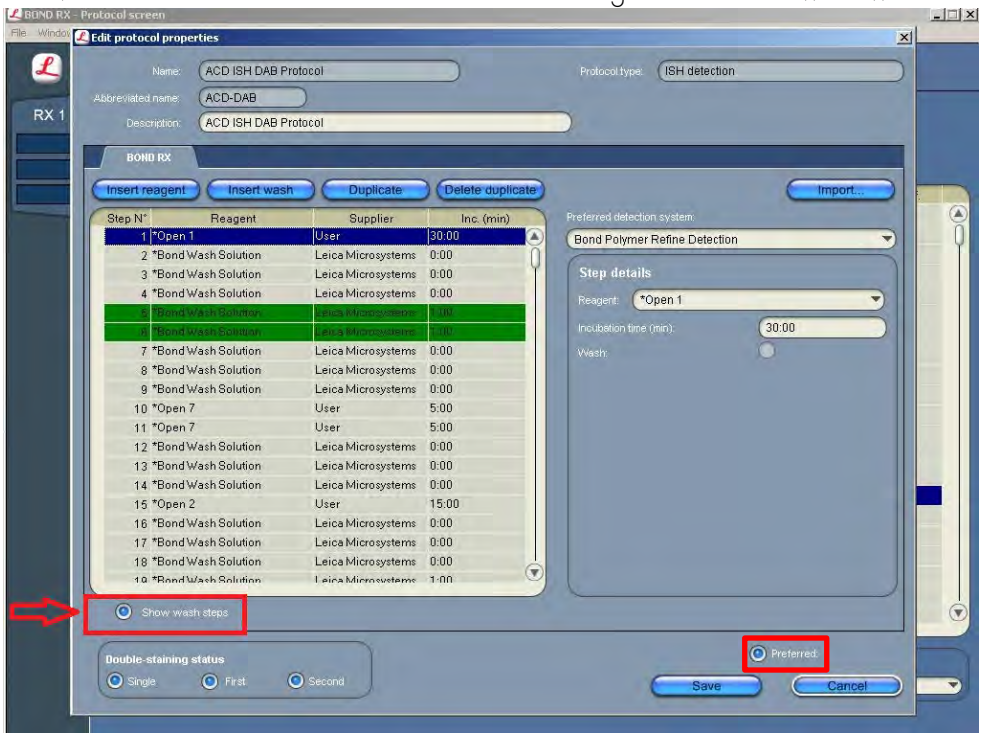
Step No.	Reagent	Step Type	Incubation Time	Temperature
1	*ACD Amp 1	Reagent	1 MIN	42°C
2	*ACD Amp 1	Reagent	30 MIN	42°C
3	*Bond Wash Solution	Wash	0 MIN	Ambient
4	*Bond Wash Solution	Wash	0 MIN	Ambient
5	*Bond Wash Solution	Wash	0 MIN	Ambient
6	*Bond Wash Solution	Wash	3 MIN	Ambient
7	*Bond Wash Solution	Wash	3 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	*ACD 1X Wash Buffer	Reagent	5 MIN	Ambient
12	*ACD 1X Wash Buffer	Reagent	5 MIN	Ambient
13	*Bond Wash Solution	Wash	0 MIN	Ambient
14	*Bond Wash Solution	Wash	0 MIN	Ambient
15	*Bond Wash Solution	Wash	0 MIN	Ambient
16	*Bond Wash Solution	Wash	0 MIN	Ambient
17	*ACD Amp 2	Reagent	1 MIN	42°C
18	*ACD Amp 2	Reagent	15 MIN	42°C
19	*Bond Wash Solution	Wash	0 MIN	Ambient
20	*Bond Wash Solution	Wash	0 MIN	Ambient
21	*Bond Wash Solution	Wash	0 MIN	Ambient
22	*Bond Wash Solution	Wash	1 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature
23	*Bond Wash Solution	Wash	1 MIN	Ambient
24	*Bond Wash Solution	Wash	1 MIN	Ambient
25	*Bond Wash Solution	Wash	1 MIN	Ambient
26	*Bond Wash Solution	Wash	1 MIN	Ambient
27	*ACD Amp 3	Reagent	1 MIN	42°C
28	*ACD Amp 3	Reagent	30 MIN	42°C
29	*Bond Wash Solution	Wash	0 MIN	Ambient
30	*Bond Wash Solution	Wash	0 MIN	Ambient
31	*Bond Wash Solution	Wash	0 MIN	Ambient
32	*Bond Wash Solution	Wash	3 MIN	Ambient
33	*Bond Wash Solution	Wash	3 MIN	Ambient
34	*Bond Wash Solution	Wash	1 MIN	Ambient
35	*Bond Wash Solution	Wash	1 MIN	Ambient
36	*Bond Wash Solution	Wash	1 MIN	Ambient
37	*ACD Amp 4	Reagent	1 MIN	42°C
38	*ACD Amp 4	Reagent	15 MIN	42°C
39	*Bond Wash Solution	Wash	0 MIN	Ambient
40	*Bond Wash Solution	Wash	0 MIN	Ambient
41	*Bond Wash Solution	Wash	0 MIN	Ambient
42	*Bond Wash Solution	Wash	1 MIN	Ambient
43	*Bond Wash Solution	Wash	1 MIN	Ambient
44	*Bond Wash Solution	Wash	1 MIN	Ambient
45	*Bond Wash Solution	Wash	1 MIN	Ambient
46	*Bond Wash Solution	Wash	1 MIN	Ambient
47	*ACD Amp 5 Brown	Reagent	1 MIN	Ambient
48	*ACD Amp 5 Brown	Reagent	30 MIN	Ambient
49	*Bond Wash Solution	Wash	0 MIN	Ambient
50	*Bond Wash Solution	Wash	0 MIN	Ambient
51	*Bond Wash Solution	Wash	0 MIN	Ambient
52	*Bond Wash Solution	Wash	1 MIN	Ambient
53	*Bond Wash Solution	Wash	1 MIN	Ambient
54	*Bond Wash Solution	Wash	1 MIN	Ambient
55	*Bond Wash Solution	Wash	1 MIN	Ambient
56	*Bond Wash Solution	Wash	1 MIN	Ambient
57	*ACD Amp 6 Brown	Reagent	1 MIN	Ambient
58	*ACD Amp 6 Brown	Reagent	15 MIN	Ambient
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

Step No.	Reagent	Step Type	Incubation Time	Temperature
63	*Bond Wash Solution	Wash	1 MIN	Ambient
64	*Bond Wash Solution	Wash	1 MIN	Ambient
65	*Bond Wash Solution	Wash	1 MIN	Ambient
66	*Bond Wash Solution	Wash	1 MIN	Ambient
67	*ACD 1X Wash Buffer	Reagent	5 MIN	Ambient
68	*ACD 1X Wash Buffer	Reagent	5 MIN	Ambient
69	*Mixed DAB Refine	Reagent	1 MIN	Ambient
70	*Mixed DAB Refine	Reagent	20 MIN	Ambient
71	*De-ionized Water	Wash	0 MIN	Ambient
72	*De-ionized Water	Wash	0 MIN	Ambient
73	*De-ionized Water	Wash	0 MIN	Ambient
74	*De-ionized Water	Wash	0 MIN	Ambient
75	*De-ionized Water	Wash	0 MIN	Ambient
76	*De-ionized Water	Wash	0 MIN	Ambient
77	*Hematoxylin	Reagent	5 MIN	Ambient
78	*De-ionized Water	Wash	0 MIN	Ambient
79	*De-ionized Water	Wash	0 MIN	Ambient
80	*De-ionized Water	Wash	0 MIN	Ambient
81	*De-ionized Water	Wash	0 MIN	Ambient

Note: The temperature for these steps cannot be changed. You may only change the incubation times.

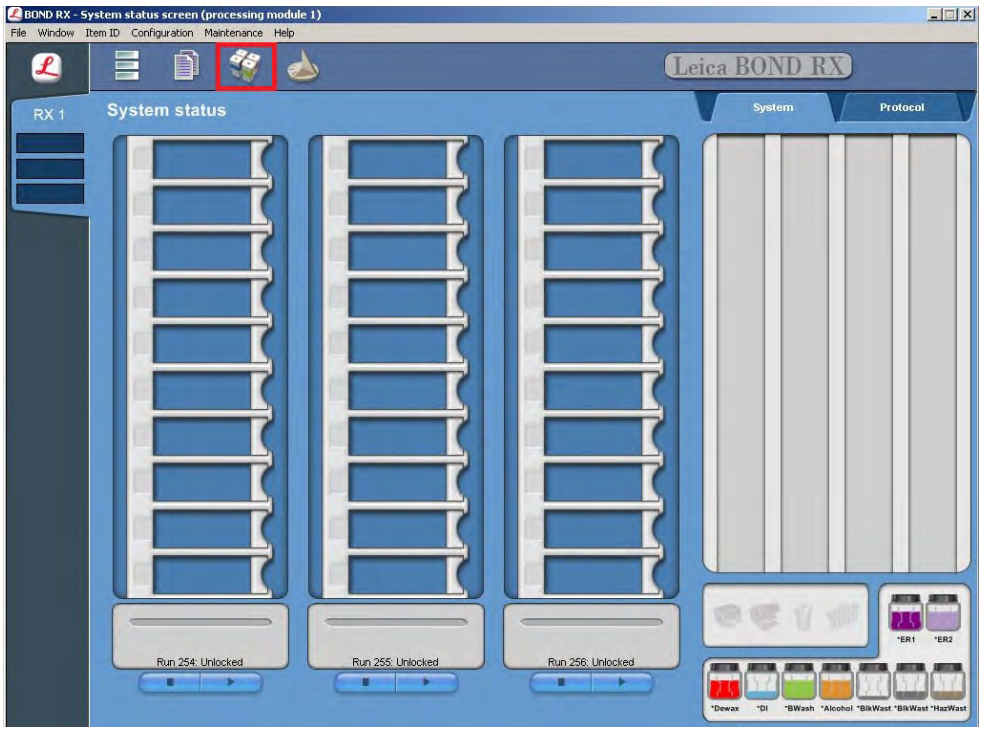
6. Click **Show wash steps** to view the washing steps in between each reagent. Insert BOND Washes to match the protocol steps shown in the table above.
7. Make sure that **Preferred** is selected at the bottom right corner of the window.



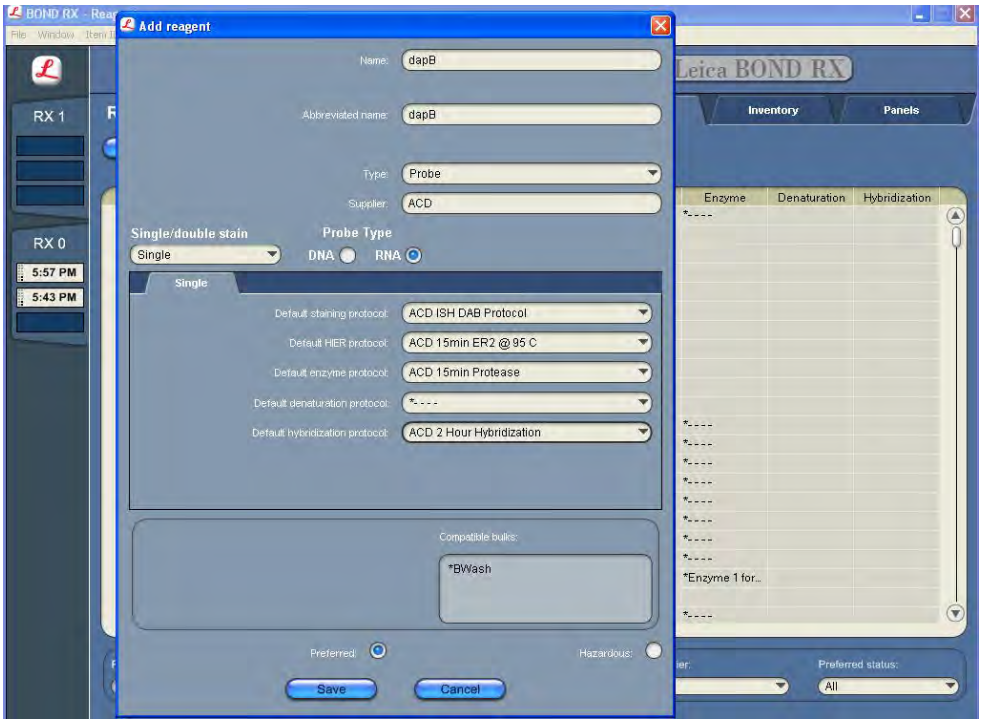
8. Select **Save**.

Register Probes

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



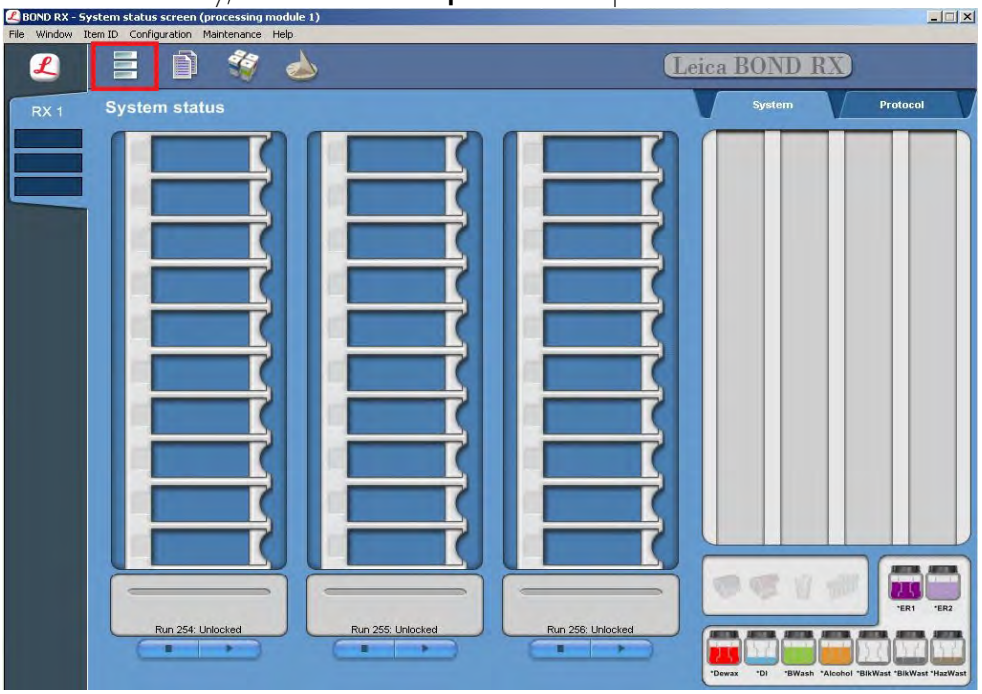
2. Select **Add**.



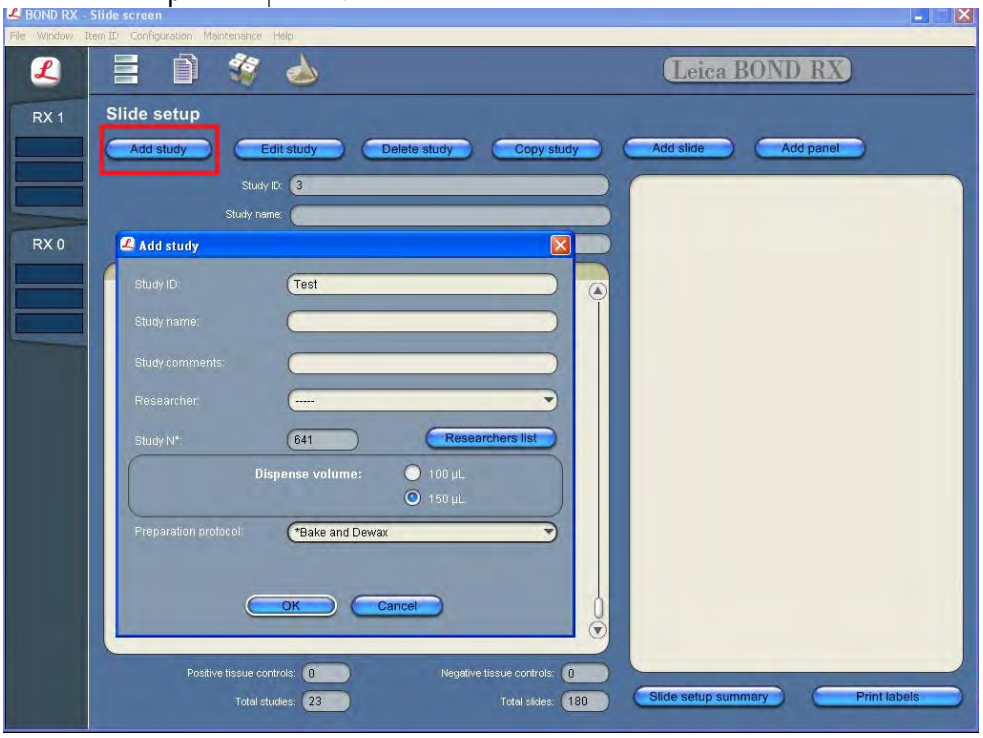
3. Enter the name of the probe in the Name and Abbreviated name text boxes.
4. Select **Probe** in the Type drop-down menu. Enter **ACD** in the Supplier text box.
5. Check **RNA** for Probe Type.
6. Select **ACD DAB** as the Default staining protocol.
7. Select **ACD 15min ER2 @ 95 C** as the Default HIER protocol.
8. Select **ACD 15min Protease** as the Default enzyme protocol.
9. Leave the Default denaturation protocol blank.
10. Select **ACD 2 Hour Hybridization** as the Default hybridization protocol.
11. Select **Save**.
12. Repeat steps 2 –11 for each additional probe.

Set up a study

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

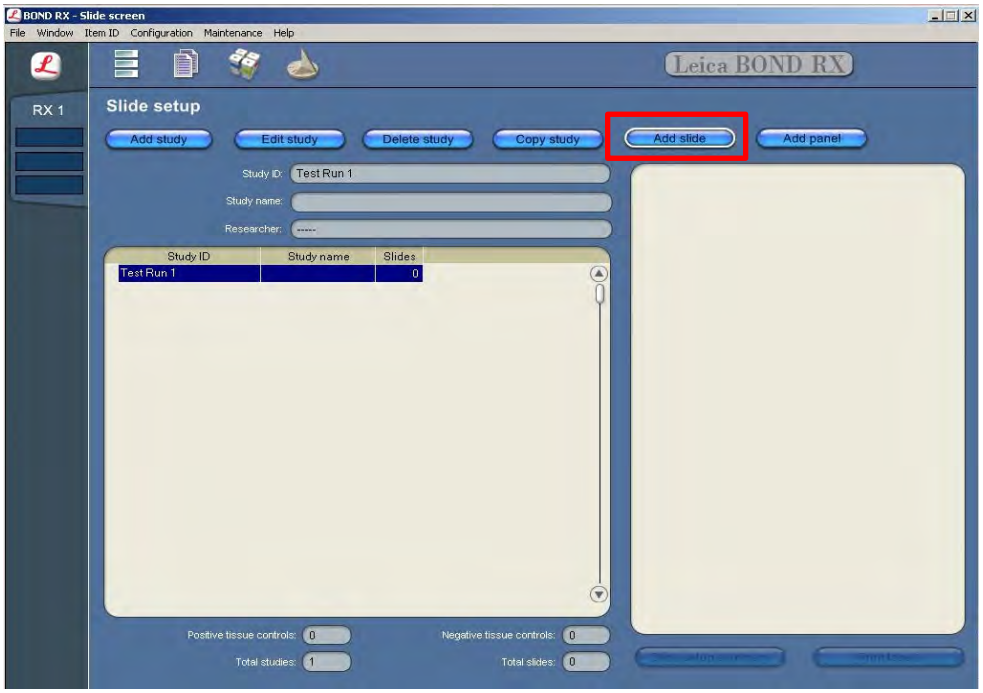


2. Select **Add study** and enter a name in the **Study ID** field.
3. Select **150 µl** for Dispense volume.

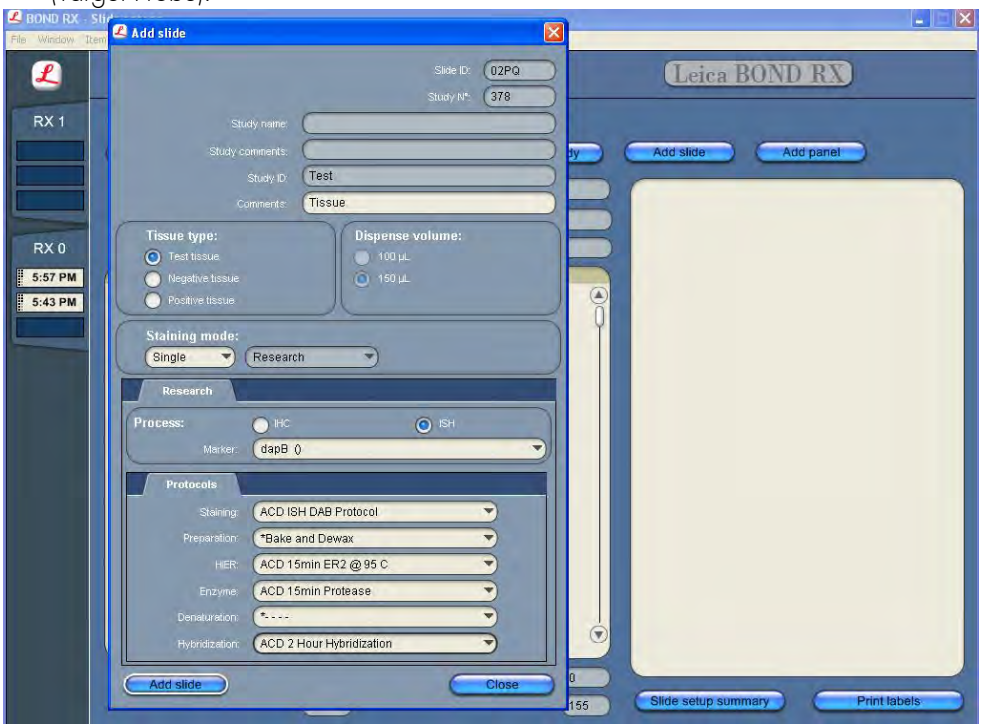


4. Select ***Bake and Dewax** for Preparation protocol.
5. Select **OK**.

6. Select **Add slide** to assign a protocol to each slide.

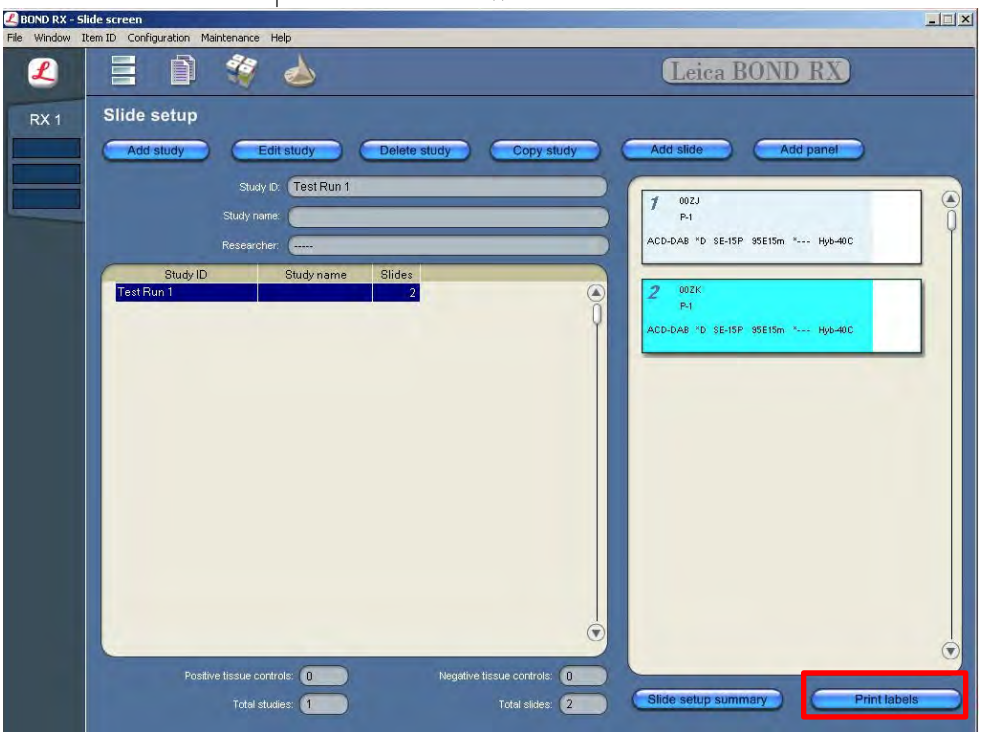


7. Enter the name of the tissue under the **Comments** field. Then select **ISH**. Select the marker (Target Probe).



8. Under the Preparation drop-down menu, select the protocol *Bake and Dewax.
9. Select **Add slide** for each target probe and the slides used for the run. After adding all the slides to the study, select **Close** to return to the Slide setup screen.

10. Select **Print labels** to print barcodes that will be attached to the slides.



Run the RNAscope® Assay

Materials required

- Distilled Water
- Fume hood
- Xylene
- 100% EtOH
- Tissue-Tek® Staining Dish (4)
- Tissue-Tek® Clearing Agent Dish, xylene-resistant (2)
- Tissue-Tek® Vertical 24 Slide Rack
- Cytoseal
- Cover Glass, 24 mm x 50 mm

Load the reagents

1. Label each Leica BOND RX container with the corresponding reagent using a water-resistant marker.
2. Use the barcode scanner to scan the barcode located on the front of each container (including Leica Bond DAB and Hematoxylin containers). A pop-up menu will appear.

IMPORTANT! Do **NOT** scan the barcode located on the top of each container.

3. Choose the appropriate Reagent Name, and enter the lot number and expiration date in their respective text boxes. Select **OK**.
4. Load the containers onto the reagent tray and slide the tray into the Leica BOND RX module.

Start the run

1. Once the barcodes have been attached to the slides, add the slides to the slide tray with the label sides facing up.

Note: Each tray can accommodate only one study. If a different protocol is used, it must be placed in a separate tray. Only **three** different parameters may be used for a complete run for a total of 30 slides.

2. Add a covertile on top of each slide. The rectangular-shaped neck of the covertile should fit into the groove of the slide tray.
3. Place the tray in the Leica Bond RX™ and press the button to load the tray onto the machine.
4. Once the slides have been scanned, select the **triangular** (PLAY) 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.

Note: The following sections may also be performed using an automated coverslipper.

Complete the run

1. After the run is complete press the button on 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 100% EtOH 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 100% EtOH for **2 MIN** with occasional agitation.
3. Move the slide rack into the third staining dish containing 100% EtOH 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 **1–2 DROPS** 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 5. Evaluate the results** on page 33.

5 Chapter 5. 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 cell nuclei 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® 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/cell (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.

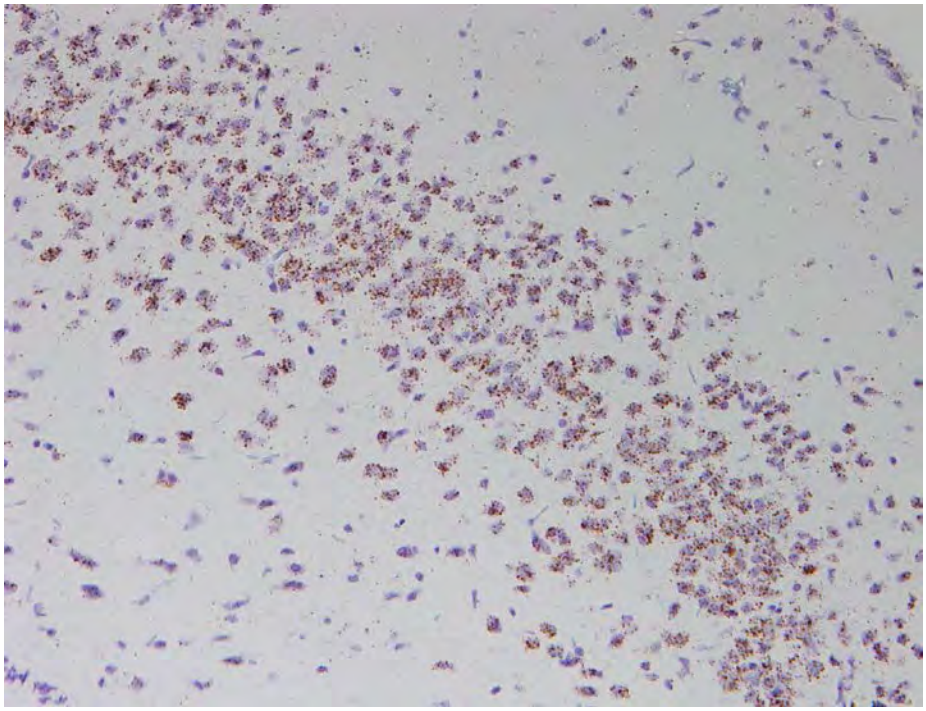
Quantitative Image Analysis

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 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 images:

Figure 2 RNAscope® Assay detection of PPIB mRNA in mouse brain 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 5 minutes and increase the Enzyme (Protease) time in increments of 10 minutes. Keep the temperatures for each step constant (e.g. 20 min ER2 at 95°C and 25 min Protease at 40°C; 25 min ER2 at 95°C and 35 min Protease at 40°C).
- Use the above process for over-fixed tissues.
- LS Amp 1, LS Amp 3, 10X LS Wash Buffer, and all target probes require warming up prior to running the assay to remove crystals that form during refrigeration. Incubate the reagents in an oven or water bath at 40°C for 30 minutes. Failure to warm the reagents properly will lead to weak or intermittent staining.
- The RNAscope® 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. To mix reagents, gently invert the dispensers several times. 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 the hematoxylin incubation time. The parameters in the staining protocol have been optimized to run the RNAscope® assay on the instrument.

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

A Appendix A. 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 Material Safety Data Sheets (MSDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain MSDSs, see **Documentation and support** in this document.
- 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:
  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:
  eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:133:0001:0043:EN:PDF

Documentation and support

Obtaining MSDSs

Material Safety Data Sheets (MSDSs) are available at: **www.acdbio.com/product_literature.html**. For the MSDSs of chemicals not distributed by Advanced Cell Diagnostics, contact the chemical manufacturer.

Obtaining support

For the latest services and support information, go to: **www.acdbio.com/product_literature.html**

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, MSDSs, application notes, citations, training videos, and other product support documents.
- Find out information about customer training events.

Contact information

Advanced Cell Diagnostics, Inc.
3960 Point Eden Way
Hayward, 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 ADC website at **www.acdbio.com/product_literature.html**. If you have any questions, please contact Advanced Cell Diagnostics at **www.acdbio.com/about/contact**.

Headquarters

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For support, email support@acdbio.com.

www.acdbio.com