



# **RNAscope™ 2.5 LS Duplex Reagent Kit – User Manual for BDZ 11**

For use with Leica Biosystems' BOND RX™ System



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**Citing RNAscope in Publications**

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

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# Chapter 1. Product Information



Before using this product, read and understand the information in **Appendix F. Safety** of this document.

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**IMPORTANT!** We recommend reading the entire user manual before beginning any protocols.

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## About this guide

This user manual provides guidelines and protocols to use the RNAscope 2.5 Duplex LS Reagent Kit for use with Leica Biosystems' BOND RX Research Advanced Staining System. RNAscope 2.5 LS Duplex Assays are compatible with a variety of sample types.

## Product description

### Background

The RNAscope 2.5 LS Duplex Assay uses a novel and proprietary method of *in situ* hybridization (ISH) to simultaneously visualize two RNA targets 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 Duplex Assay allows users to automate the highly sensitive RNAscope 2.5 Duplex Assay using Leica Biosystems' BOND RX System.

### Overview

Figure 1 on page 7 illustrates the RNAscope 2.5 LS Duplex Assay procedure, which can be completed in ~14 hours. Starting with properly prepared samples, sections are first pretreated, and then RNA-specific probes are hybridized to target RNA. The RNAscope Duplex LS Assay employs two independent signal amplification systems each using a different chromogenic enzyme, enabling users to investigate expression as well as positional relationship between two different genes within a cellular context. Single RNA transcripts for two target genes appear as punctate dots of two distinctly colored chromogen precipitates, 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.	Use two independent signal amplification systems to detect both target RNAs. Probes are hybridized to a cascade of signal amplification molecules, culminating in binding of HRP- or AP-labeled probes. Add two chromogenic substrates to detect RNAs.	Visualize target RNA using a standard bright field microscope.

## Kit contents and storage

The RNAscope 2.5 LS Duplex Assay requires the RNAscope 2.5 LS Probes and the RNAscope 2.5 LS Duplex Reagents, available from Advanced Cell Diagnostics.

### RNAscope 2.5 LS Probes

The RNAscope 2.5 LS Probes consist of the user-specified Target Probe(s) and the Positive and Negative Control Probes. Visit <https://acdbio.com/products> to find a gene-specific target probe or appropriate control probes.

Each target probe contains a mixture of short oligonucleotides designed to bind to a specific target RNA and detectable in one of two color channels, C1 or C2.

**Note:** Different colors are assigned to the C1 and C2 color channels depending on the particular RNAscope Assay. The color channels for the RNAscope 2.5 LS Duplex Assay are shown in the following table:

Probe Channel ID	Chromogenic Labels	
	Enzyme	Color
C1*	HRP	Dark Brown†
C2	AP	RED

\* Default channel

†You may replace the brown chromogen with green chromogen. See **Appendix B**.

Channel C1 target probes are Ready-To-Use (RTU), while channel C2 probes are shipped as a 50X concentrated stock. To independently detect two target RNAs in a Duplex assay, each target probe must be in a different color channel and there must be a C1 probe in the mixture. If you wish to use only the C2 probe, use the RNAscope 2.5 LS Blank Probe Diluent (Cat. No. 300048) in place of the C1 probe.



Each probe is sufficient 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 tables:

Target Probes					
<input checked="" type="checkbox"/>	Reagent	Cat. No.	Content	Quantity	Storage
	RNAscope 2.5 LS Duplex Target Probe – [species] – [gene]	Various	Ready-To-Use (RTU) probe for color channel 1	16 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS Duplex Target Probe – [species] – [gene] – C2	Various	50X probe for color channel 2	320 µL x 1 tube	2–8°C
Control Probes					
<input checked="" type="checkbox"/>	Reagent	Cat. No.	Content	Quantity	Storage
	RNAscope 2.5 LS Duplex Control Probes	Various	RTU mixture of two probes targeting PPIB in channel C1 and POLR2A in channel C2.	16 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS Negative Control – <i>dapB</i>	320758	RTU probe targeting a bacterial gene in channel C1 and in channel C2.	16 mL x 1 bottle	2–8°C

## RNAscope 2.5 LS Duplex Reagents

The RNAscope 2.5 LS Duplex Reagent Kit (Cat. No. 322440) contains all the reagents needed to run the RNAscope 2.5 LS Duplex 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 Duplex Reagent Kit (Cat. No. 322440)			
<input checked="" type="checkbox"/>	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 Rinse	29 mL x 2 bottles	2–8°C
	RNAscope 2.5 LS Duplex Wash	21 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS Duplex AMP 1	21 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS Duplex AMP 2	21 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS Duplex AMP 3	21 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS Duplex AMP 4	21 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS Duplex AMP 5	21 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS Duplex AMP 6	21 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS Duplex AMP 7	21 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS Duplex AMP 8	21 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS Duplex AMP 9	21 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS Duplex AMP 10	21 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS Bluing Reagent*	21 mL x 1 bottle	2–8°C

\* Do not use if performing semi-automated green staining. See **Appendix B**.



## Required materials from Leica BOND RX

The RNAscope 2.5 LS Duplex 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
	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 Polymer Refine Red Detection*	DS9390	2–8°C
	BOND DAB Enhancer 30mL†	AR9432	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.

† If performing semi-automated green staining, use the materials in the RNAscope 2.5 LS Green Accessory Pack.

## 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
	100% alcohol (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	–



☑	Description	Supplier	Cat. No.
	Water bath or incubator, capable of holding temperature at 40 +/- 1°C	MLS	—
	Vector Labs VectaMount™ or EcoMount or PERTEX™	ACD (or directly from manufacturer) Biocare Medical MLS	321584 EM897L
	Tissue-Tek® Vertical 24 Slide Rack	American Master Tech Scientific/MLS	LWS2124
	Tissue-Tek Staining Dishes	American Master Tech Scientific/MLS	LWS20WH
	Tissue-Tek Clearing Agent Dishes, xylene resistant	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.

# Chapter 2. Before You Begin

Prior to running the RNAscope 2.5 LS Duplex 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** for preparation of FFPE slides. For preparation of other sample types, contact [support.acd@bio-techne.com](mailto: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 F. Safety** for more information.

# Chapter 3. Prepare and Pretreat Samples

**IMPORTANT!** Only run a maximum of two trays. Running three trays will result in significant instrument errors including loss of dispensers.

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
- 100% alcohol (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 Duplex Assay.

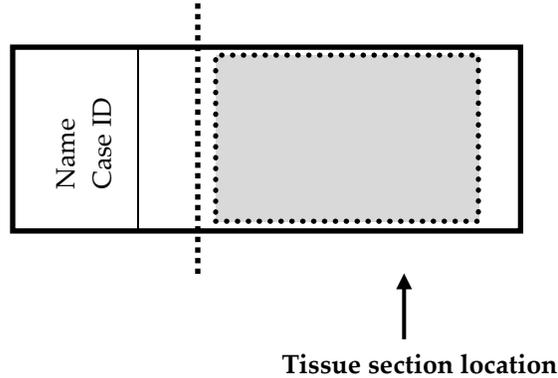
### Dehydrate, embed, and cut the sample

**IMPORTANT!** Use fresh reagents.

4. Wash sample with 1X PBS.
5. Dehydrate sample using a standard ethanol series, followed by xylene.
6. 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.

7. Trim paraffin blocks as needed and **cut** embedded tissue into 5 +/- 1 µm sections using a microtome.
8. 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.

---

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

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

---

# 4

## Chapter 4. Set Up the BDZ 11 Software

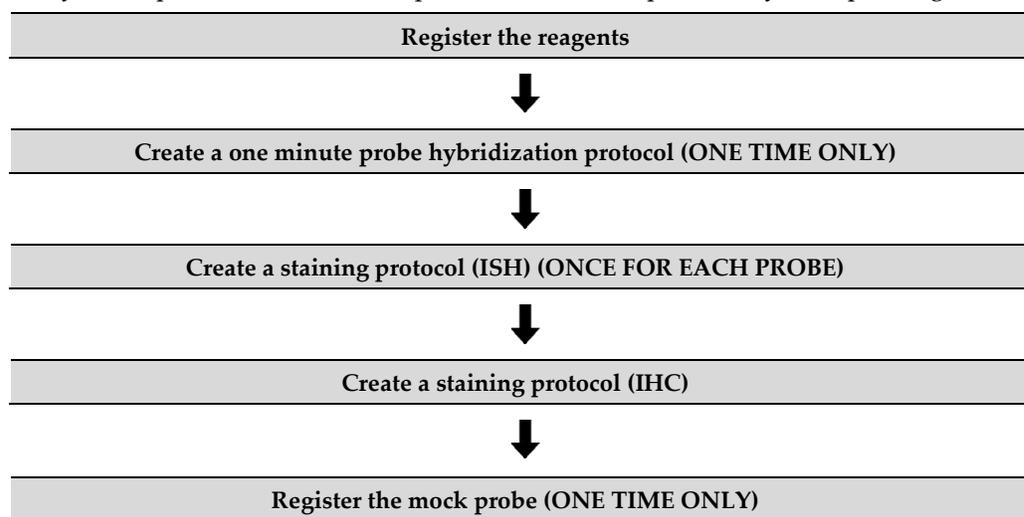
---

**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.

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### Workflow

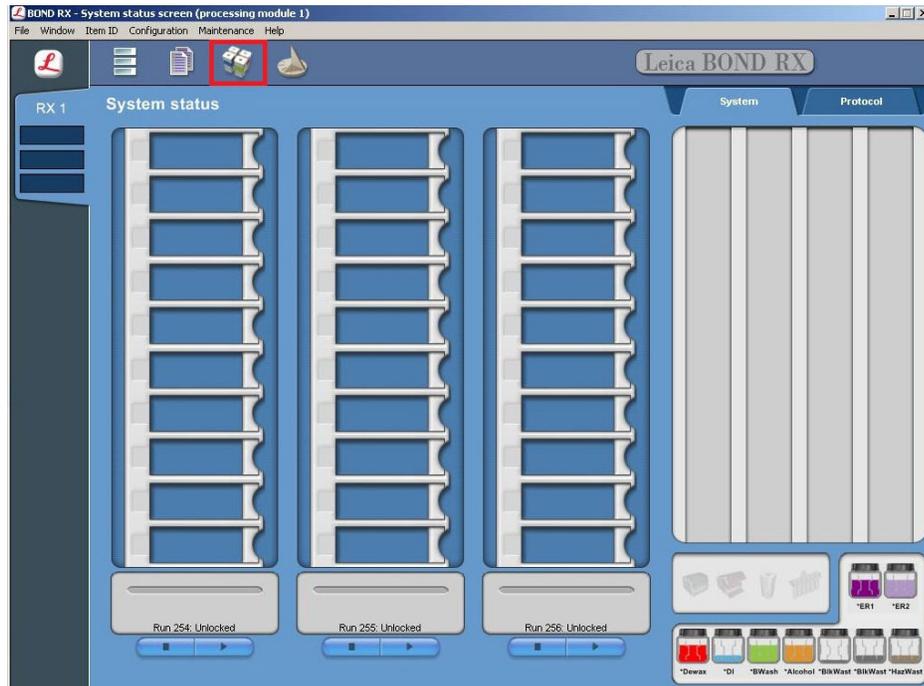
**Note:** Many of the procedures in this chapter need to be completed only once per reagent.



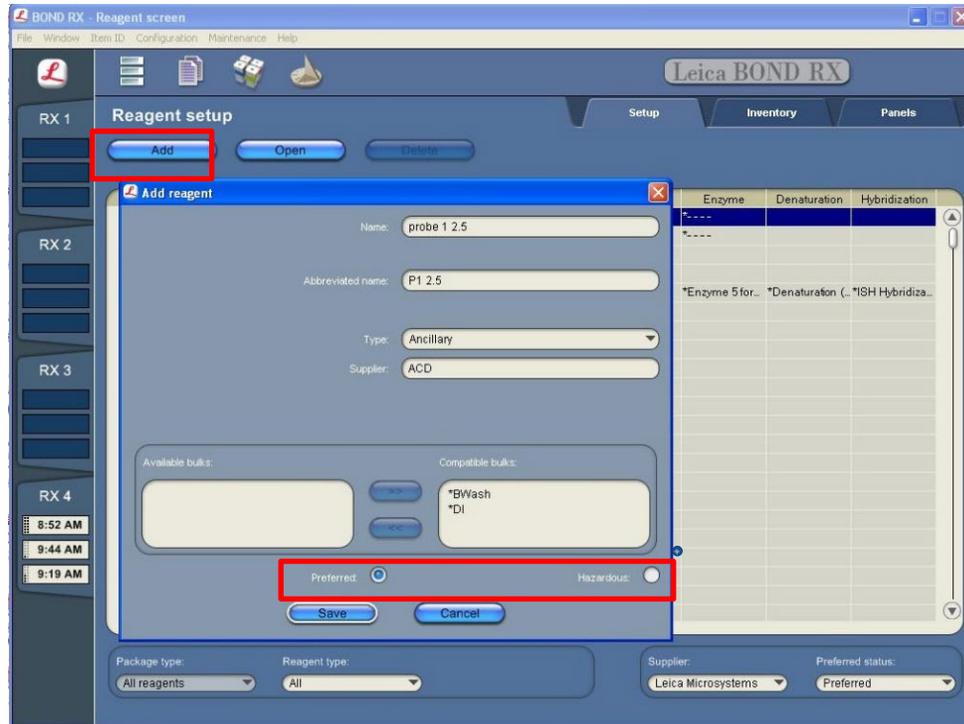
## Register the reagents

This step is a “work around” to the existing BDZ 11 software to accommodate the RNAscope 2.5 LS Duplex 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 in the Name text box.



4. Enter a name 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.

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8. Select **Save**.
9. Repeat Steps 2–7 to register the rest of the reagents using the container names in the following table:

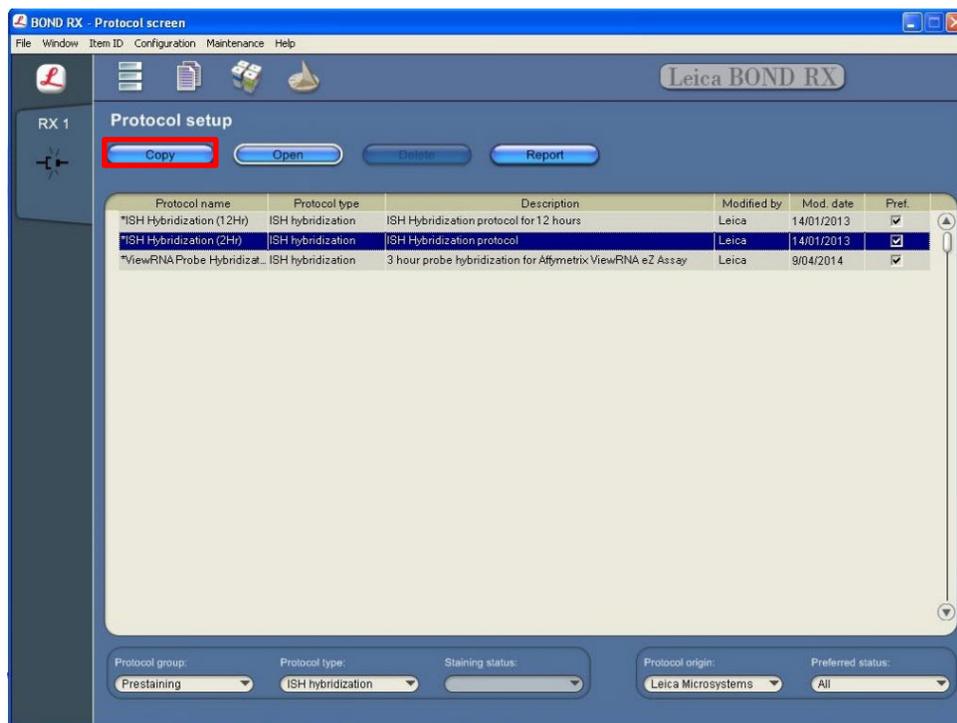
Reagents	Container Name
RNAscope 2.5 LS Duplex Wash	Duplex Wash
RNAscope 2.5 LS Duplex AMP 1	ACD Duplex Amp 1
RNAscope 2.5 LS Duplex AMP 2	ACD Duplex Amp 2
RNAscope 2.5 LS Duplex AMP 3	ACD Duplex Amp 3
RNAscope 2.5 LS Duplex AMP 4	ACD Duplex Amp 4
RNAscope 2.5 LS Duplex AMP 5	ACD Duplex Amp 5
RNAscope 2.5 LS Duplex AMP 6	ACD Duplex Amp 6
RNAscope 2.5 LS Duplex AMP 7	ACD Duplex Amp 7
RNAscope 2.5 LS Duplex AMP 8	ACD Duplex Amp 8
RNAscope 2.5 LS Duplex AMP 9	ACD Duplex Amp 9
RNAscope 2.5 LS Duplex AMP 10	ACD Duplex Amp 10

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

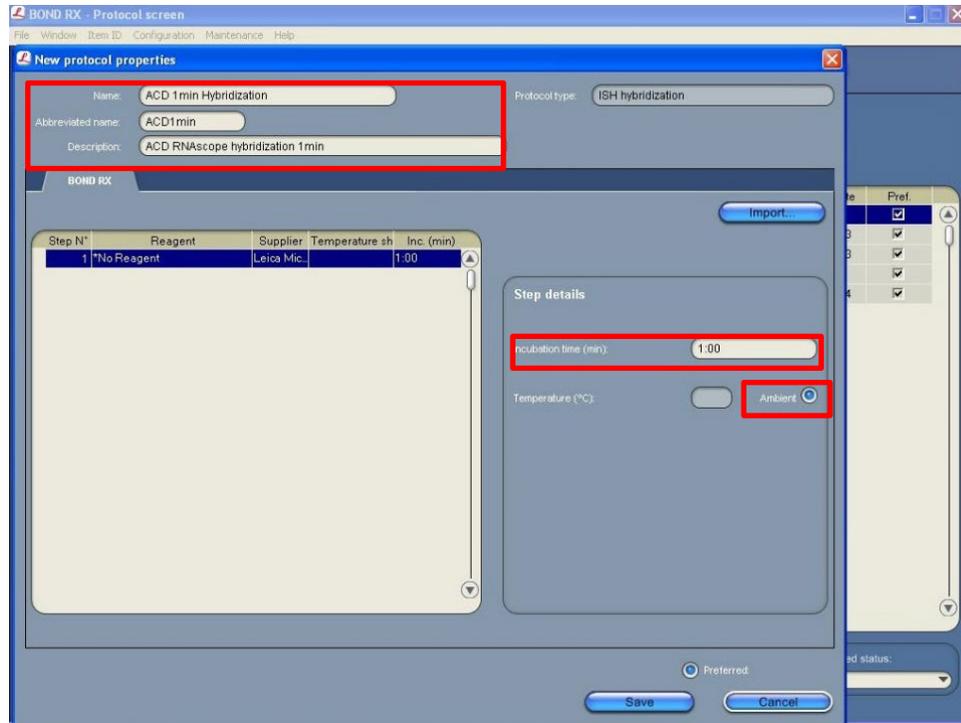
## Create a one minute probe hybridization protocol

A mock probe hybridization step must be created as part of BDZ 11 software workaround for the RNAscope 2.5 LS Duplex 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**.

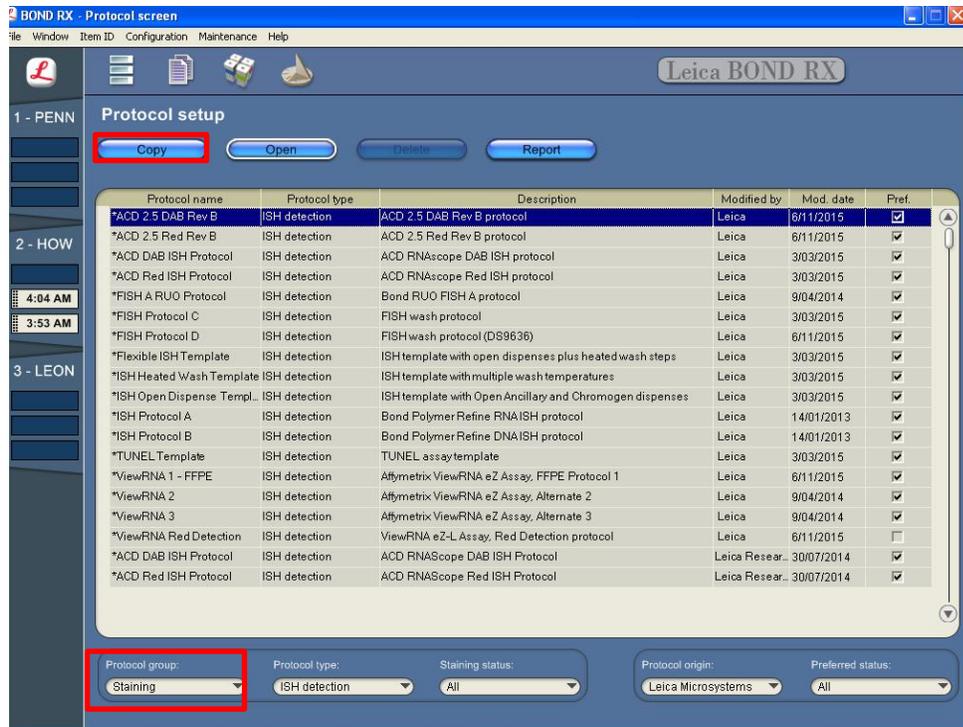


4. Highlight the **\*No Reagent** step.
5. Change the incubation time to **1 MIN** and select **Ambient** as Temperature (°C).
6. Select **Save**.

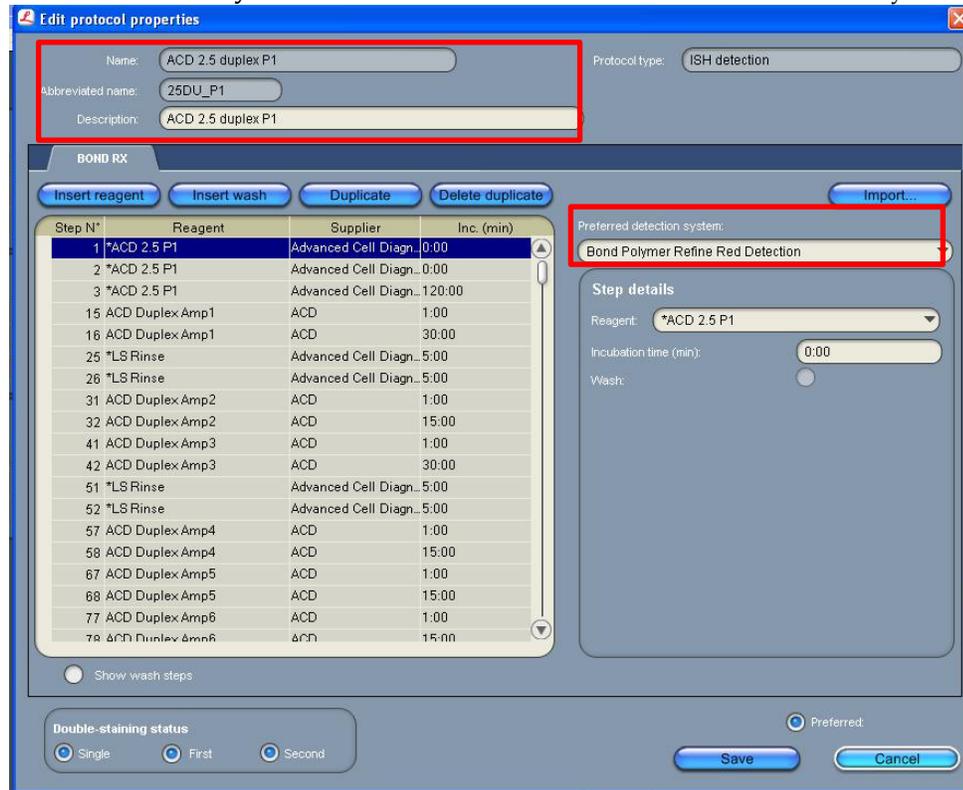
## Create a staining protocol for In Situ Hybridization (ISH)

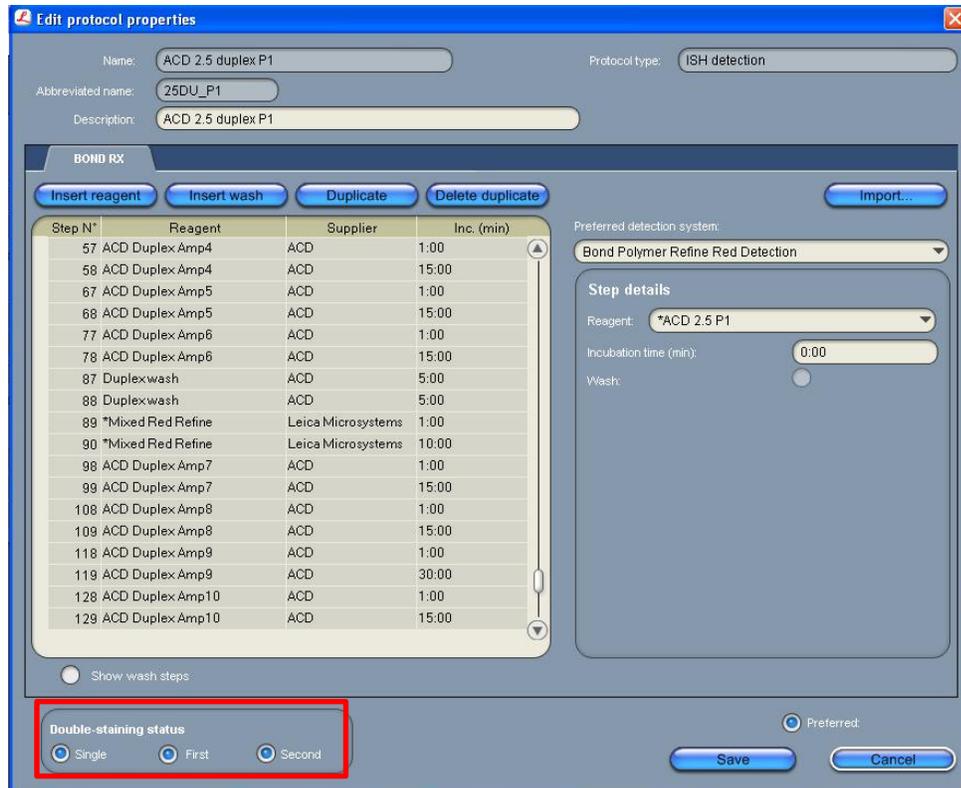
Due to the BDZ 11 software workaround for the RNAscope 2.5 LS Duplex assay, unique staining protocols *must be created for each probe*.

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 duplex P1** in the Name text box, **25DU\_P1** in the Abbreviated name text box, and **ACD 2.5 duplex P1** in the Description text box.
4. Select **Bond Polymer Refine Red Detection** from the Preferred detection system menu.





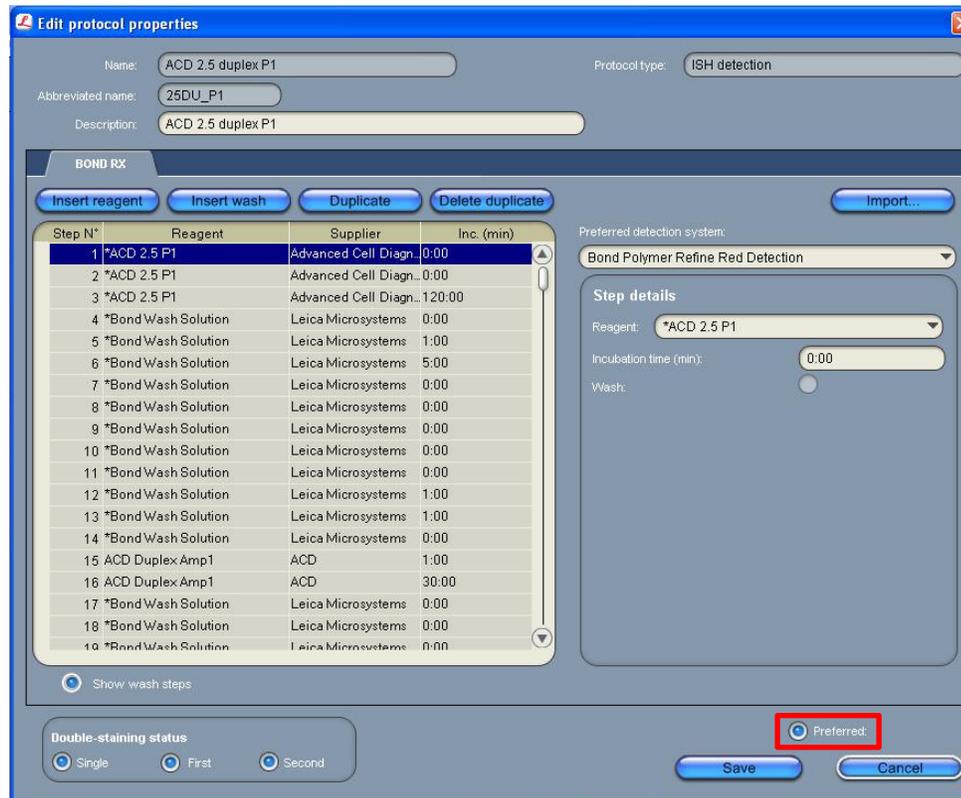
**IMPORTANT!** The **First** button must be selected for the ISH protocol. Other buttons are optional.

**Note:** The preceding two figures display all reagent steps.

5. Highlight and select each Reagent step to edit.

**IMPORTANT!** You can only change the incubation times, not the temperatures, of these steps.

6. Click **Show wash steps** (see the following figure) to also view the wash steps. Insert BOND Washes to match each of the protocol steps shown.
7. Choose the workflow you want to stain with and compare and confirm screen protocol with the full protocol listed in **Appendix A, B** or **C**.
8. Select **Preferred** in the bottom right corner of the window.



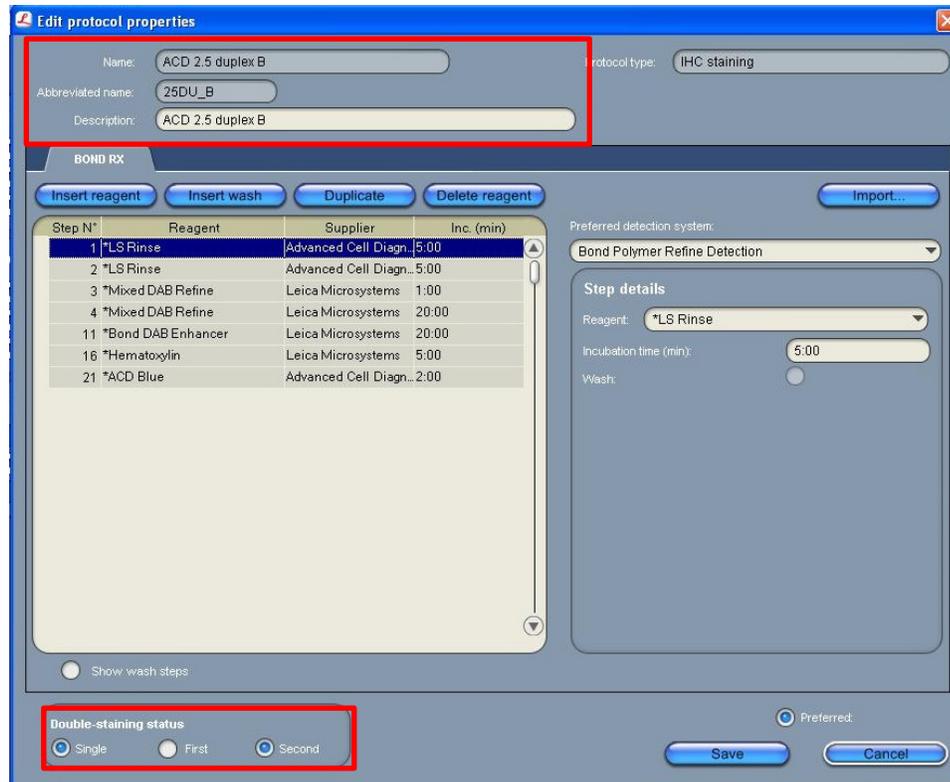
9. Select **Save**.
10. Click **Next** to proceed. Ignore pop-ups that say Step 3 exceeds the recommended time and that the protocol is not validated by Leica. If you see any other pop-ups please contact your FAS or technical support at ACD.
11. Create a new probe protocol.

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

## Create a staining protocol for Immunohistochemistry (IHC)

To develop the second chromogen, create an IHC protocol in the software following guidance in Appendix A or Appendix C depending on reagent/color choice.

1. Highlight the **\*IHC Protocol F** protocol. Select **Copy**.
2. Name the protocol (for example, **ACD 2.5 duplex B**) in the Name text box, **25DU\_B** in the Abbreviated name text box, and **ACD 2.5 duplex B** in the Description text box.
3. Select **Second** in the Double-staining status menu.

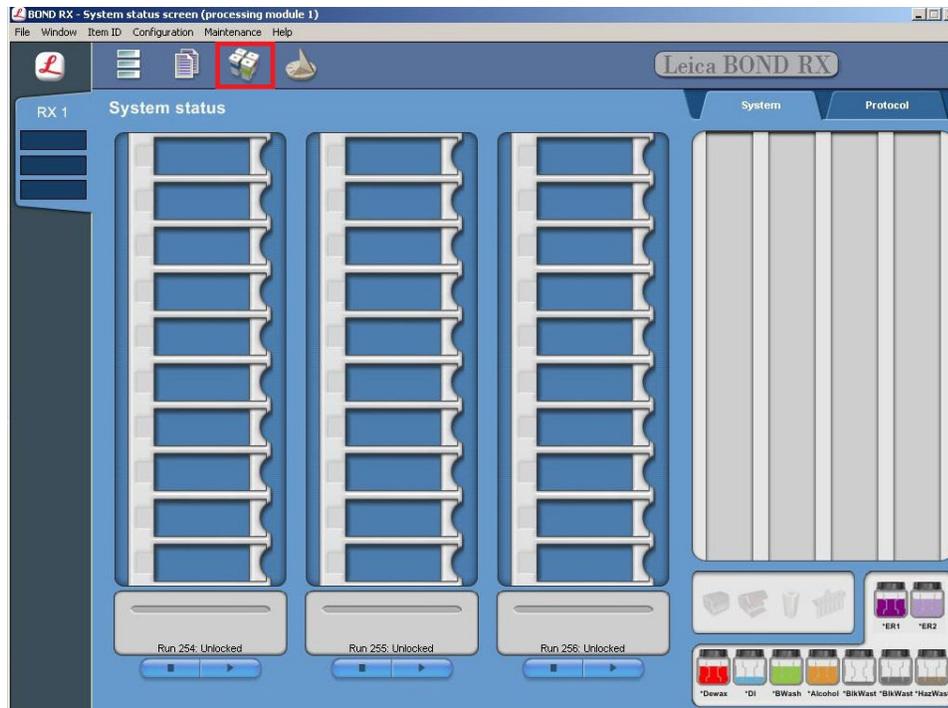


**IMPORTANT!** The **Second** button must be selected in the Double-staining status menu.

## 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. Select **Probe** in the Type drop-down menu. Enter **ACD** in the Supplier text box.
5. Select **RNA** for Probe Type.



6. Select **\*ACD 2.5 DAB Rev B** as the Default staining protocol.
7. Select **\*ACD HIER 15min with ER2 (95)** 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 1 min Hybridization** as the Default hybridization protocol.
11. To set up the default protocols for duplex, select **Sequential DS** under the Single/double stain drop down menu.
12. Repeat steps 5-12 to set up the default protocols to use for duplex.
13. Select **Save**.

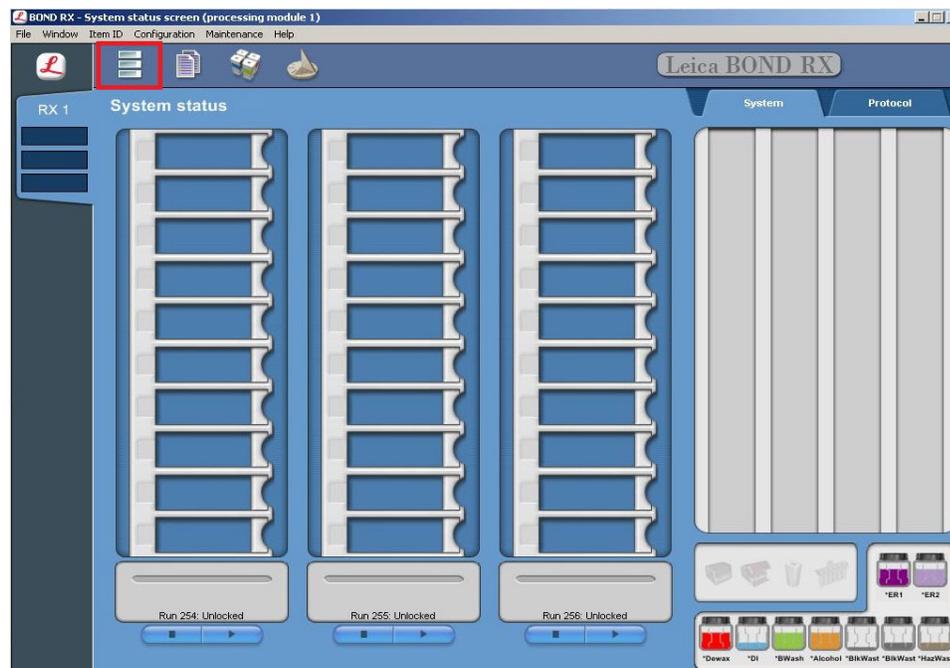
## Set up a sequential dual stain (ds) study for ISH and IHC

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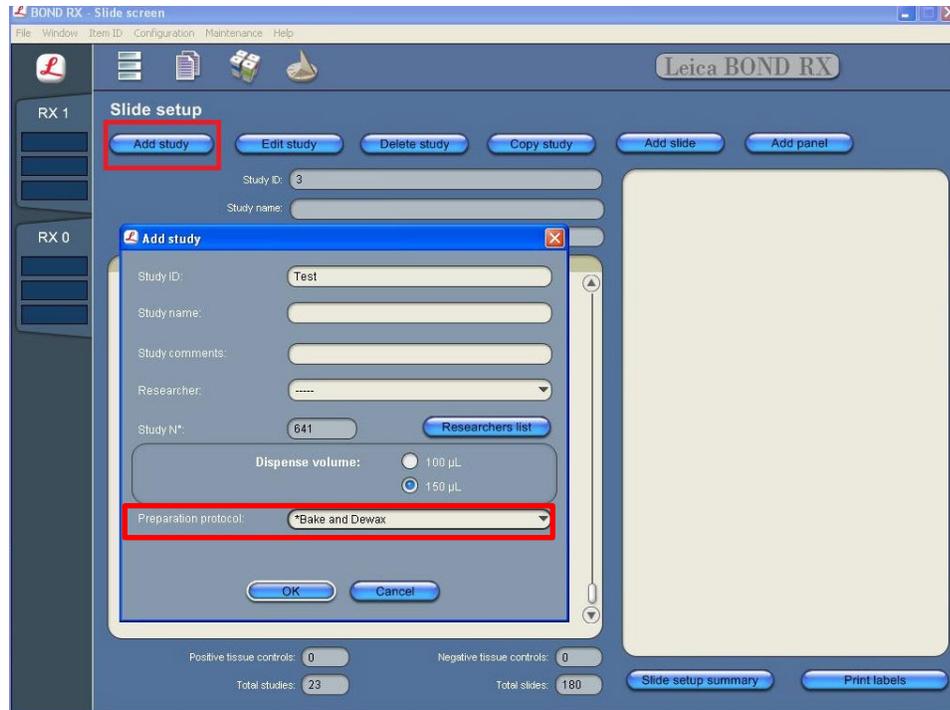
If running a fully automated duplex assay (Red and Brown), only run a maximum of two trays. Running three trays will result in significant instrument errors including loss of dispensers. If running a semi-automated duplex assay (Red and Green), you can use all three trays (see **Appendix B**).

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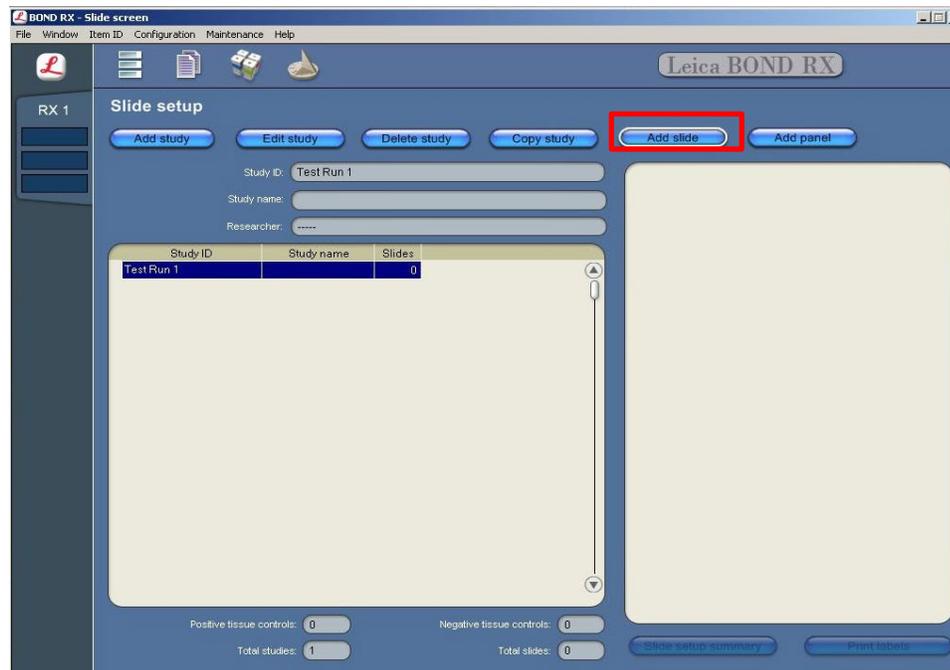
1. To build a study, select the **Slide setup** icon at the top of the screen.



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



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. Select **Sequential DS** from the Staining mode drop down menu

- In the First tab, select **ISH** under Process and **mock probe (ACD)** from the Marker drop down menu.

9. For RNAscope 2.5 LS duplex assays , under the Protocols tab:
  - a. Make sure that each probe is associated with a different protocol (for example, ACD 2.5 duplex P1), 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.

10. In the Second tab, select **IHC** under Process and **\*Negative** from the Marker menu.  
**Note:** This marker is not required on the instrument to start the run.
11. In the Protocols tab, select **ACD 2.5 duplex B** from the Staining menu, and leave HIER and Enzyme empty.

Slide ID: 0LCD  
Study N°: 2110

Study name: \_\_\_\_\_  
Study comments: \_\_\_\_\_  
Study ID: ACD 2.5 duplex  
Comments: tissue probe

**Tissue type:**  
 Test tissue  
 Negative tissue  
 Positive tissue

**Dispense volume:**  
 100 µL  
 150 µL

**Staining mode:**  
 Sequential D [v] Research [v]

**First** **Second**

**Process:**  IHC  ISH

**Marker:** \*Negative [v]

**Protocols**

**Staining:** ACD 2.5 duplex B [v]

**HIER:** ---- [v]

**Enzyme:** ---- [v]

Add slide Close

12. For samples using a different probe, IHC settings (in the second tab) will remain the same. Only ISH settings (in the first tab) need to be changed.
13. Select **Add slide** for each target probe, and for each of the slides used for the run.
14. After adding all the slides to the study, select **Close** to return to the Slide setup screen.
15. Select **Print labels** to print barcodes to attach to the slides.

1 - PENN  
4:35 PM

2 - HOW  
7:48 AM

3 - LEON

Slide setup

Add study Edit study Delete study Copy study Add slide Add panel

Study ID: ACD 2.5 duplex  
Study name: \_\_\_\_\_  
Researcher: \_\_\_\_\_

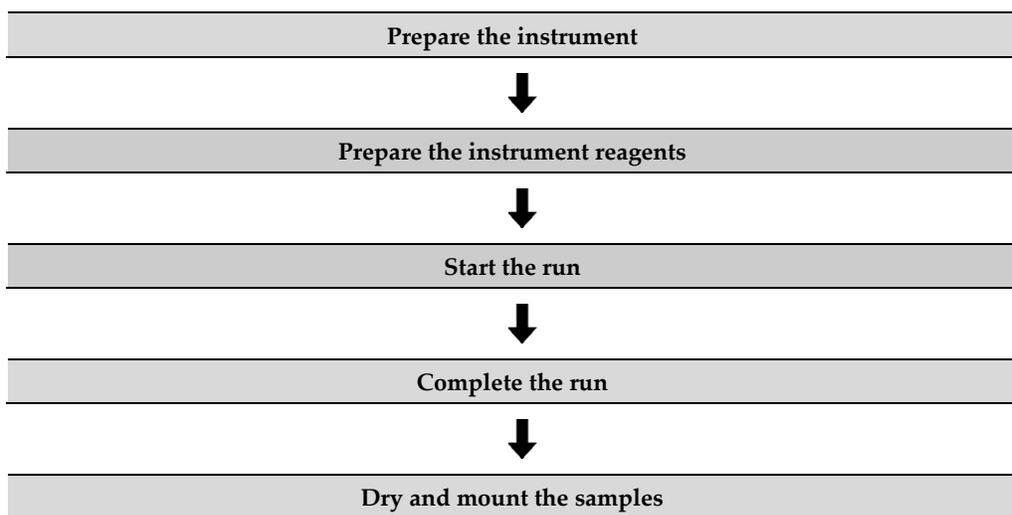
Study ID	Study name	Slides
ACD 2.5 duplex		2

Positive tissue controls: 0 Negative tissue controls: 0  
Total studies: 167 Total slides: 2058

Slide setup summary **Print labels**

# Chapter 5. Run the RNAscope 2.5 LS Duplex 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"> <li>• RNAscope 2.5 LS C1 Target Probe</li> <li>• RNAscope 2.5 LS C2 Target Probe (50X)</li> <li>• RNAscope 2.5 LS Duplex Positive Control Probe</li> <li>• RNAscope 2.5 LS Duplex Negative Control Probe</li> <li>• RNAscope 2.5 LS Hydrogen Peroxide</li> <li>• RNAscope 2.5 LS Protease III</li> <li>• RNAscope 2.5 LS Duplex AMP 1</li> <li>• RNAscope 2.5 LS Duplex AMP 2</li> <li>• RNAscope 2.5 LS Duplex AMP 3</li> <li>• RNAscope 2.5 LS Duplex AMP 4</li> <li>• RNAscope 2.5 LS Duplex AMP 5</li> <li>• RNAscope 2.5 LS Duplex AMP 6</li> <li>• RNAscope 2.5 LS Duplex AMP 7</li> <li>• RNAscope 2.5 LS Duplex AMP 8</li> <li>• RNAscope 2.5 LS Duplex AMP 9</li> <li>• RNAscope 2.5 LS Duplex AMP 10</li> <li>• RNAscope 2.5 LS Duplex Wash</li> <li>• RNAscope 2.5 LS Rinse</li> <li>• RNAscope 2.5 LS Bluing Reagent</li> </ul> <p><b>RNAscope 2.5 LS Green Accessory Pack (Appendix B. Semi-automated Reg/green)</b></p> <ul style="list-style-type: none"> <li>• 50X Wash Buffer</li> <li>• Green A</li> <li>• Green B</li> </ul>	<p>Leica Biosystems' BOND RX System</p> <p>Bulk Reagents</p> <ul style="list-style-type: none"> <li>• BOND Wash Solution, 10X</li> <li>• BOND Dewax Solution</li> <li>• BOND Epitope Retrieval Solution 1</li> <li>• BOND Epitope Retrieval Solution 2</li> </ul> <p>Reagents</p> <ul style="list-style-type: none"> <li>• BOND Polymer Refine Detection (DAB) plus Hematoxylin</li> <li>• BOND Polymer Refine Red Detection</li> <li>• Bond DAB enhancer (Red/DAB workflow only)</li> <li>• Leica Green Chromogen (Appendix C. Fullu automated red/green workflow)</li> </ul>	<ul style="list-style-type: none"> <li>• Distilled water</li> <li>• Xylene</li> <li>• Fume hood</li> <li>• Tissue-Tek Staining Dish</li> <li>• Tissue-Tek Clearing Agent Dish, xylene-resistant (2)</li> <li>• Tissue-Tek Vertical 24 Slide Rack</li> <li>• Drying oven</li> <li>• EcoMount or Vectamount or PERTEX® for Brown/Red</li> <li>• Vectamount ONLY for GREEN/Red</li> <li>• Cover Glass, 24 mm x 50 mm</li> </ul>

## Prepare the instrument

If running a fully automated duplex assay (Red and Brown), only run a maximum of two trays. Running three trays will result in significant instrument errors including loss of dispensers. If running a semi-automated duplex assay (Red and Green), you can use all three trays (see **Appendix B**).

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. Obtain one empty 30 mL 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. Prepare LS Duplex probe mix:
  - a. Determine the volume of duplex probe needed.
  - b. In an LS 30 mL container, dilute the 50X C2 probe stock 1:50 with the Ready-To-Use C1 probe. For example, add 320 µL 50X C2 probe to 16 mL C1 probe.
  - c. The 2.5 LS Duplex probe mix is stable for one year at 2–8°C.

**Note:** If using other open containers for probe delivery, ensure you account for dead volume required in each container with the suggested following volumes.

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

5. Using the Barcode Scanner, scan the front barcode on the 30 mL Open Bond container. A window will appear.
6. From the drop down menu, select the corresponding name of the reagent as shown in the following table under **Container Name**:

Reagents	Container Name
RNAscope 2.5 LS Hydrogen Peroxide	*Open 0 Haz
RNAscope 2.5 LS Protease III	*ACD Enzyme
RNAscope 2.5 LS Rinse	*LS Rinse
RNAscope 2.5 LS Duplex Wash	Duplex Wash
RNAscope 2.5 LS Duplex AMP 1	ACD Duplex Amp 1
RNAscope 2.5 LS Duplex AMP 2	ACD Duplex Amp 2
RNAscope 2.5 LS Duplex AMP 3	ACD Duplex Amp 3
RNAscope 2.5 LS Duplex AMP 4	ACD Duplex Amp 4
RNAscope 2.5 LS Duplex AMP 5	ACD Duplex Amp 5



Reagents	Container Name
RNAscope 2.5 LS Duplex AMP 6	ACD Duplex Amp 6
RNAscope 2.5 LS Duplex AMP 7	ACD Duplex Amp 7
RNAscope 2.5 LS Duplex AMP 8	ACD Duplex Amp 8
RNAscope 2.5 LS Duplex AMP 9	ACD Duplex Amp 9
RNAscope 2.5 LS Duplex AMP 10	ACD Duplex Amp 10
RNAscope 2.5 LS Bluing Reagent	*ACD blue
1X BOND Wash	Mock probe
2.5 LS Duplex probe mix	variable

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

**Note:** Leica BOND DAB plus Hematoxylin, Leica BOND Red Detection, and Leica BOND DAB enhancer come in pre-filled Leica BOND RX containers.

7. Enter the RNAscope 2.5 LS Duplex 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 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 placement and seating of covertile.

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

---

**IMPORTANT!** Before leaving the instrument unattended, ensure that the instrument is running successfully. In the event of a problem, please contact ACD or Leica support to resolve.

---

**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 and remove covertiles
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.



## Dry and mount the samples

1. Remove the slide rack from the staining dish and dry slides in a **60°C** dry oven for **30 MIN.**

---

**IMPORTANT!** The RED substrate is alcohol sensitive. Do not dehydrate the slides in alcohol.

---

2. Cool the slides for **5 MIN** at **RT.**
3. Briefly dip one slide into fresh pure xylene and *immediately* place 1–2 drops of EcoMount or Vectamount on the slide before the xylene dries.

---

**IMPORTANT!** Use EcoMount or Vectamount or PERTEX mounting media.

---

4. Carefully place 24 mm x 50 mm coverslip over the tissue section. Avoid trapping air bubbles.
5. Repeat steps 3 and 4 for each slide.
6. Air dry slides for **5 MIN.**
7. Proceed to **Chapter 6. Evaluate the Results.**

# 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 Duplex 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:

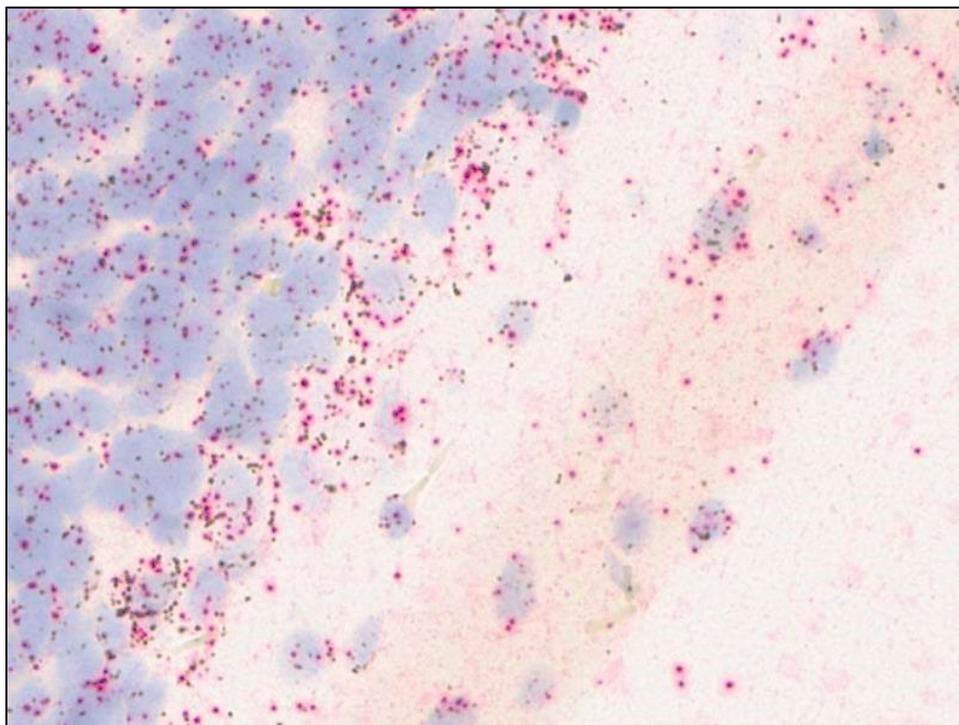
Staining Score	Microscope Objective Scoring*
0	No staining, or less than 1 dot for every 10 cells (40X magnification)
1	1–3 dots/cell (visible at 20–40X magnification)
2	4–9 dots/cell. No, or very few dot clusters (visible at 20–40X magnification)
3	10–15 dots/cell and /or< 10% positive cells have dot 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 LS Duplex Assay detection of PPIB/POLR2A mRNA in liver tumor 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 or have over-fixed samples, increase the Epitope Retrieval 2 (ER2) in increments of five minutes and increase the Enzyme time in increments of ten minutes (see **Appendix E**).
- The RNAscope 2.5 LS Duplex Assay utilizes Leica Biosystems' BOND Polymer Refine Detection and Bond Polymer Refine Red Detection kits. 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](mailto:support.acd@bio-techne.com).



# Appendix A. Red/Brown Duplex 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.

## Part I: ISH protocol (ACD 2.5 DAB Rev B)

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 Solution	Reagent	0 MIN	42°C
5	*Bond Wash Solution	Reagent	1 MIN	42°C
6	*Bond Wash Solution	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 Duplex Amp 1	Reagent	1 MIN	42°C
16	ACD Duplex 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



Step No.	Reagent	Step Type	Incubation Time	Temperature
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
31	ACD Duplex Amp 2	Reagent	1 MIN	42°C
32	ACD Duplex 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	Open Wash	1 MIN	Ambient
40	*Bond Wash Solution	Wash	1 MIN	Ambient
41	ACD Duplex Amp 3	Reagent	1 MIN	42°C
42	ACD Duplex 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 Duplex Amp 4	Reagent	1 MIN	42°C
58	ACD Duplex 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



Step No.	Reagent	Step Type	Incubation Time	Temperature
65	*Bond Wash Solution	Open Wash	1 MIN	Ambient
66	*Bond Wash Solution	Wash	1 MIN	Ambient
67	ACD Duplex Amp 5	Reagent	1 MIN	Ambient
68	ACD Duplex Amp 5	Reagent	15 MIN	Ambient
69	*Bond Wash Solution	Wash	0 MIN	Ambient
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 Duplex Amp 6	Reagent	1 MIN	Ambient
78	ACD Duplex Amp 6	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	Duplex wash	Reagent	5 MIN	Ambient
88	Duplex wash	Reagent	5 MIN	Ambient
89	*Mixed Red Refine	Reagent	1 MIN	Ambient
90	*Mixed Red Refine	Reagent	10 MIN	Ambient
91	*Bond Wash Solution	Wash	0 MIN	Ambient
92	*Bond Wash Solution	Wash	0 MIN	Ambient
93	*Bond Wash Solution	Wash	0 MIN	Ambient
94	*Bond Wash Solution	Wash	1 MIN	Ambient
95	*Bond Wash Solution	Wash	1 MIN	Ambient
96	*Bond Wash Solution	Wash	1 MIN	Ambient
97	*Bond Wash Solution	Wash	1 MIN	Ambient
98	*Bond Wash Solution	Wash	1 MIN	Ambient
99	ACD Duplex Amp 7	Reagent	1 MIN	42°C
100	ACD Duplex Amp 7	Reagent	15 MIN	42°C
101	*Bond Wash Solution	Wash	0 MIN	Ambient



Step No.	Reagent	Step Type	Incubation Time	Temperature
102	*Bond Wash Solution	Wash	0 MIN	Ambient
103	*Bond Wash Solution	Wash	0 MIN	Ambient
104	*Bond Wash Solution	Wash	1 MIN	Ambient
105	*Bond Wash Solution	Wash	1 MIN	Ambient
106	*Bond Wash Solution	Wash	1 MIN	Ambient
107	*Bond Wash Solution	Wash	1 MIN	Ambient
108	*Bond Wash Solution	Wash	1 MIN	Ambient
109	ACD Duplex Amp 8	Reagent	1 MIN	42°C
110	ACD Duplex Amp 8	Reagent	15 MIN	42°C
111	*Bond Wash Solution	Wash	0 MIN	Ambient
112	*Bond Wash Solution	Wash	0 MIN	Ambient
113	*Bond Wash Solution	Wash	0 MIN	Ambient
114	*Bond Wash Solution	Wash	1 MIN	Ambient
115	*Bond Wash Solution	Wash	1 MIN	Ambient
116	*Bond Wash Solution	Wash	1 MIN	Ambient
117	*Bond Wash Solution	Wash	1 MIN	Ambient
118	*Bond Wash Solution	Wash	1 MIN	Ambient
119	ACD Duplex Amp 9	Reagent	1 MIN	Ambient
120	ACD Duplex Amp 9	Reagent	30 MIN	Ambient
121	*Bond Wash Solution	Wash	0 MIN	Ambient
122	*Bond Wash Solution	Wash	0 MIN	Ambient
123	*Bond Wash Solution	Wash	0 MIN	Ambient
124	*Bond Wash Solution	Wash	1 MIN	Ambient
125	*Bond Wash Solution	Wash	1 MIN	Ambient
126	*Bond Wash Solution	Wash	1 MIN	Ambient
127	*Bond Wash Solution	Wash	1 MIN	Ambient
128	*Bond Wash Solution	Wash	1 MIN	Ambient
129	ACD Duplex Amp 10	Reagent	1 MIN	Ambient
130	ACD Duplex Amp 10	Reagent	15 MIN	Ambient
131	*Bond Wash Solution	Wash	0 MIN	Ambient
132	*Bond Wash Solution	Wash	0 MIN	Ambient
133	*Bond Wash Solution	Wash	0 MIN	Ambient
134	*Bond Wash Solution	Wash	1 MIN	Ambient
135	*Bond Wash Solution	Wash	1 MIN	Ambient
136	*Bond Wash Solution	Wash	1 MIN	Ambient
137	*Bond Wash Solution	Wash	1 MIN	Ambient
138	*Bond Wash Solution	Wash	1 MIN	Ambient

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



---

**IMPORTANT!** You cannot directly edit the temperatures of these steps. Only the incubation times can be edited.

---

## Part II: IHC protocol (IHC Protocol F)

Step No.	Reagent	Step Type	Incubation Time	Temperature
1	*LS Rinse	Reagent	5 MIN	Ambient
2	*LS Rinse	Reagent	5 MIN	Ambient
3	*Mixed DAB Refine	Reagent	1 MIN	Ambient
4	*Mixed DAB Refine	Reagent	20 MIN	Ambient
5	*De-ionized Water	Wash	0 MIN	Ambient
6	*De-ionized Water	Wash	0 MIN	Ambient
7	*De-ionized Water	Wash	0 MIN	Ambient
8	*De-ionized Water	Wash	0 MIN	Ambient
9	*De-ionized Water	Wash	0 MIN	Ambient
10	*De-ionized Water	Wash	0 MIN	Ambient
11	*Bond DAB Enhancer	Reagent	20min	Ambient
12	*De-ionized Water	Wash	0 MIN	Ambient
13	*De-ionized Water	Wash	0 MIN	Ambient
14	*De-ionized Water	Wash	0 MIN	Ambient
15	*De-ionized Water	Wash	0 MIN	Ambient
16	*Hematoxylin	Reagent	5 MIN	Ambient
17	*De-ionized Water	Wash	0 MIN	Ambient
18	*De-ionized Water	Wash	0 MIN	Ambient
19	*De-ionized Water	Wash	0 MIN	Ambient
20	*De-ionized Water	Wash	0 MIN	Ambient
21	*ACD blue	Reagent	2 MIN	Ambient
22	*De-ionized Water	Wash	0 MIN	Ambient
23	*De-ionized Water	Wash	0 MIN	Ambient
24	*De-ionized Water	Wash	0 MIN	Ambient
25	*De-ionized Water	Wash	0 MIN	Ambient

# B

## Appendix B. Semi-Automated Red/Green Duplex Staining

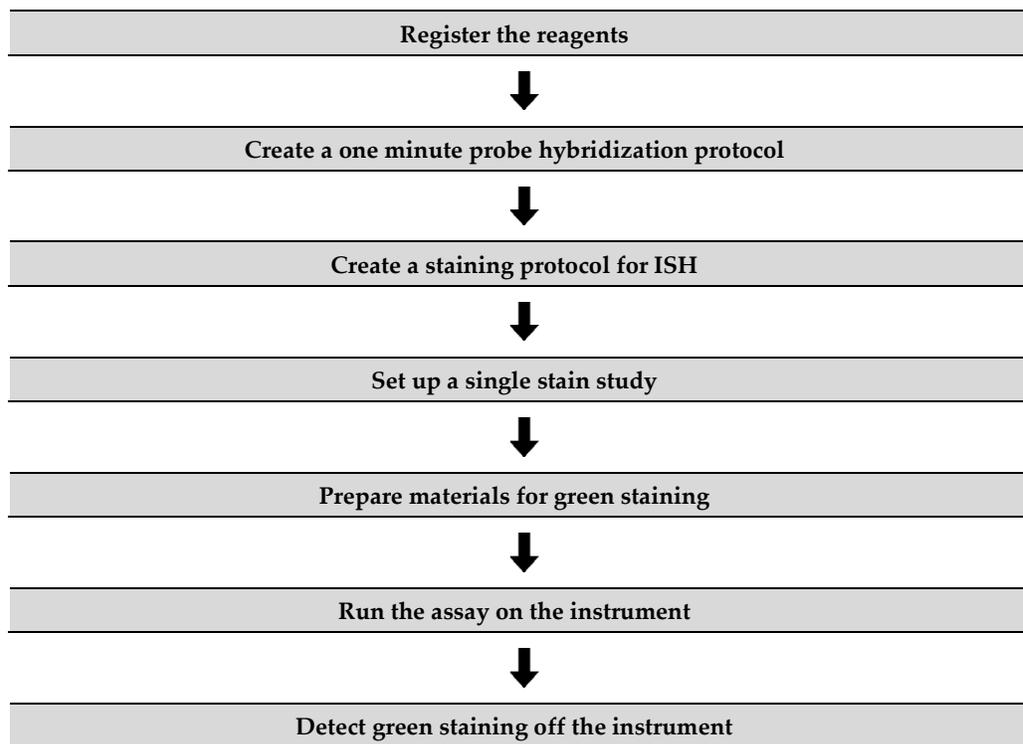
Use the following procedure to replace the brown chromogen with green using the RNAscope 2.5 LS Green Accessory Pack (Cat. No. 322550). Green staining is performed off the instrument.

---

**IMPORTANT!** If running a fully automated duplex assay (Red and Brown), only run a maximum of two trays. Running three trays will result in significant instrument errors including loss of dispensers. If running a semi-automated duplex assay (Red and Green), you can use all three trays.

---

### Workflow





## Materials required

Materials provided by Advanced Cell Diagnostics	Materials provided by Leica Biosystems	Other materials and equipment
RNAscope 2.5 LS Probes <ul style="list-style-type: none"> <li>• RNAscope 2.5 LS C1 Target Probe</li> <li>• RNAscope 2.5 LS C2 Target Probe (50X)</li> <li>• RNAscope 2.5 LS Duplex Positive Control Probe</li> <li>• RNAscope 2.5 LS Duplex Negative Control Probe</li> <li>• RNAscope 2.5 LS Duplex Reagents</li> <li>• RNAscope 2.5 LS Hydrogen Peroxide</li> <li>• RNAscope 2.5 LS Protease III</li> <li>• RNAscope 2.5 LS Duplex AMP 1</li> <li>• RNAscope 2.5 LS Duplex AMP 2</li> <li>• RNAscope 2.5 LS Duplex AMP 3</li> <li>• RNAscope 2.5 LS Duplex AMP 4</li> <li>• RNAscope 2.5 LS Duplex AMP 5</li> <li>• RNAscope 2.5 LS Duplex AMP 6</li> <li>• RNAscope 2.5 LS Duplex AMP 7</li> <li>• RNAscope 2.5 LS Duplex AMP 8</li> <li>• RNAscope 2.5 LS Duplex AMP 9</li> <li>• RNAscope 2.5 LS Duplex AMP 10</li> <li>• RNAscope 2.5 LS Duplex Wash</li> <li>• RNAscope 2.5 LS Rinse</li> </ul> <b>RNAscope 2.5 LS Green Accessory Pack</b> <ul style="list-style-type: none"> <li>• 50X Wash Buffer</li> <li>• Green A</li> <li>• Green B</li> </ul>	Leica Biosystems' BOND RX System Bulk Reagents <ul style="list-style-type: none"> <li>• BOND Wash Solution, 10X</li> <li>• BOND Dewax Solution</li> <li>• BOND Epitope Retrieval Solution 1</li> <li>• BOND Epitope Retrieval Solution 2 Reagents</li> <li>• BOND Polymer Refine Red Detection</li> </ul>	<ul style="list-style-type: none"> <li>• Distilled water</li> <li>• Carboy (&gt;3L)</li> <li>• Fume hood</li> <li>• Xylene</li> <li>• 100% alcohol (EtOH)</li> <li>• Tissue-Tek Staining Dishes</li> <li>• Tissue-Tek Clearing Agent Dishes</li> <li>• Gill's Hematoxylin</li> <li>• (Optional) 20X SSC</li> <li>• Graduated cylinder</li> <li>• Parafilm</li> <li>• Water bath or incubator</li> <li>• Tissue-Tek Vertical 24 Slide Rack</li> <li>• Tubes (various sizes)</li> <li>• Paper towel or absorbent paper</li> <li>• Pipettors and tips, 1–1000 µL</li> <li>• Microcentrifuge</li> <li>• Dry oven</li> <li>• Cover Glass, 24 mm x 50 mm</li> <li>• VectaMount</li> <li>• 28% Ammonium Hydroxide</li> </ul>

### RNAscope 2.5 LS Green Accessory Reagents

The RNAscope 2.5 LS Green Accessory Pack (Cat. No. 322550) contains the reagents needed to replace the brown chromogen with green. The kits provide enough reagents to stain ~60 standard slides.



The reagents are stored as indicated in the following table:

RNAscope 2.5 LS Green Accessory Pack (Cat. No. 322550)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	RNAscope 2.5 LS Duplex Green A	12 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS Duplex Green B	240 µL x 1 vial	2–8°C
	RNAscope 50X Wash Buffer	60 mL x 4 bottles	Room temperature (15–30°C)

## VectaMount Mounting Medium

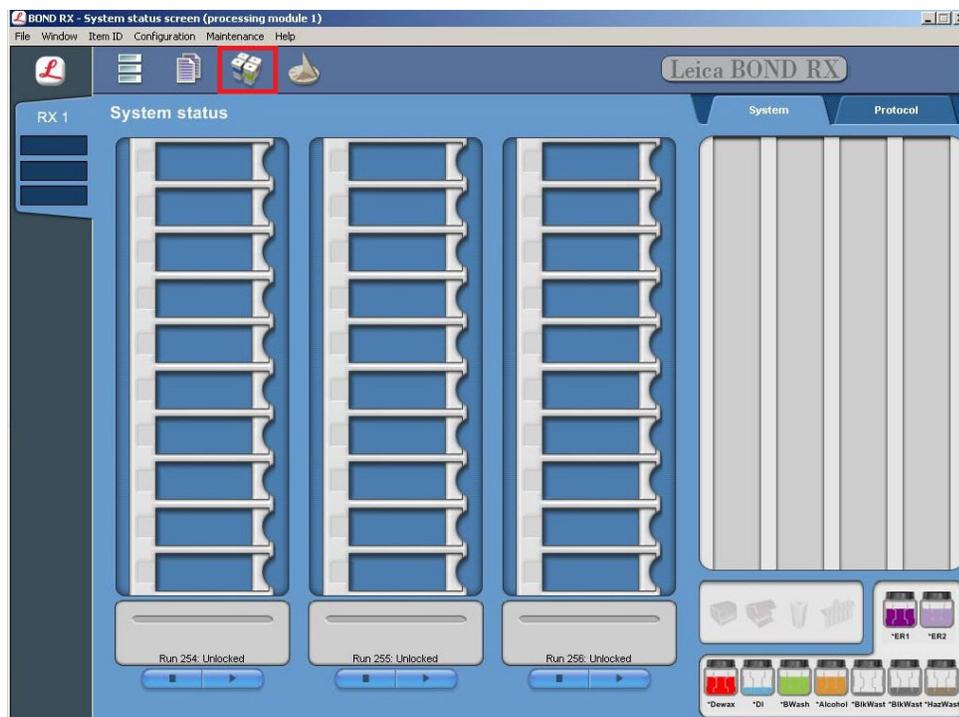
VectaMount Mounting Medium (Cat. No. 321584) is also required to perform the RNAscope 2.5 LS Red/Green Duplex Assay. The reagent is stored as indicated in the following table:

VectaMount Mounting Medium (Cat. No. 321584)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	VectaMount Mounting Medium	60 mL x 1 bottle	Room temperature (15–30°C)

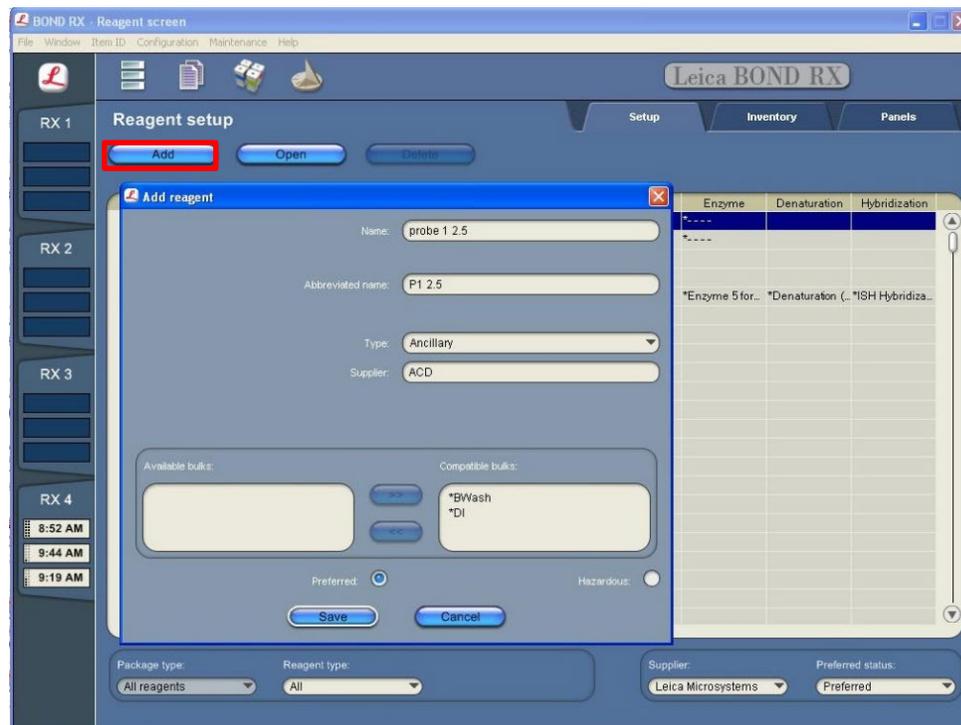
## Register the reagents

This step is a “work around” to the existing BDZ 11 software to accommodate the RNAscope 2.5 LS Duplex assay. Your ACD Field Application Specialist (FAS) should implement this procedure.

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 in the Name text box.



4. Enter a name 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. Repeat Steps 2–7 to register the rest of the reagents using the container names in the following table:

Reagents	Container Name
RNAscope 2.5 LS Duplex Wash	Duplex Wash
RNAscope 2.5 LS Duplex AMP 1	ACD Duplex Amp 1
RNAscope 2.5 LS Duplex AMP 2	ACD Duplex Amp 2
RNAscope 2.5 LS Duplex AMP 3	ACD Duplex Amp 3
RNAscope 2.5 LS Duplex AMP 4	ACD Duplex Amp 4
RNAscope 2.5 LS Duplex AMP 5	ACD Duplex Amp 5
RNAscope 2.5 LS Duplex AMP 6	ACD Duplex Amp 6
RNAscope 2.5 LS Duplex AMP 7	ACD Duplex Amp 7
RNAscope 2.5 LS Duplex AMP 8	ACD Duplex Amp 8

Reagents	Container Name
RNAscope 2.5 LS Duplex AMP 9	ACD Duplex Amp 9
RNAscope 2.5 LS Duplex AMP 10	ACD Duplex Amp 10

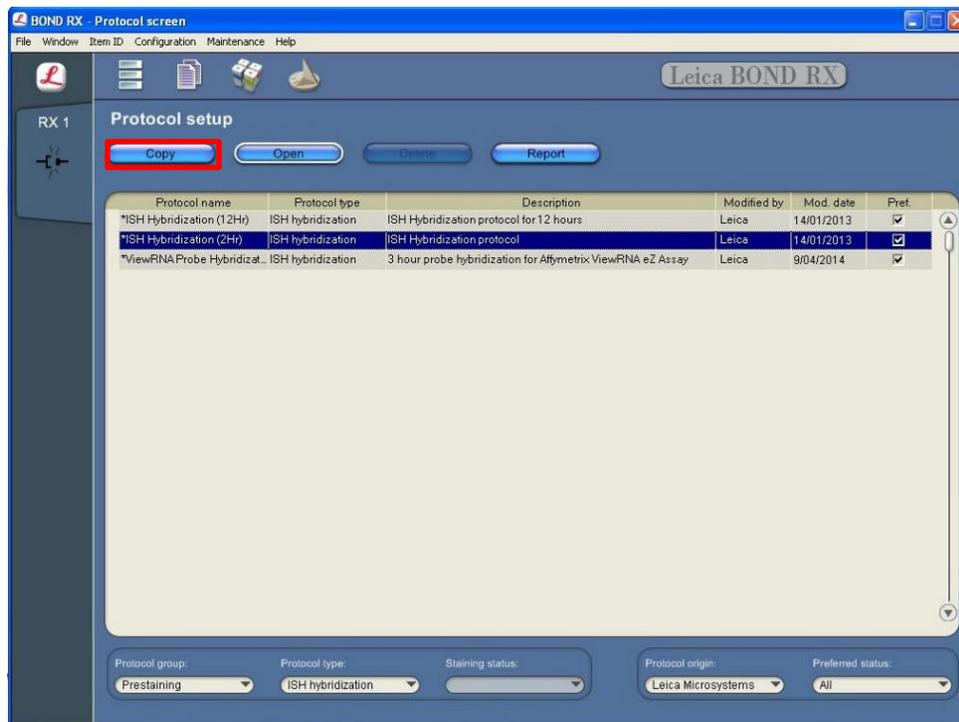
\* Indicates step is hard-coded in software by Leica Biosystems.

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

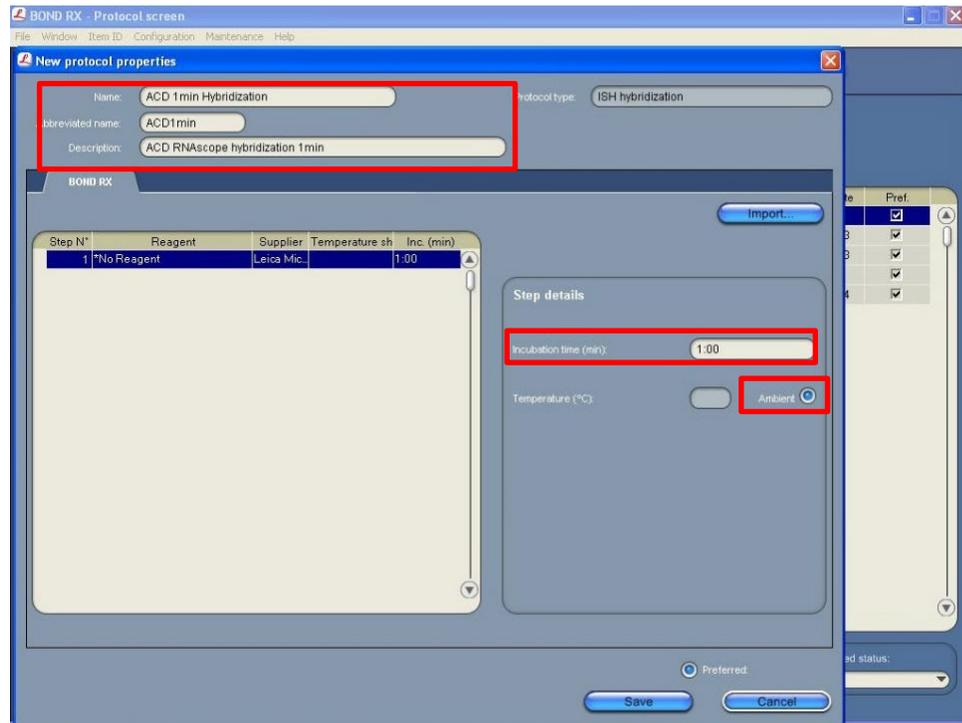
## Create a one-minute probe hybridization protocol

A mock probe hybridization step must be created as part of BDZ 11 software workaround for the RNAscope 2.5 LS Duplex assay. 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**.

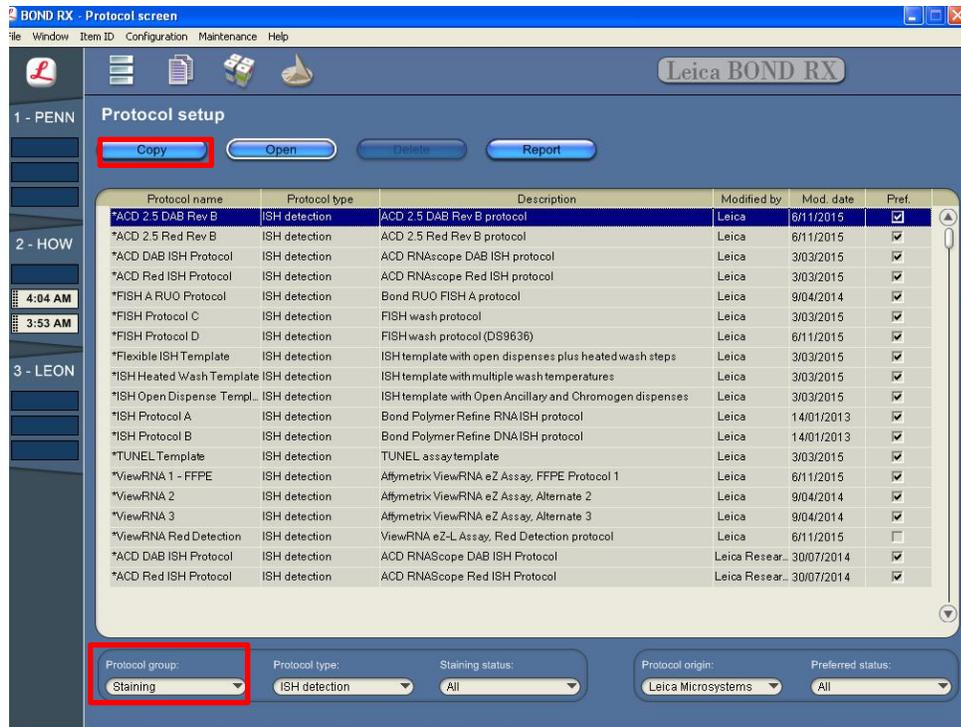


4. Highlight the **\*No Reagent** step.
5. Change the incubation time to **1 MIN** and select **Ambient** as Temperature (°C).
6. Select **Save**.

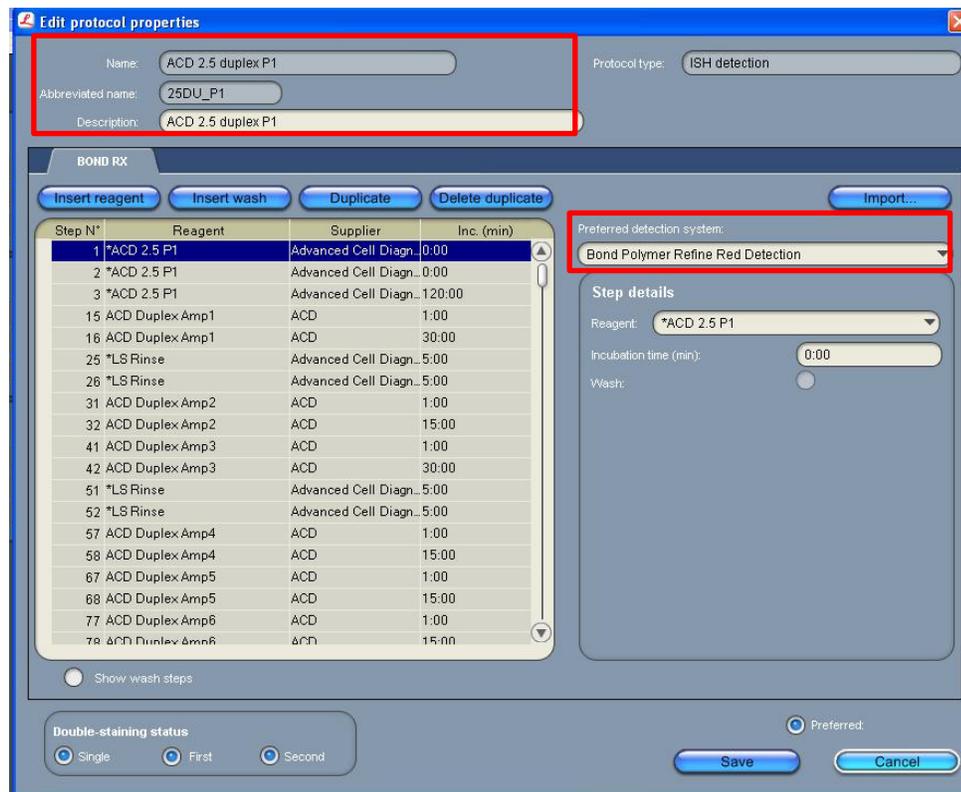
## Create a staining protocol for In Situ Hybridization (ISH)

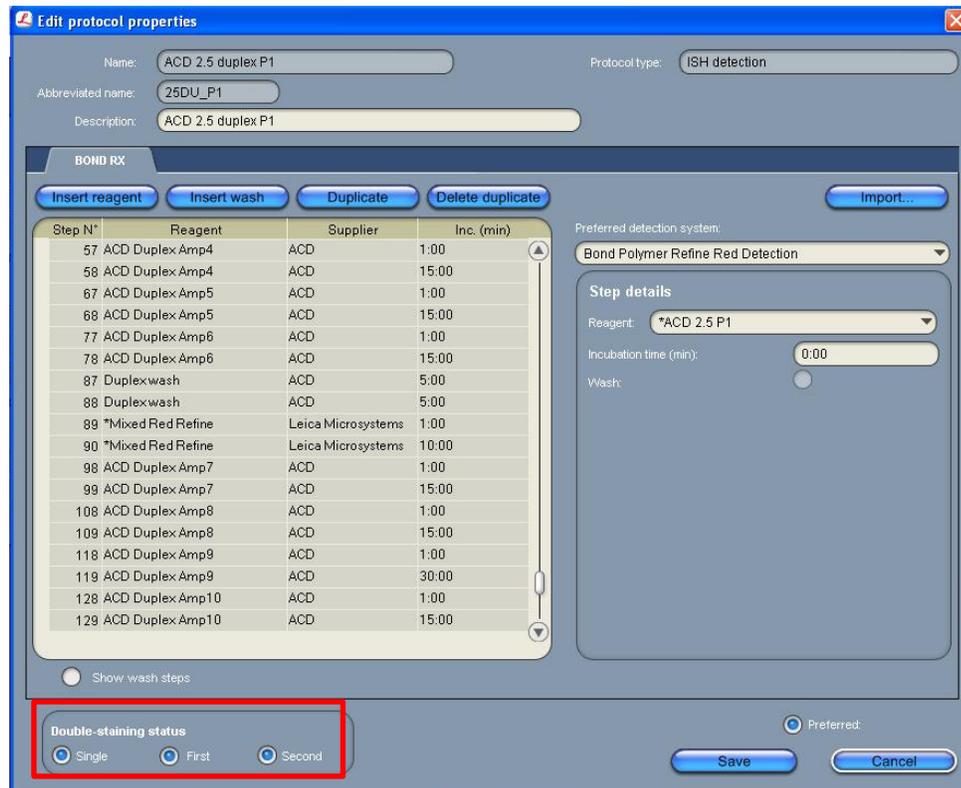
Due to the BDZ 11 software workaround for the RNAscope 2.5 LS Duplex assay, unique staining protocols *must be created for each probe*.

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 duplex P1** in the Name text box, **25DU\_P1** in the Abbreviated name text box, and **ACD 2.5 duplex P1** in the Description text box.
4. Select **Bond Polymer Refine Red Detection** from the Preferred detection system menu.





**Note:** The preceding two figures display all reagent steps.

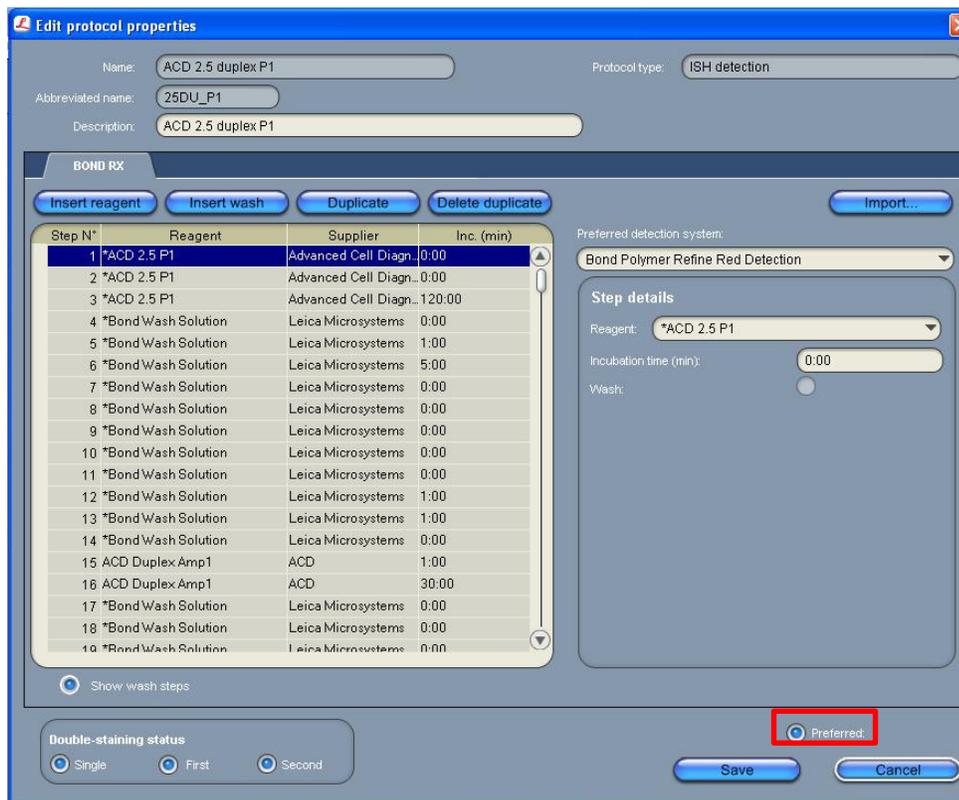
5. Highlight and select each Reagent step to edit.

---

**IMPORTANT!** You can only change the incubation times, not the temperatures, of these steps. To be able to use all three trays for the semi-automated red and green duplex assay, strictly follow the protocol steps listed in **Appendix A**. Adding any other steps will prevent the system from running all three trays.

---

6. Click **Show wash steps** (see the following figure) to also view the wash steps. Insert BOND Washes to match each of the protocol steps shown.
7. Compare and confirm screen protocol with the full protocol listed in **Appendix A**.
8. Select **Preferred** in the bottom right corner of the window.

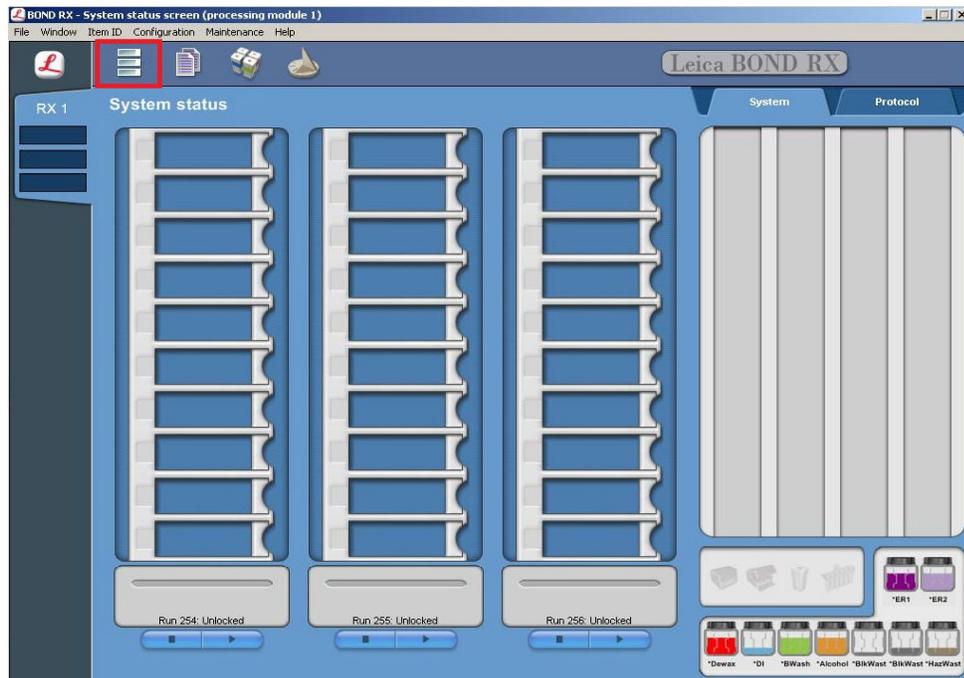


9. Select **Save**.
10. Click **Next** to proceed. Ignore any pop ups that may appear on the screen.
11. Create a new probe protocol.

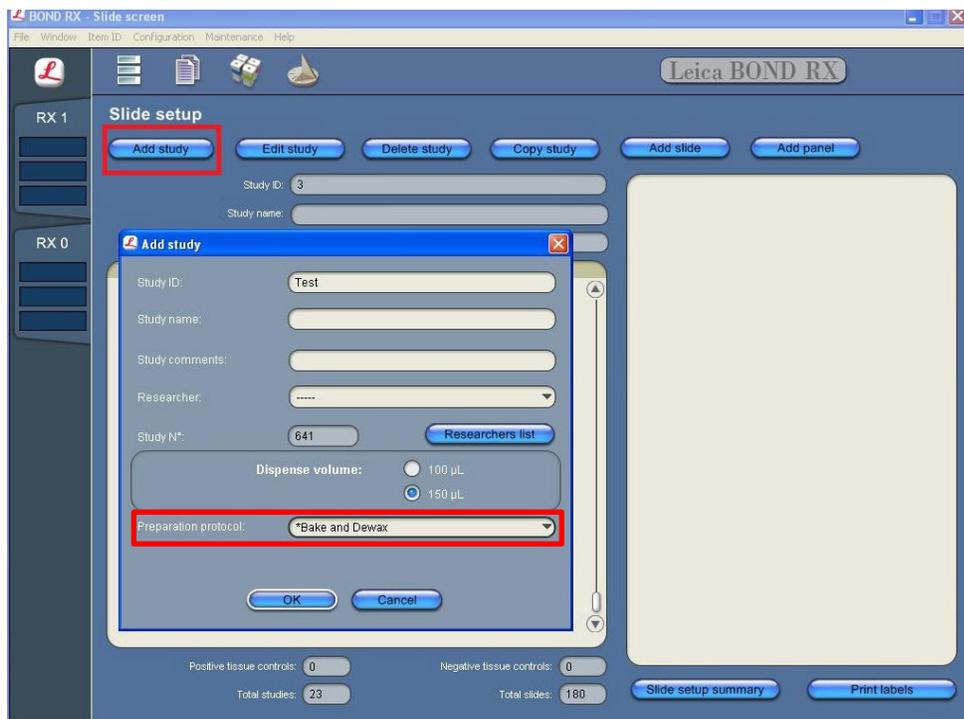
**Note:** You must create a new protocol for each new probe you use.

## Set up a single stain study

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

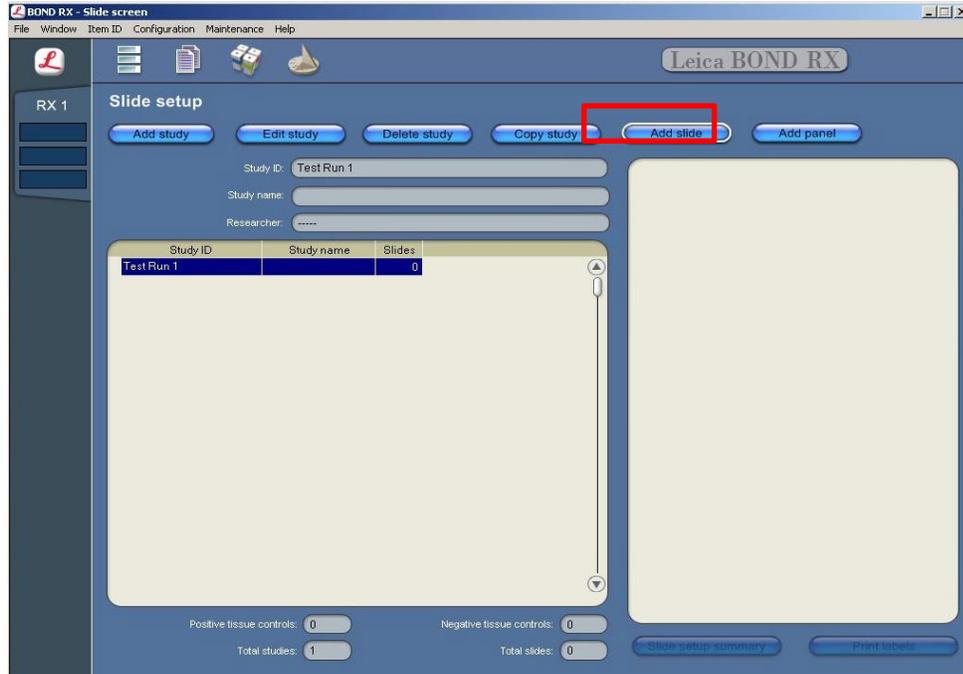


2. Select **Add study** and enter a name in the Study ID field (keep the Dispense volume at 150  $\mu$ L as shown).



3. Select **\*Bake and Dewax** as the Preparation protocol.

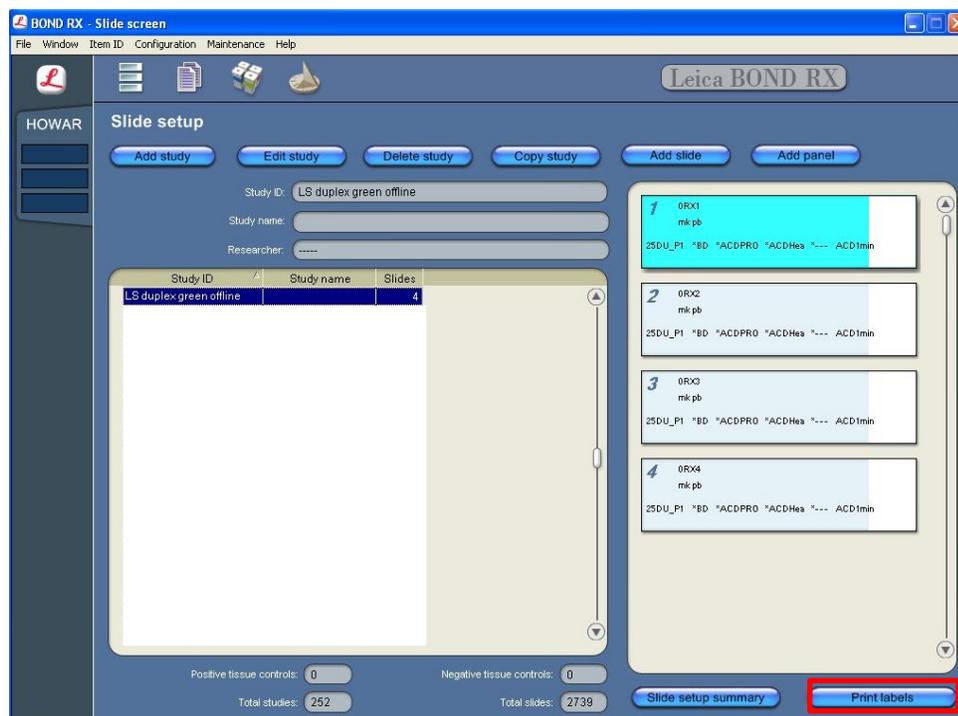
4. Select **OK**.
5. Select **Add slide** to assign a protocol to each slide.



6. Enter the tissue type and probe name under the Comments field.
7. Make sure the selection is **Single** from the Staining mode selection drop down menu.
  - a. Make sure that each probe is associated with a different protocol (for example, ACD 2.5 duplex P1), 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.



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



## Prepare materials for green staining

### (Optional) Prepare 5X SSC to store slides

- Prepare 200 mL of 5X SSC by diluting 50 mL of 20X SSC with 150 mL distilled water. Mix well.

**Note:** Prepare 5X SSC if you are planning on storing your slides before performing green staining off of the instrument. The slides can be stored up to 1h before performing the green staining offline.

### Prepare 1X Wash Buffer

**Note:** Warm 50X Wash Buffer up to 40°C for 10–20 MIN before making 1X Wash Buffer. 1X Wash Buffer may be prepared ahead of time and stored at room temperature for up to one month.

- Prepare 3 L of 1X Wash Buffer by adding 2.94 L distilled water and 1 bottle (60 mL) of 50X Wash Buffer to a large carboy. Mix well.

### Prepare counterstaining reagents

1. In the fume hood, prepare 50% **Gill's-Hematoxylin I** staining solution by adding 100 mL Gill's Hematoxylin I to 100 mL distilled water in a staining dish.
2. Seal the cylinder with parafilm. Mix well three to five times.



3. For Optional Bluing: In the fume hood, prepare 0.02% (w/v) Ammonia water (bluing reagent) by adding 1.43 mL of 1N Ammonium Hydroxide to 250 mL distilled water in a graduated cylinder or other container
4. Seal the cylinder with parafilm. Mix well **3–5 times**.

**Note:** 50% Hematoxylin staining solution can be reused for up to one week.

**Note:** For assay quantitation, it is critical to use Ammonium Hydroxide.

## Run the assay on the instrument

### 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. Obtain one empty 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. Prepare LS Duplex probe mix:
  - a. Determine the volume of duplex probe needed.
  - b. In an LS 30 mL container, dilute the 50X C2 probe stock 1:50 into the Ready-To-Use C1 probe. For example, add 320  $\mu$ L 50X C2 probe to 16 mL C1 probe.

**Note:** The 2.5 LS Duplex probe mix is stable for one year at 2–8°C.

**Note:** If using other open containers for probe delivery, ensure you account for dead volume required in each container with volumes suggested in table below.

Open Container	Suggested Dead-Volume
30 mL	2.5 mL
7 mL	1 mL
6 mL	600 $\mu$ L

5. Using the Barcode Scanner, scan the front barcode on the 30 mL Open Bond container. A window will appear.
6. From the drop down menu, select the corresponding name of the reagent as shown in the following table under **Container Name**:



Reagents	Container Name
RNAscope 2.5 LS Hydrogen Peroxide	*Open 0 Haz
RNAscope 2.5 LS Protease III	*ACD Enzyme
RNAscope 2.5 LS Rinse	*LS Rinse
RNAscope 2.5 LS Duplex Wash	Duplex Wash
RNAscope 2.5 LS Duplex AMP 1	ACD Duplex Amp 1
RNAscope 2.5 LS Duplex AMP 2	ACD Duplex Amp 2
RNAscope 2.5 LS Duplex AMP 3	ACD Duplex Amp 3
RNAscope 2.5 LS Duplex AMP 4	ACD Duplex Amp 4
RNAscope 2.5 LS Duplex AMP 5	ACD Duplex Amp 5
RNAscope 2.5 LS Duplex AMP 6	ACD Duplex Amp 6
RNAscope 2.5 LS Duplex AMP 7	ACD Duplex Amp 7
RNAscope 2.5 LS Duplex AMP 8	ACD Duplex Amp 8
RNAscope 2.5 LS Duplex AMP 9	ACD Duplex Amp 9
RNAscope 2.5 LS Duplex AMP 10	ACD Duplex Amp 10
1X BOND Wash	Mock probe
2.5 LS Duplex probe mix	variable

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

**Note:** Leica BOND Red Detection comes in a pre-filled Leica BOND RX container.

7. Enter the RNAscope 2.5 LS Duplex 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 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 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 35.

---

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



## Complete the run

1. As soon as the run is complete, press the button on the front of the instrument and unload the slides *immediately*.
2. Proceed to the following section.

---

**IMPORTANT!** If you do not perform the green assay immediately, store the slides in 5X SSC at room temperature. After the run is completed, do not leave the slides loaded on the instrument. The slides are automatically re-hydrated by the instrument. Do not store the slides in water or wash buffer.

---

## Detect green staining off the instrument

---

**IMPORTANT!** Do *not* let sections dry out between incubation steps. Work *quickly* and make sure the sections are hydrated at all times.

---

**IMPORTANT!** View the wash step video at [www.acdbio.com/technical-support/learn-more](http://www.acdbio.com/technical-support/learn-more) by clicking on the Training Videos Tab before proceeding.

---

## Prepare reagents and equipment

- Remove Green A and Green B reagents from refrigerator, and warm to ambient temperature.

## Detect the green signal

1. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Agitate slides by moving the slide rack up and down in the staining dish.
2. Repeat Step 1 with fresh 1X Wash Buffer.
3. Briefly spin down the contents of the Green B tube to be sure content is at the bottom of the tube before opening the cap.
4. Prepare 200  $\mu\text{L}$  of GREEN working solution per slide using a 1:50 ratio of Green B to Green A. Mix well.

---

**IMPORTANT!** Use the GREEN solution within **5 MIN**. Do not expose to direct sunlight or UV light.

---

5. Take each slide one at a time from the Tissue-Tek Slide Rack and tap and/or flick to remove the excess liquid.
6. Pipette ~200  $\mu\text{L}$  GREEN solution onto each tissue section. Ensure sections are covered.
7. Incubate the slides for **15 MIN** at **RT**.
8. To remove the GREEN working solution from the slides, tilt each slide one at a time over a waste container and tap the corner on the edge of the container. *Immediately* insert the slide into a Tissue-Tek Slide Rack submerged in a Tissue-Tek Staining Dish filled with 1X wash buffer.
9. Wash the slides with 1X wash buffer for **5 MIN** followed by a quick rinse in water to remove excess wash buffer.



## Counterstain the slides

1. Move the Tissue-Tek Slide Rack into the staining dish containing 50% Hematoxylin-I staining solution for **30 SEC** at **RT**. Slides will be purple.

---

**IMPORTANT!** Proceed quickly to the next step. Green substrate may fade if in Hematoxylin for longer than 30 seconds.

---

2. *Immediately* transfer the slide rack into a staining dish filled with tap water. Do not let the slides remain in the water for more than 30 seconds. Repeat wash step once or twice.
3. Replace tap water in the staining dish with 0.02% ammonia water. Move the rack up and down five times. Sections should turn blue.
4. Replace ammonia water with tap water, and wash the slides 3–5 times.

---

**IMPORTANT!** Use *only* 0.02% ammonia water for the bluing step. Commercial bluing solutions may degrade the green signal.

---

**IMPORTANT!** Adhere closely to these instructions. Extended exposure to water and ammonia water can degrade green signal.

---

## Dry and mount the samples

1. Remove the slide rack from the staining dish and dry slides in a **60°C** dry oven for **30 MIN**.

---

**IMPORTANT!** The RED and GREEN substrates are alcohol sensitive. Do not dehydrate the slides in alcohol.

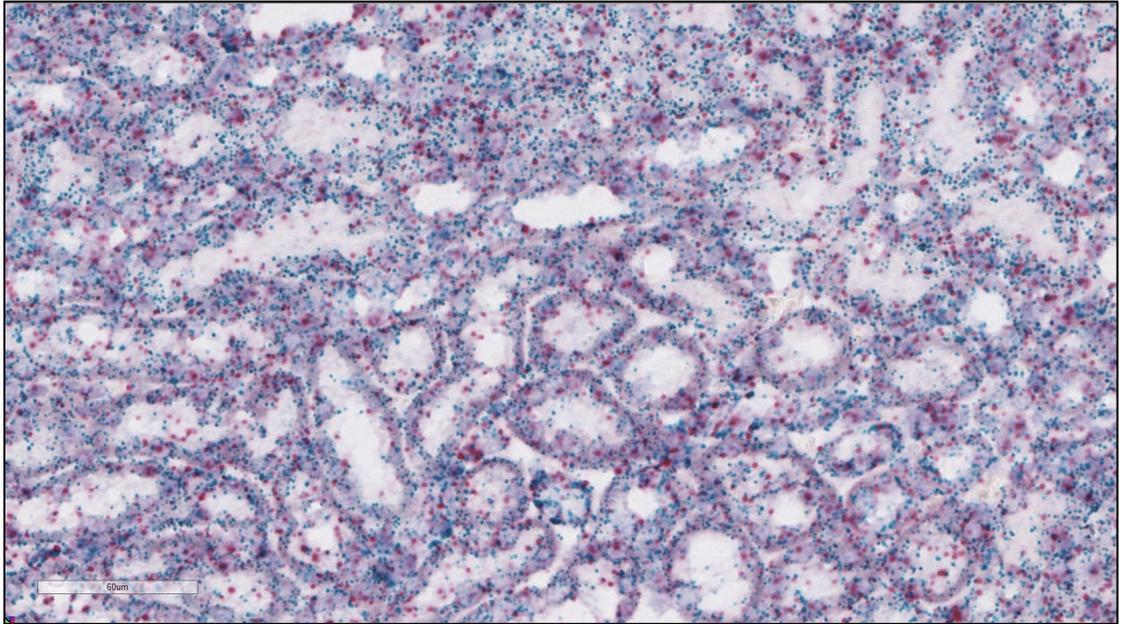
---

2. Cool the slides for **5 MIN** at **RT**.
3. Briefly dip one slide into FRESH pure xylene and *immediately* place 1–2 drops of VectaMount Mounting Medium on the slide before the xylene dries.
4. Carefully place 24 mm x 50 mm coverslip over the tissue section. Avoid trapping air bubbles.
5. Repeat steps 3 and 4 for each slide.
6. Air dry slides for **5 MIN**.
7. Proceed to **Chapter 6. Evaluate the Results**.

## Control example

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

**Figure 3.** Detection of Ppib/Polr2a mRNA in mouse kidney FFPE tissue section using RNAscope 2.5 LS Duplex Assay





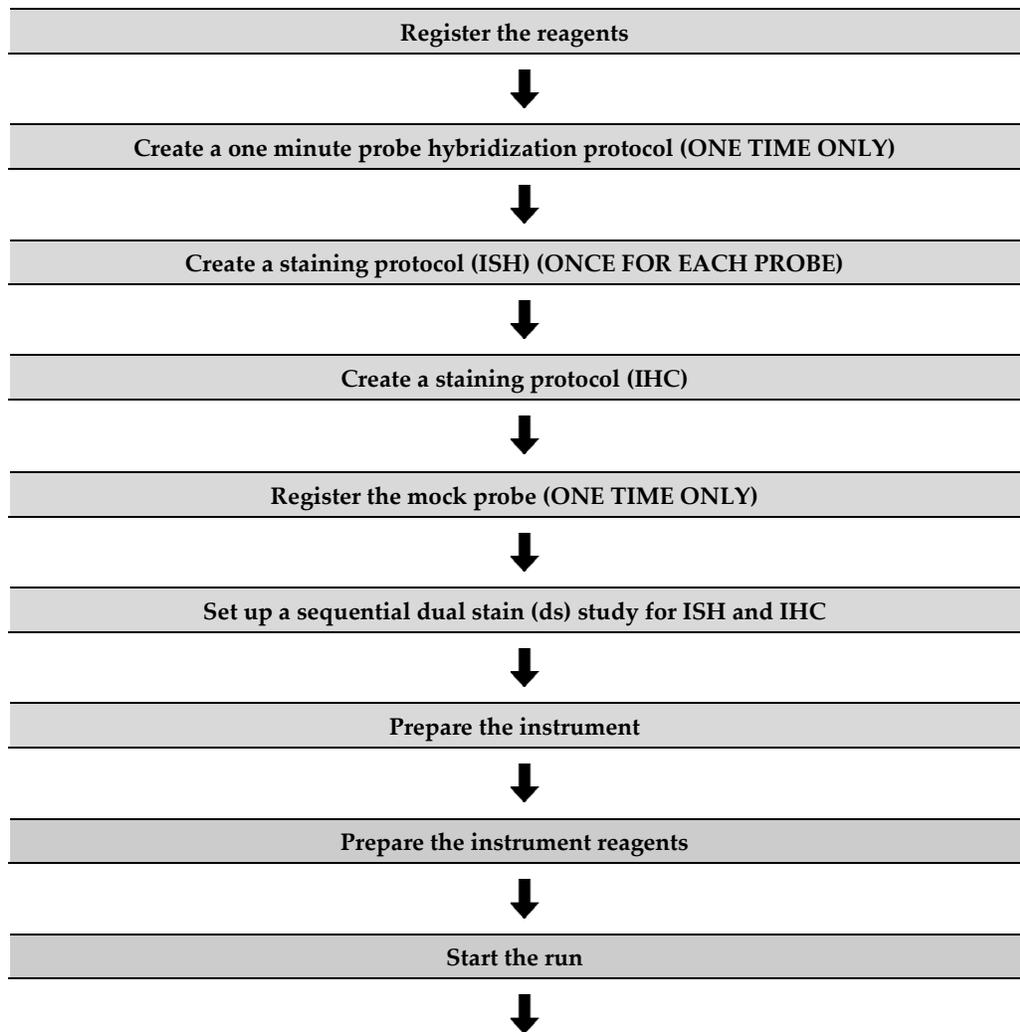
# Appendix C. Fully Automated Red/Green Duplex Protocol

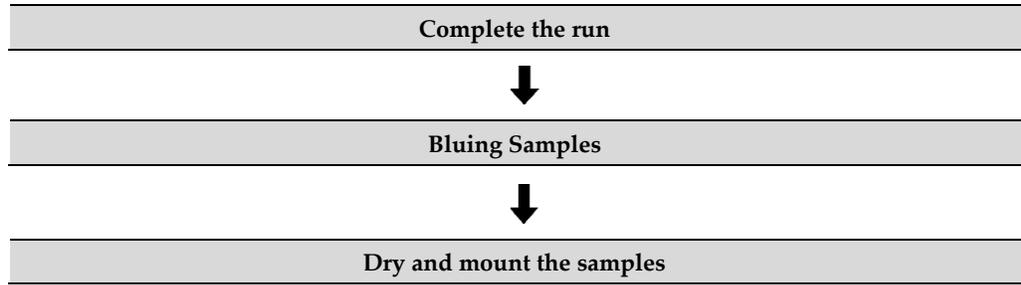
Use the following procedure to replace the brown chromogen with green using the Leica Biosystem's Green Chromogen (Cat. No. DC9913).

**IMPORTANT!** Sample bluing with ammonia water is performed off the instrument due to the incompatibility with green chromogen. Please do not use RNAscope 2.5 LS Bluing Reagent included in the reagent kit when using green chromogen.

**IMPORTANT!** By strictly following the updated protocol steps listed in this appendix, you can use all three trays to run the fully automated red and green duplex assay. Adding any other steps will prevent the system from running all three trays.

## Workflow





Please follow the instructions in **Chapter 4**: "Register the reagents," "Create a one minute probe hybridization protocol" and "Create a staining protocol ISH", if needed. Compare and confirm screen protocol with the full protocol listed in this appendix. The table below displays the complete updated 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 can delete other wash steps.



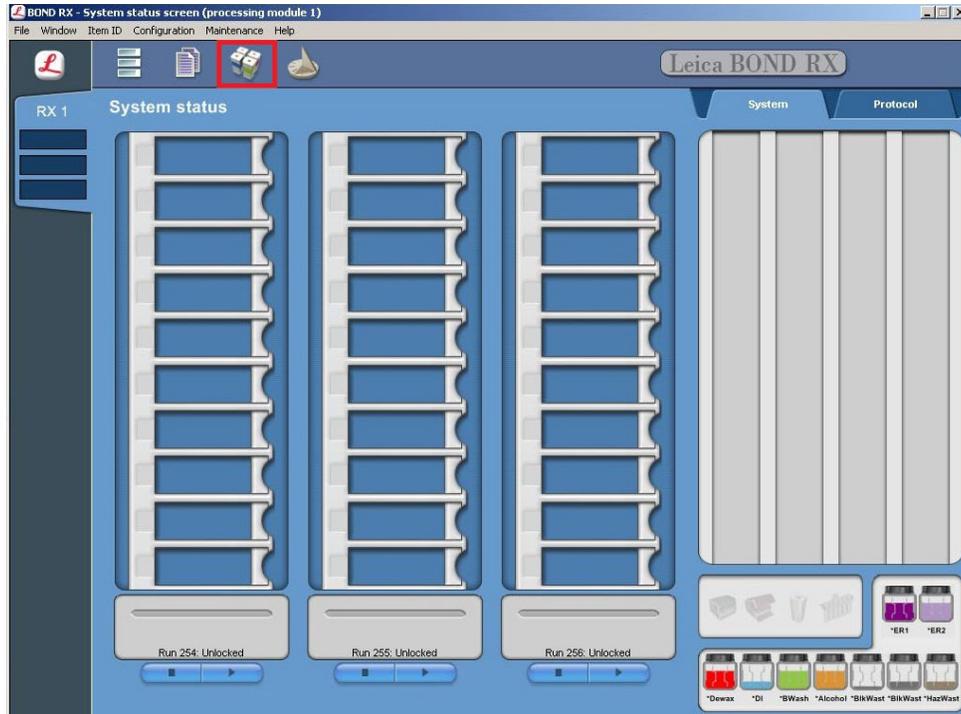
## Materials required

Materials provided by Advanced Cell Diagnostics	Materials provided by Leica Biosystems	Materials provided by User
<ul style="list-style-type: none"> <li>• RNAscope 2.5 LS C1 Target Probe</li> <li>• RNAscope 2.5 LS C2 Target Probe (50X)</li> <li>• RNAscope 2.5 LS Duplex Positive Control Probe</li> <li>• RNAscope 2.5 LS Duplex LS Negative Control Probe</li> <li>• RNAscope 2.5 LS Hydrogen Peroxide</li> <li>• RNAscope 2.5 LS Protease III</li> <li>• RNAscope 2.5 LS Duplex AMP 1</li> <li>• RNAscope 2.5 LS Duplex AMP 2</li> <li>• RNAscope 2.5 LS Duplex AMP 3</li> <li>• RNAscope 2.5 LS Duplex AMP 4</li> <li>• RNAscope 2.5 LS Duplex AMP 5</li> <li>• RNAscope 2.5 LS Duplex AMP 6</li> <li>• RNAscope 2.5 LS AMP 7</li> <li>• RNAscope 2.5 LS Duplex AMP 8</li> <li>• RNAscope 2.5 LS Duplex AMP 9</li> <li>• RNAscope 2.5 LS Duplex AMP 10</li> <li>• RNAscope 2.5 LS Duplex Wash</li> <li>• RNAscope 2.5 LS Rinse</li> <li>• RNAscope 2.5 LS Bluing Reagent--</li> </ul>	<p>Leica Biosystems' BOND RX System</p> <p>Bulk Reagents</p> <ul style="list-style-type: none"> <li>• BOND Wash Solution, 10X</li> <li>• BOND Dewax Solution</li> <li>• BOND Epitope Retrieval Solution 1</li> <li>• BOND Epitope Retrieval Solution 2</li> </ul> <p>Reagents</p> <ul style="list-style-type: none"> <li>• BOND Polymer Refine Red Detection</li> <li>• Green Chromogen Cat. No. DC9913)</li> </ul>	<ul style="list-style-type: none"> <li>• Distilled water</li> <li>• Xylene</li> <li>• Fume hood</li> <li>• Tissue-Tek Staining Dish</li> <li>• Tissue-Tek Clearing Agent Dish, xylene-resistant (2)</li> <li>• Tissue-Tek Vertical 24 Slide Rack</li> <li>• Drying oven</li> <li>• EcoMount or VectaMount or PERTEX for Brown/Red</li> <li>• VectaMount ONLY for GREEN/Red</li> <li>• Cover Glass, 24 mm x 50 mm</li> <li>• 0.02% ammonia water</li> </ul>

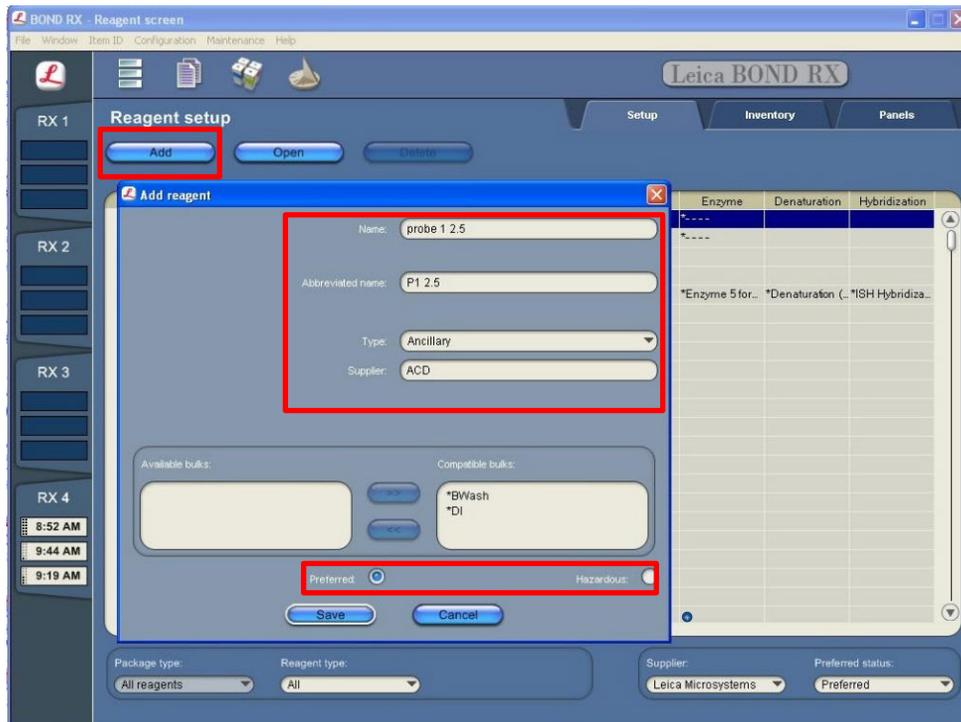
## Register the reagents

This step is a “work around” to the existing BDZ 11 software to accommodate the RNAscope 2.5 LS Duplex assay. Your ACD Field Application Specialist (FAS) should implement this procedure.

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 in the Name text box.



4. Enter a name 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. Repeat Steps 2–7 to register the rest of the reagents using the container names in the following table:

Reagents	Container Name
RNAscope 2.5 LS Duplex Wash	Duplex Wash
RNAscope 2.5 LS Duplex AMP 1	ACD Duplex Amp 1
RNAscope 2.5 LS Duplex AMP 2	ACD Duplex Amp 2
RNAscope 2.5 LS Duplex AMP 3	ACD Duplex Amp 3
RNAscope 2.5 LS Duplex AMP 4	ACD Duplex Amp 4
RNAscope 2.5 LS Duplex AMP 5	ACD Duplex Amp 5
RNAscope 2.5 LS Duplex AMP 6	ACD Duplex Amp 6
RNAscope 2.5 LS Duplex AMP 7	ACD Duplex Amp 7
RNAscope 2.5 LS Duplex AMP 8	ACD Duplex Amp 8
RNAscope 2.5 LS Duplex AMP 9	ACD Duplex Amp 9
RNAscope 2.5 LS Duplex AMP 10	ACD Duplex Amp 10

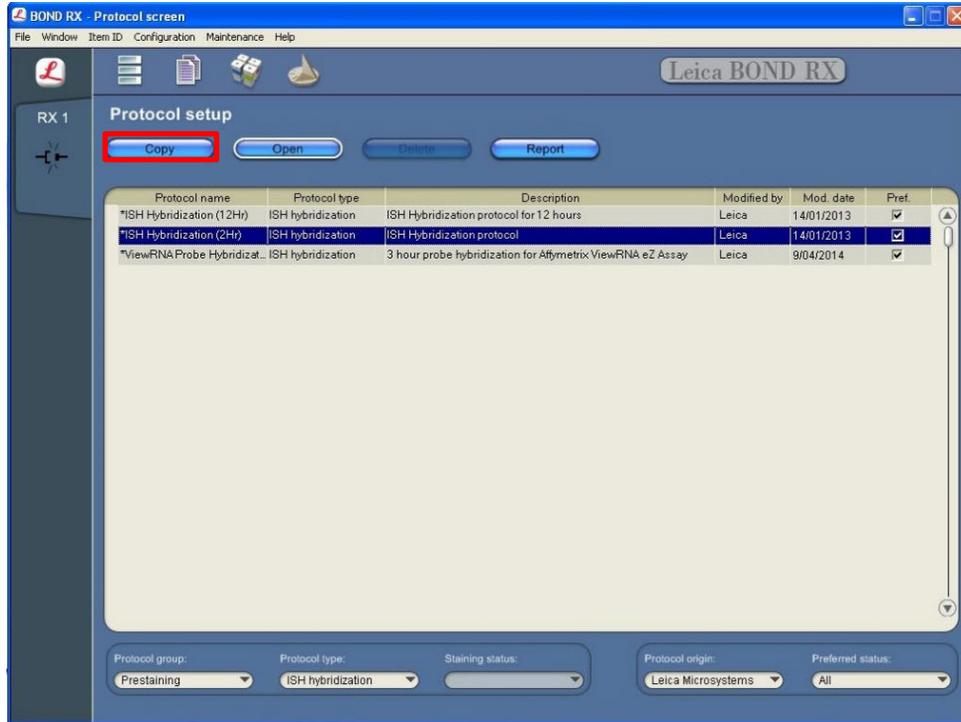
\*

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

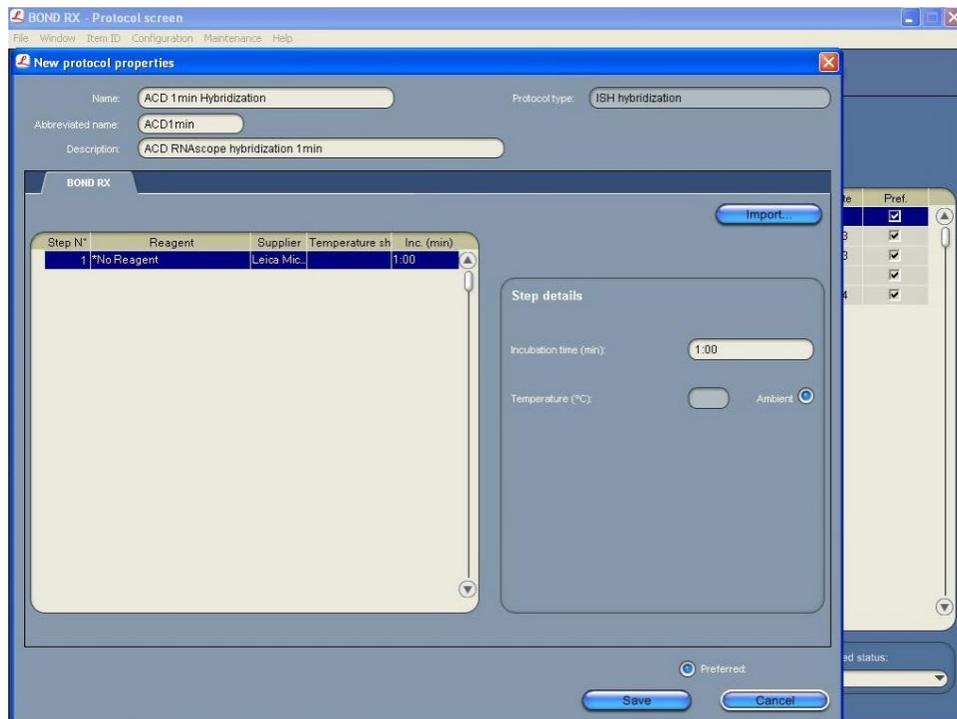
## Create a one-minute probe hybridization protocol

A mock probe hybridization step must be created as part of BDZ 11 software workaround for the RNAscope 2.5 LS Duplex assay. 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**.

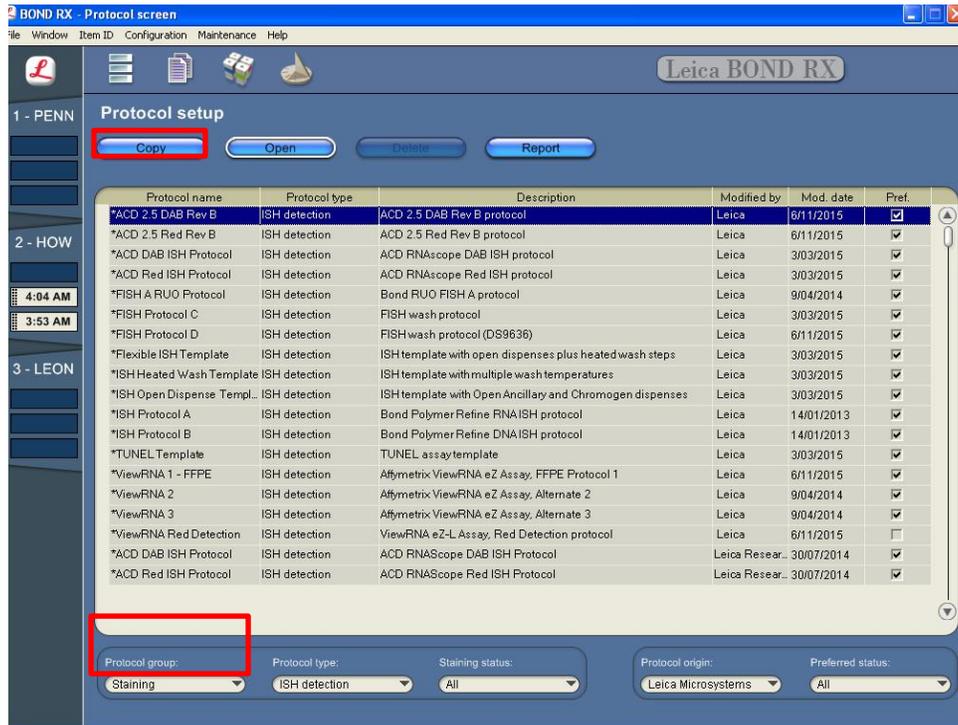


4. Highlight the **\*No Reagent** step.
5. Change the incubation time to **1 MIN** and select **Ambient** as Temperature (°C).
6. Select **Save**.

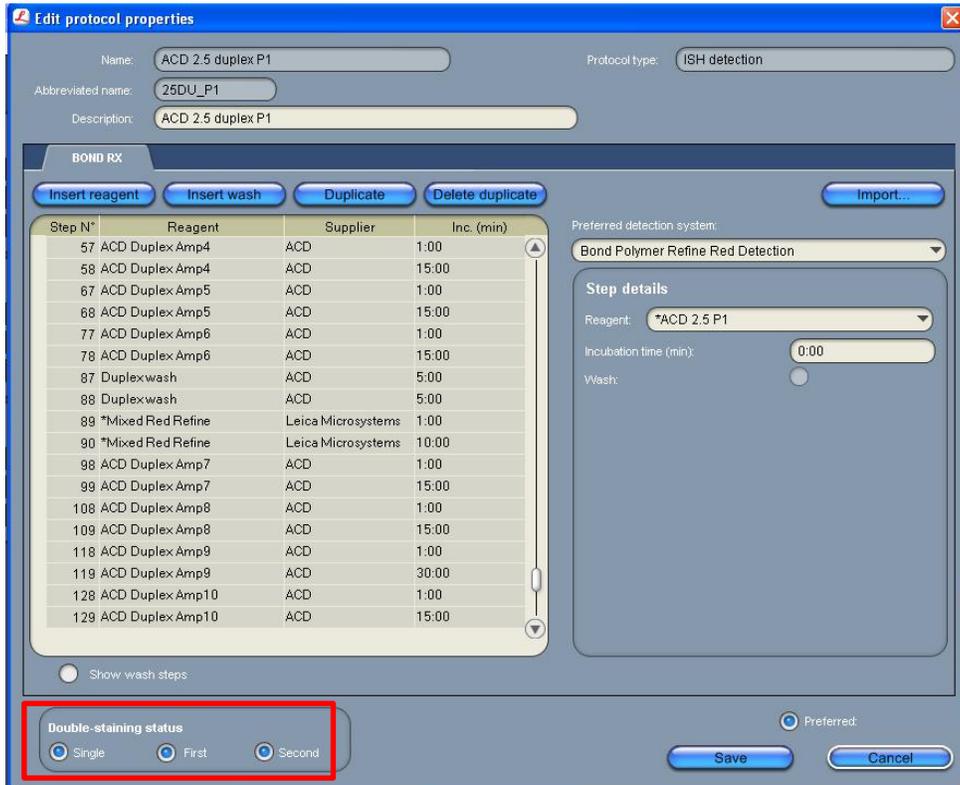
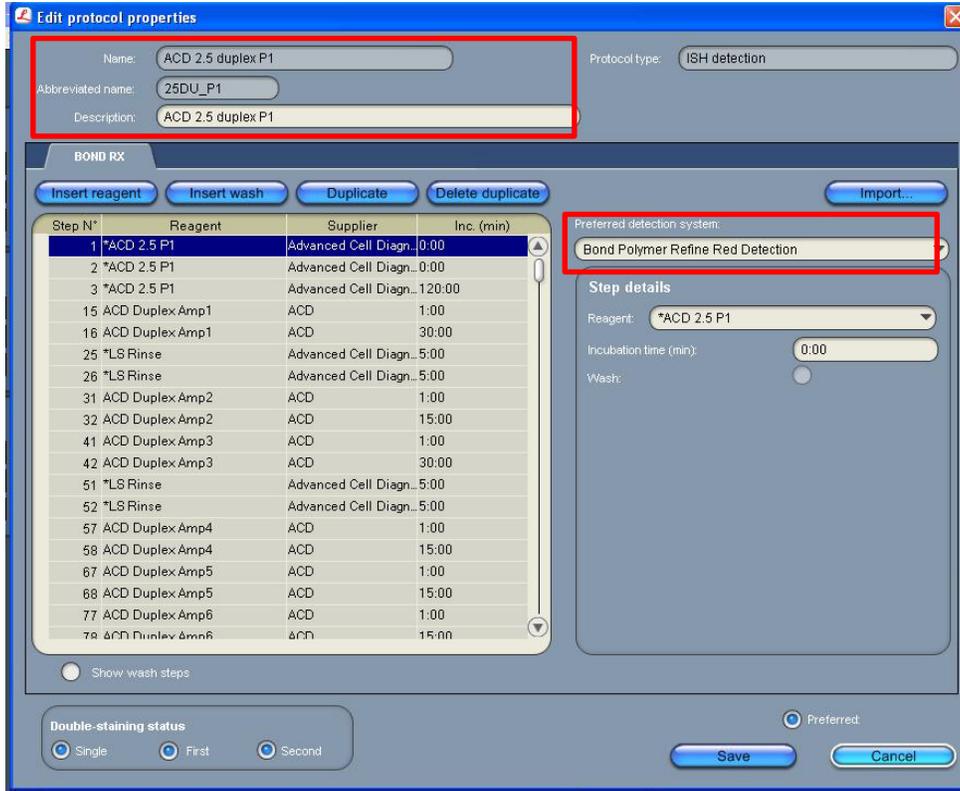
## Create a staining protocol for In Situ Hybridization (ISH)

Due to the BDZ 11 software workaround for the RNAscope 2.5 LS Duplex 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 duplex P1** in the Name text box, **25DU\_P1** in the Abbreviated name text box, and **ACD 2.5 duplex P1** in the Description text box.
4. Select **Bond Polymer Refine Red Detection** from the Preferred detection system menu.

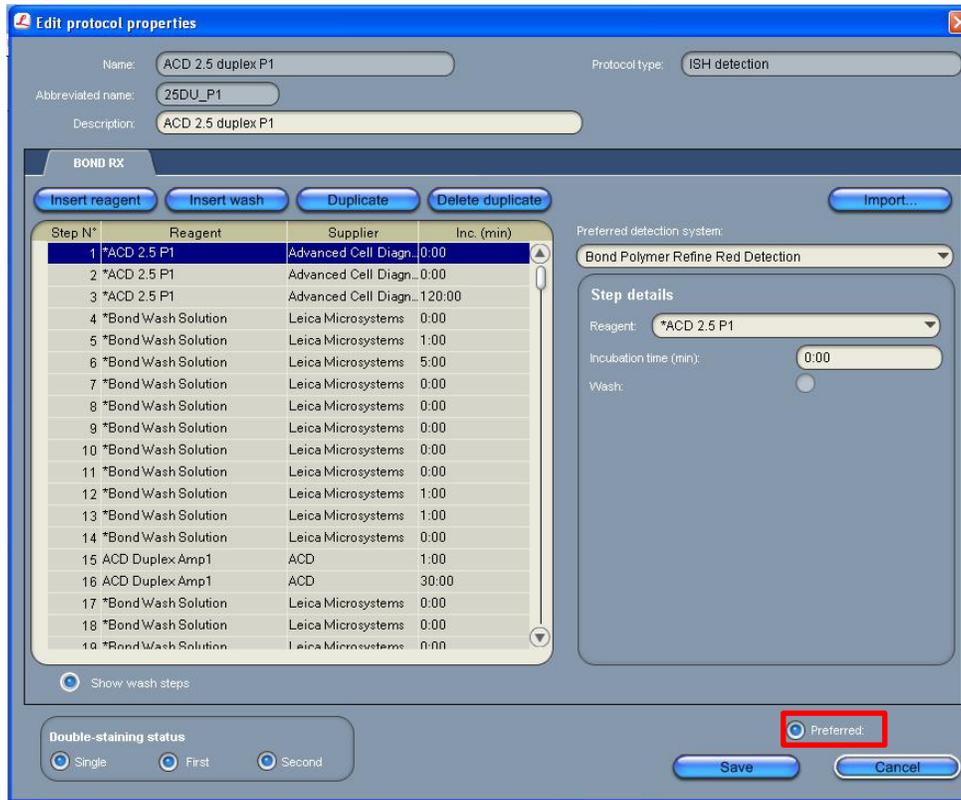


**Note:** The preceding two figures display all reagent steps.

- Highlight and select each Reagent step to edit.



6. Click **Show wash steps** (see the following figure) to also view the wash steps. Insert BOND Washes to match each of the protocol steps shown.
7. Compare and confirm screen protocol with the full protocol listed in this appendix.
8. Select **Preferred** in the bottom right corner of the window.



9. Select **Save**.
10. Click **Next** to proceed. Ignore any pop ups that may appear on the screen.
11. Create a new probe protocol.

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

## Part I: ISH protocol (ACD 2.5 DAB Rev B)

**IMPORTANT!** By strictly following the updated protocol steps listed in this appendix, you can use all three trays to run the fully automated red and green duplex assay. Adding any other steps will prevent the system from running all three trays.

**Note:** Change the step type of the steps with yellow lines according to the table.

Step No.	Reagent	Step Type	Incubation Time	Temperature	Updated Step No.
1	Probe 1 2.5	Reagent	0 MIN	Ambient	1
2	Probe 1 2.5	Reagent	0 MIN	Ambient	2
3	Probe 1 2.5	Reagent	120 MIN	42°C	3
4	*Bond Wash Solution	Reagent	0 MIN	42°C	4
5	*Bond Wash Solution	Reagent	1 MIN	42°C	5



Step No.	Reagent	Step Type	Incubation Time	Temperature	Updated Step No.
6	*Bond Wash Solution	Reagent	5 MIN	42°C	6
7	*Bond Wash Solution	Wash	0 MIN	Ambient	7
8	*Bond Wash Solution	Wash	0 MIN	Ambient	8
9	*Bond Wash Solution	Wash	0 MIN	Ambient	9
10	*Bond Wash Solution	Wash	0 MIN	Ambient	10
11	*Bond Wash Solution	Wash	0 MIN	Ambient	11
12	*Bond Wash Solution	Wash	1 MIN	Ambient	12
13	*Bond Wash Solution	Wash	1 MIN	Ambient	13
14	*Bond Wash Solution	Wash	0 MIN	Ambient	14
15	ACD Duplex Amp 1	Reagent	1 MIN	42°C	15
16	ACD Duplex Amp 1	Reagent	30 MIN	42°C	16
17	*Bond Wash Solution	Wash	0 MIN	Ambient	17
18	*Bond Wash Solution	Wash	0 MIN	Ambient	18
19	*Bond Wash Solution	Wash	0 MIN	Ambient	19
20	*Bond Wash Solution	Wash	3 MIN	Ambient	20
21	*Bond Wash Solution	Wash	3 MIN	Ambient	21
22	*Bond Wash Solution	Wash	0 MIN	Ambient	22
23	*Bond Wash Solution	Wash	0 MIN	Ambient	23
24	*Bond Wash Solution	Wash	0 MIN	Ambient	24
25	*LS Rinse	Reagent	5 MIN	Ambient	25
26	*LS Rinse	Reagent	5 MIN	Ambient	26
27	*Bond Wash Solution	Wash	0 MIN	Ambient	27
28	*Bond Wash Solution	Wash	0 MIN	Ambient	28
29	*Bond Wash Solution	Open wash	0 MIN	Ambient	29
30	*Bond Wash Solution	Wash	0 MIN	Ambient	30
31	ACD Duplex Amp 2	Reagent	1 MIN	42°C	31
32	ACD Duplex Amp 2	Reagent	15 MIN	42°C	32
33	*Bond Wash Solution	Wash	0 MIN	Ambient	33
34	*Bond Wash Solution	Wash	0 MIN	Ambient	34
35	*Bond Wash Solution	Wash	0 MIN	Ambient	35
36	*Bond Wash Solution	Wash	1 MIN	Ambient	36
37	*Bond Wash Solution	Wash	1 MIN	Ambient	37
<del>38</del>	<del>*Bond Wash Solution</del>	<del>Wash</del>	<del>1 MIN</del>	<del>Ambient</del>	
39	*Bond Wash Solution	Open Wash	1 MIN	Ambient	38
40	*Bond Wash Solution	Wash	1 MIN	Ambient	39
41	ACD Duplex Amp 3	Reagent	1 MIN	42°C	40
42	ACD Duplex Amp 3	Reagent	30 MIN	42°C	41
43	*Bond Wash Solution	Wash	0 MIN	Ambient	42



Step No.	Reagent	Step Type	Incubation Time	Temperature	Updated Step No.
44	*Bond Wash Solution	Wash	0 MIN	Ambient	43
45	*Bond Wash Solution	Wash	0 MIN	Ambient	44
46	*Bond Wash Solution	Wash	3 MIN	Ambient	45
47	*Bond Wash Solution	Wash	3 MIN	Ambient	46
48	*Bond Wash Solution	Wash	0 MIN	Ambient	47
49	*Bond Wash Solution	Wash	0 MIN	Ambient	48
<del>50</del>	<del>*Bond Wash Solution</del>	<del>Wash</del>	<del>0 MIN</del>	<del>Ambient</del>	
51	*LS Rinse	Reagent	5 MIN	Ambient	49
52	*LS Rinse	Reagent	5 MIN	Ambient	50
53	*Bond Wash Solution	Wash	0 MIN	Ambient	51
54	*Bond Wash Solution	Wash	1 MIN	Ambient	52
55	*Bond Wash Solution	Open wash	1 MIN	Ambient	53
56	*Bond Wash Solution	Wash	1 MIN	Ambient	54
57	ACD Duplex Amp 4	Reagent	1 MIN	42°C	55
58	ACD Duplex Amp 4	Reagent	15 MIN	42°C	56
59	*Bond Wash Solution	Wash	0 MIN	Ambient	57
60	*Bond Wash Solution	Wash	0 MIN	Ambient	58
61	*Bond Wash Solution	Wash	0 MIN	Ambient	59
62	*Bond Wash Solution	Wash	1 MIN	Ambient	60
<del>63</del>	<del>*Bond Wash Solution</del>	<del>Wash</del>	<del>1 MIN</del>	<del>Ambient</del>	
<del>64</del>	<del>*Bond Wash Solution</del>	<del>Wash</del>	<del>1 MIN</del>	<del>Ambient</del>	
65	*Bond Wash Solution	Open Wash	1 MIN	Ambient	61
66	*Bond Wash Solution	Wash	1 MIN	Ambient	62
67	ACD Duplex Amp 5	Reagent	1 MIN	Ambient	63
68	ACD Duplex Amp 5	Reagent	15 MIN	Ambient	64
69	*Bond Wash Solution	Wash	0 MIN	Ambient	65
70	*Bond Wash Solution	Wash	0 MIN	Ambient	66
71	*Bond Wash Solution	Wash	0 MIN	Ambient	67
72	*Bond Wash Solution	Wash	1 MIN	Ambient	68
73	*Bond Wash Solution	Wash	1 MIN	Ambient	69
74	*Bond Wash Solution	Wash	1 MIN	Ambient	70
<del>75</del>	<del>*Bond Wash Solution</del>	<del>Wash</del>	<del>1 MIN</del>	<del>Ambient</del>	
<del>76</del>	<del>*Bond Wash Solution</del>	<del>Wash</del>	<del>1 MIN</del>	<del>Ambient</del>	
77	ACD Duplex Amp 6	Reagent	1 MIN	Ambient	71
78	ACD Duplex Amp 6	Reagent	15 MIN	Ambient	72
79	*Bond Wash Solution	Wash	0 MIN	Ambient	73
80	*Bond Wash Solution	Wash	0 MIN	Ambient	74
81	*Bond Wash Solution	Wash	0 MIN	Ambient	75



Step No.	Reagent	Step Type	Incubation Time	Temperature	Updated Step No.
82	*Bond Wash Solution	Wash	1 MIN	Ambient	76
83	*Bond Wash Solution	Wash	1 MIN	Ambient	77
84	*Bond Wash Solution	Wash	1 MIN	Ambient	78
<del>85</del>	<del>*Bond Wash Solution</del>	<del>Wash</del>	<del>1 MIN</del>	<del>Ambient</del>	
<del>86</del>	<del>*Bond Wash Solution</del>	<del>Wash</del>	<del>1 MIN</del>	<del>Ambient</del>	
87	Duplex wash	Reagent	5 MIN	Ambient	79
88	Duplex wash	Reagent	5 MIN	Ambient	80
89	*Mixed Red Refine	Reagent	1 MIN	Ambient	81
90	*Mixed Red Refine	Reagent	10 MIN	Ambient	82
91	*Bond Wash Solution	Wash	0 MIN	Ambient	83
92	*Bond Wash Solution	Wash	0 MIN	Ambient	84
93	*Bond Wash Solution	Wash	0 MIN	Ambient	85
94	*Bond Wash Solution	Wash	1 MIN	Ambient	86
95	*Bond Wash Solution	<del>Wash</del> Open Wash	1 MIN	Ambient	87
96	*Bond Wash Solution	Wash	1 MIN	Ambient	88
<del>97</del>	<del>*Bond Wash Solution</del>	<del>Wash</del>	<del>1 MIN</del>	<del>Ambient</del>	
<del>98</del>	<del>*Bond Wash Solution</del>	<del>Wash</del>	<del>1 MIN</del>	<del>Ambient</del>	
99	ACD Duplex Amp 7	Reagent	1 MIN	42°C	89
100	ACD Duplex Amp 7	Reagent	15 MIN	42°C	90
101	*Bond Wash Solution	Wash	0 MIN	Ambient	91
102	*Bond Wash Solution	Wash	0 MIN	Ambient	92
103	*Bond Wash Solution	Wash	0 MIN	Ambient	93
104	*Bond Wash Solution	<del>Wash</del> Open Wash	1 MIN	Ambient	94
105	*Bond Wash Solution	Wash	1 MIN	Ambient	95
<del>106</del>	<del>*Bond Wash Solution</del>	<del>Wash</del>	<del>1 MIN</del>	<del>Ambient</del>	
<del>107</del>	<del>*Bond Wash Solution</del>	<del>Wash</del>	<del>1 MIN</del>	<del>Ambient</del>	
108	*Bond Wash Solution	Wash	1 MIN	Ambient	96
109	ACD Duplex Amp 8	Reagent	1 MIN	42°C	97
110	ACD Duplex Amp 8	Reagent	15 MIN	42°C	98
111	*Bond Wash Solution	Wash	0 MIN	Ambient	99
112	*Bond Wash Solution	Wash	0 MIN	Ambient	100
113	*Bond Wash Solution	Wash	0 MIN	Ambient	101
114	*Bond Wash Solution	Wash	1 MIN	Ambient	102
115	*Bond Wash Solution	Wash	1 MIN	Ambient	103
116	*Bond Wash Solution	Wash	1 MIN	Ambient	104
<del>117</del>	<del>*Bond Wash Solution</del>	<del>Wash</del>	<del>1 MIN</del>	<del>Ambient</del>	



Step No.	Reagent	Step Type	Incubation Time	Temperature	Updated Step No.
<del>118</del>	<del>*Bond Wash Solution</del>	<del>Wash</del>	<del>1 MIN</del>	<del>Ambient</del>	
119	ACD Duplex Amp 9	Reagent	1 MIN	Ambient	105
120	ACD Duplex Amp 9	Reagent	30 MIN	Ambient	106
121	*Bond Wash Solution	Wash	0 MIN	Ambient	107
122	*Bond Wash Solution	Wash	0 MIN	Ambient	108
123	*Bond Wash Solution	Wash	0 MIN	Ambient	109
124	*Bond Wash Solution	Wash	1 MIN	Ambient	110
125	*Bond Wash Solution	Wash	1 MIN	Ambient	111
126	*Bond Wash Solution	Wash	1 MIN	Ambient	112
<del>127</del>	<del>*Bond Wash Solution</del>	<del>Wash</del>	<del>1 MIN</del>	<del>Ambient</del>	
<del>128</del>	<del>*Bond Wash Solution</del>	<del>Wash</del>	<del>1 MIN</del>	<del>Ambient</del>	
129	ACD Duplex Amp 10	Reagent	1 MIN	Ambient	113
130	ACD Duplex Amp 10	Reagent	15 MIN	Ambient	114
131	*Bond Wash Solution	Wash	0 MIN	Ambient	115
132	*Bond Wash Solution	Wash	0 MIN	Ambient	116
133	*Bond Wash Solution	Wash	0 MIN	Ambient	117
134	*Bond Wash Solution	Wash	1 MIN	Ambient	118
135	*Bond Wash Solution	Wash	1 MIN	Ambient	119
136	*Bond Wash Solution	Wash	1 MIN	Ambient	120
<del>137</del>	<del>*Bond Wash Solution</del>	<del>Wash</del>	<del>1 MIN</del>	<del>Ambient</del>	
<del>138</del>	<del>*Bond Wash Solution</del>	<del>Wash</del>	<del>1 MIN</del>	<del>Ambient</del>	

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

## Part II: Create a staining protocol for Immunohistochemistry (IHC)

### IHC protocol (ACD 2.5 duplex B Green)

To develop the second chromagen, create an IHC protocol in the software.

1. Highlight the **\*IHC Protocol F** protocol. **Select Copy**.
2. Enter a protocol name, an abbreviated protocol name, and a description.
3. Select **Second** in the Double-staining status menu.

New protocol properties

Name:

Abbreviated name:

Description:

Staining method:  Single  First  Second  Preferred

[Import protocol](#) Protocol type: IHC staining

Preferred detection system:

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
1		*Peroxide Block	Leica Microsystems	✓		5:00	150 µL
5		*MARKER	Leica Microsystems	✓		15:00	150 µL
9		*Post Primary	Leica Microsystems	✓		8:00	150 µL
13		*Polymer	Leica Microsystems	✓		8:00	150 µL
17		*Mixed DAB Refine	Leica Microsystems	✓		0:00	150 µL
18		*Mixed DAB Refine	Leica Microsystems	✓		10:00	150 µL
22		*Hematoxylin	Leica Microsystems	✓		5:00	150 µL

Show wash steps [Insert wash](#) | [Insert reagent](#) | [Delete step](#)

**IMPORTANT!** The Second button must be selected in the Double-staining status menu.

- Select **Bond Polymer Refine Red Detection** from the Preferred detection system menu. The \*Mixed DAB Refine and other Reagents will disappear from the protocol. Click on the empty box, and in the pull-down menu select **Mixed Green Chromogen**.

New protocol properties

Name:

Abbreviated name:

Description:

Staining method:  Single  First  Second  Preferred

[Import protocol](#) Protocol type: IHC staining

Preferred detection system:

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
1				✓		5:00	150 µL
5		*MARKER	Leica Microsystems	✓		15:00	150 µL
9				✓		8:00	150 µL
13				✓		8:00	150 µL
17				✓		0:00	150 µL
18				✓		10:00	150 µL
22		*Hematoxylin	Leica Microsystems	✓		5:00	150 µL

Show wash steps [Insert wash](#) | [Insert reagent](#) | [Delete step](#)

- Add the steps listed in the following table. Click **Show wash steps** (see the following figure) to also view the wash steps. Insert BOND Washes to match each of the protocol steps shown.

Name:

Abbreviated name:

Description:

Staining method:  Single  First  Second  Preferred

[Import protocol](#) Protocol type: IHC staining

Preferred detection system:

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
1		*LS Rinse	Advanced Cell Diagnostics	✓		5:00	150 µL
2		*LS Rinse	Advanced Cell Diagnostics	✓		5:00	150 µL
3		*Mixed Green Chromogen	OEM	✓		1:00	150 µL
4		*Mixed Green Chromogen	OEM	✓		15:00	150 µL
5	✓	*Deionized Water		✓		0:00	150 µL
6	✓	*Deionized Water		✓		0:00	150 µL
7	✓	*Deionized Water		✓		0:00	150 µL
8	✓	*Deionized Water		✓		0:00	150 µL
9		*Bond DAB Enhancer	Leica Microsystems	✓		5:00	150 µL

Show wash steps [Insert wash](#) | [Insert reagent](#) | [Delete step](#)

6. Compare and confirm screen protocol with the full protocol listed in the following table.

**IMPORTANT!** Please confirm that the protocol does not include any bluing steps.

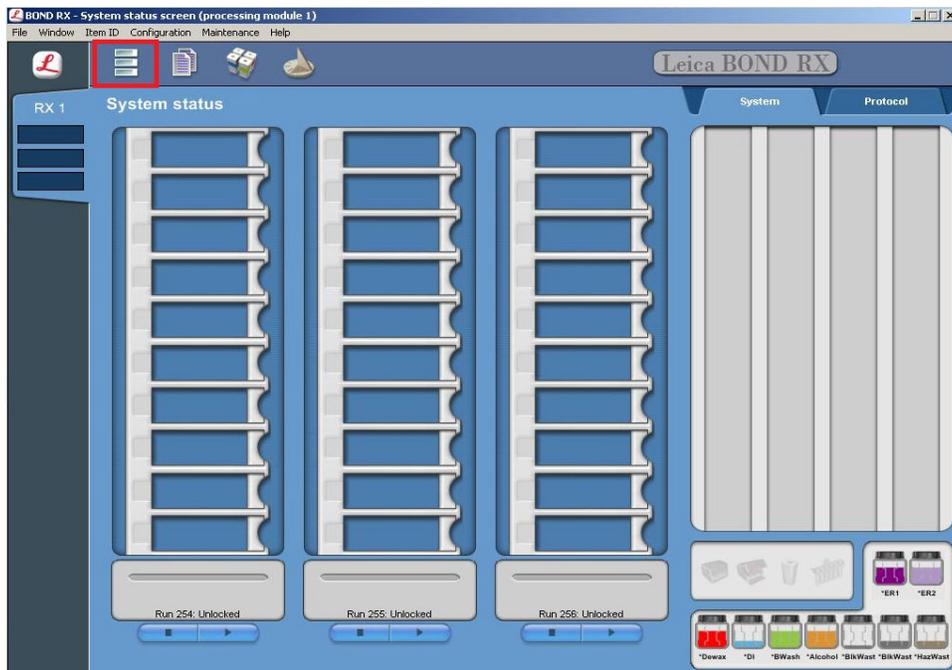
Step No.	Reagent	Step Type	Incubation Time	Temperature	Updated Step No.
1	*LS Rinse	Reagent	5 MIN	Ambient	1
<del>2</del>	<del>*LS Rinse</del>	<del>Reagent</del>	<del>5 MIN</del>	<del>Ambient</del>	
3	*Mixed DAB Refine * Mixed Green Chromogen	Reagent	1 MIN	Ambient	2
4	*Mixed DAB Refine * Mixed Green Chromogen	Reagent	20 MIN 15 MIN	Ambient	3
5	*De-ionized Water	Wash	0 MIN	Ambient	4
6	*De-ionized Water	Wash	0 MIN	Ambient	5
7	*De-ionized Water	Wash	0 MIN	Ambient	6
8	*De-ionized Water	Wash	0 MIN	Ambient	7
9	*De-ionized Water	Wash	0 MIN	Ambient	8
10	*De-ionized Water	Wash	0 MIN	Ambient	9
<del>11</del>	<del>*Bond DAB Enhancer</del>	<del>Reagent</del>	<del>20min</del>	<del>Ambient</del>	
12	*De-ionized Water	Wash	0 MIN	Ambient	10
13	*De-ionized Water	Wash	0 MIN	Ambient	11
14	*De-ionized Water	Wash	0 MIN	Ambient	12
15	*De-ionized Water	Wash	0 MIN	Ambient	13

16	*Hematoxylin	Reagent	5 MIN	Ambient	14
17	*De-ionized Water	Wash	0 MIN	Ambient	15
18	*De-ionized Water	Wash	0 MIN	Ambient	16
<del>19</del>	<del>*De-ionized Water</del>	<del>Wash</del>	<del>0 MIN</del>	<del>Ambient</del>	
<del>20</del>	<del>*De-ionized Water</del>	<del>Wash</del>	<del>0 MIN</del>	<del>Ambient</del>	
<del>21</del>	<del>*ACD blac</del>	<del>Reagent</del>	<del>2 MIN</del>	<del>Ambient</del>	
<del>22</del>	<del>*De-ionized Water</del>	<del>Wash</del>	<del>0 MIN</del>	<del>Ambient</del>	
<del>23</del>	<del>*De-ionized Water</del>	<del>Wash</del>	<del>0 MIN</del>	<del>Ambient</del>	
<del>24</del>	<del>*De-ionized Water</del>	<del>Wash</del>	<del>0 MIN</del>	<del>Ambient</del>	
<del>25</del>	<del>*De-ionized Water</del>	<del>Wash</del>	<del>0 MIN</del>	<del>Ambient</del>	

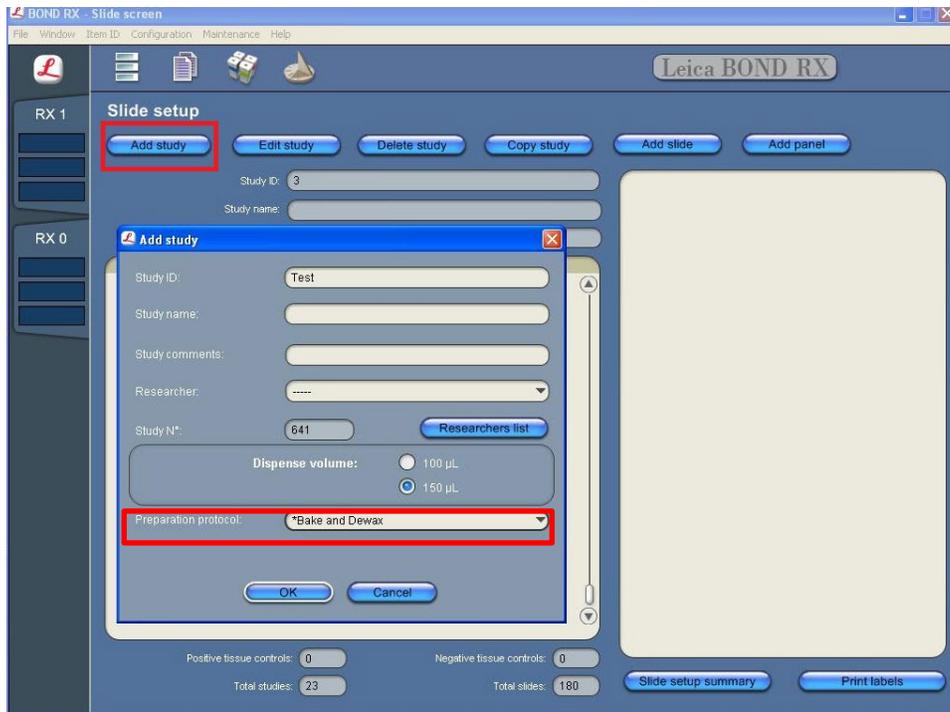
7. Select **Save**.
8. Click **Next** to proceed. Ignore pop-ups that state that the protocol is not validated by Leica. If you see any other pop-ups please contact your FAS or technical support at ACD.

## Set up a sequential dual stain (ds) study for ISH and IHC

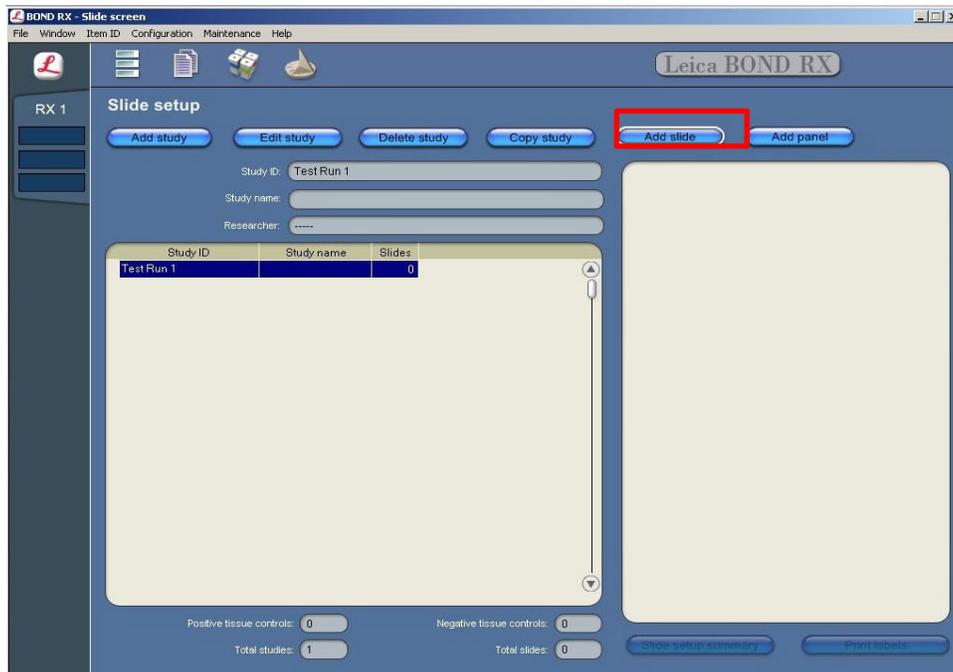
1. To build a study, select the **Slide setup** icon at the top of the screen.



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



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. Select **Sequential DS** from the Staining mode drop down menu

8. In the First tab, select **ISH** under Process and **mock probe (ACD)** from the Marker drop down menu.
9. Do the following on the Protocols tab:
  - a. Make sure that each probe is associated with a different protocol (for example, ACD 2.5 duplex P1), 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.
10. In the Second tab, select **IHC** under Process and **\*Negative** from the Marker menu. This marker is not required on the instrument to start the run.
11. In the Protocols tab, select **ACD 2.5 duplex B Green** from the Staining menu, and leave HIER and Enzyme empty.

x

Add slide

Slide comments

**Tissue type:**

Test tissue     
  Negative tissue     
  Positive tissue

**Dispense volume:**

100 µL     
  150 µL

**Staining mode:**

Sequential DS ▼     
 Routine ▼

First     
Second

**Process:**

IHC     
 ISH

**Marker:** \*Negative ▼

**Protocols**

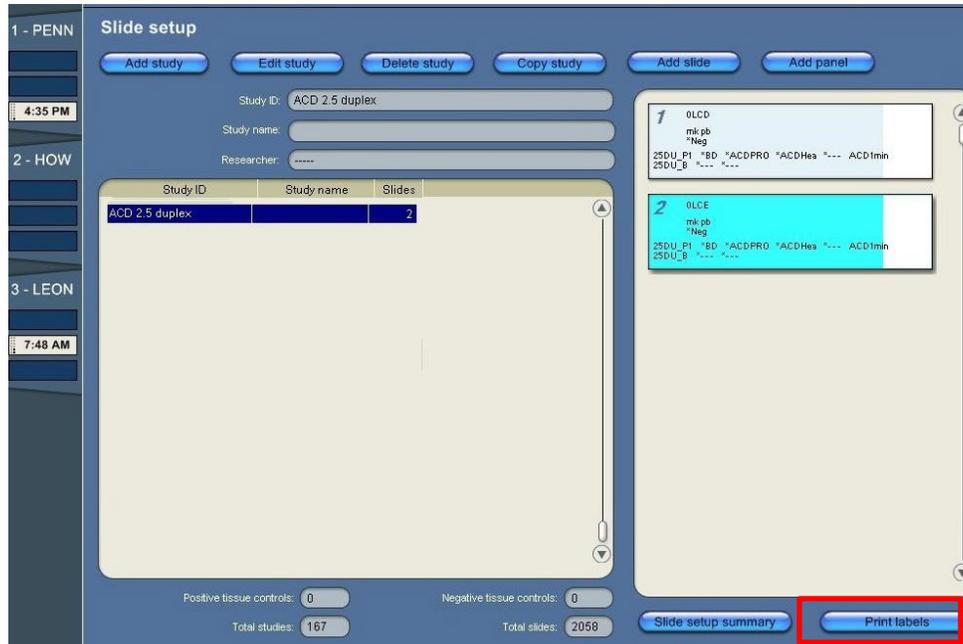
**Staining:** ACD 2.5 duplex B Green ▼

**HIER:** \*---- ▼

**Enzyme:** \*---- ▼

Add slide
Close

12. For samples using a different probe, IHC settings (in the second tab) will remain the same. Only ISH settings (in the first tab) need to be changed.
13. Select **Add slide** for each target probe, and for each of the slides used for the run.
14. After adding all the slides to the study, select **Close** to return to the Slide setup screen.
15. Select **Print labels** to print barcodes to attach to the slides.



## 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. Obtain one empty 30 mL 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. Prepare LS Duplex probe mix:
  - a. Determine the volume of duplex probe needed.
  - b. In an LS 30 mL container, dilute the 50X C2 probe stock 1:50 with the Ready-To-Use C1 probe. For example, add 320  $\mu$ L 50X C2 probe to 16 mL C1probe.
  - c. The 2.5 LS Duplex probe mix is stable for one year at 2–8°C.

**Note:** The 2.5 LS Duplex probe mix is stable for one year at 2–8°C.

**Note:** If using other open containers for probe delivery, ensure you account for dead volume required in each container with volumes suggested in table below.



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

- Using the barcode scanner, scan the front barcode on the 30 mL Open Bond container. A window will appear.
- 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 Rinse	*LS Rinse
RNAscope 2.5 LS Duplex Wash	Duplex Wash
RNAscope 2.5 LS Duplex AMP 1	ACD Duplex Amp 1
RNAscope 2.5 LS Duplex AMP 2	ACD Duplex Amp 2
RNAscope 2.5 LS Duplex AMP 3	ACD Duplex Amp 3
RNAscope 2.5 LS Duplex AMP 4	ACD Duplex Amp 4
RNAscope 2.5 LS Duplex AMP 5	ACD Duplex Amp 5
RNAscope 2.5 LS Duplex AMP 6	ACD Duplex Amp 6
RNAscope 2.5 LS Duplex AMP 7	ACD Duplex Amp 7
RNAscope 2.5 LS Duplex AMP 8	ACD Duplex Amp 8
RNAscope 2.5 LS Duplex AMP 9	ACD Duplex Amp 9
RNAscope 2.5 LS Duplex AMP 10	ACD Duplex Amp 10
RNAscope 2.5 LS Bluing Reagent	*ACD blue
1X BOND Wash	Mock probe
2.5 LS Duplex probe mix	variable

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

**Note:** Leica BOND Red Detection and Green Chromogen come in pre-filled Leica BOND RX containers.

- Enter the RNAscope 2.5 LS Duplex 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

- 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 placement and seating of covertile.



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

---

**IMPORTANT!** Before leaving the instrument unattended, ensure that the instrument is running successfully. In the event of a problem, please contact ACD or Leica support to resolve any issues. You can also use an automated coverslipper in the following sections.

---

## Complete the run

- After the run is complete, press the button on the front of the instrument to unload the slides and remove covertiles

## Bluing Samples

Do not use RNAscope 2.5 LS Bluing Reagent included in the reagent kit when using green chromogen. We suggest using 0.02% ammonia water for bluing (see page 58). Be cautious when using other bluing reagents as they could be incompatible with green chromogen.

1. Place the slides onto the Tissue-Tek Slide Rack, move the rack into a staining dish containing 0.02% ammonia water, and incubate for **2 MIN** at **RT**. Section should turn blue.
2. Replace Ammonia water with tap water. Wash slides 3–5 times.

## Dry and mount the samples

1. Remove the slide rack from the staining dish and dry slides in a **60°C** dry oven for **30 MIN**.

---

**IMPORTANT!** The RED substrate is alcohol sensitive. Do not dehydrate the slides in alcohol.

---

2. Cool the slides for **5 MIN** at **RT**.
3. Briefly dip one slide into fresh pure xylene and *immediately* place 1–2 drops of EcoMount or Vectamount on the slide before the xylene dries.

---

**IMPORTANT!** Use EcoMount (Biocare Medical), Vectamount (VectaLabs), or PERTEX mounting media.

---

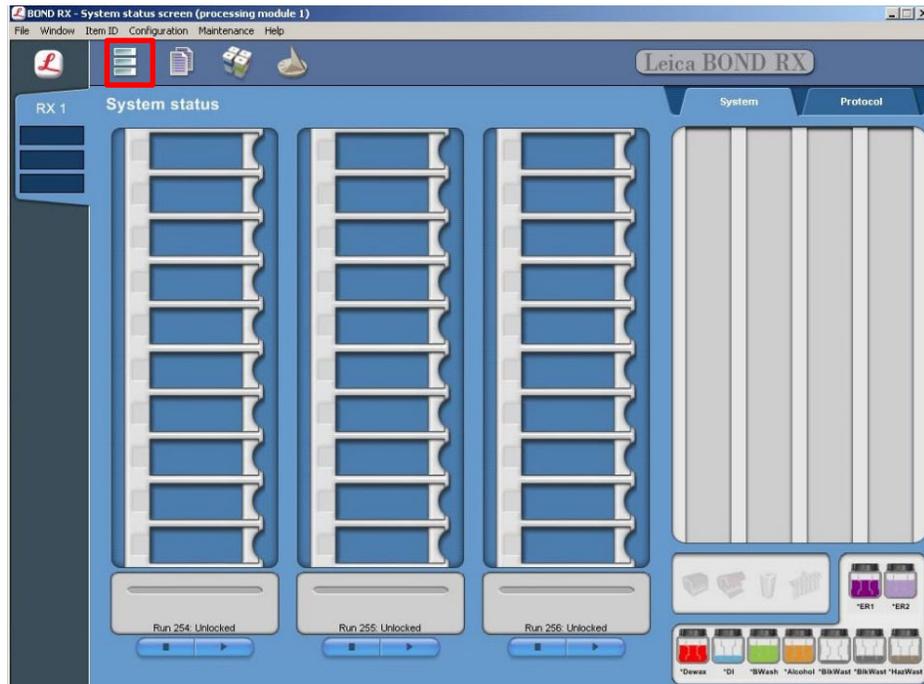
4. Carefully place 24 mm x 50 mm coverslip over the tissue section. Avoid trapping air bubbles.
5. Repeat steps 3 and 4 for each slide.
6. Air dry slides for **5 MIN**.
7. Proceed to **Chapter 6. Evaluate the Results**.

# Appendix D. Edit the Epitope Retrieval Protocol

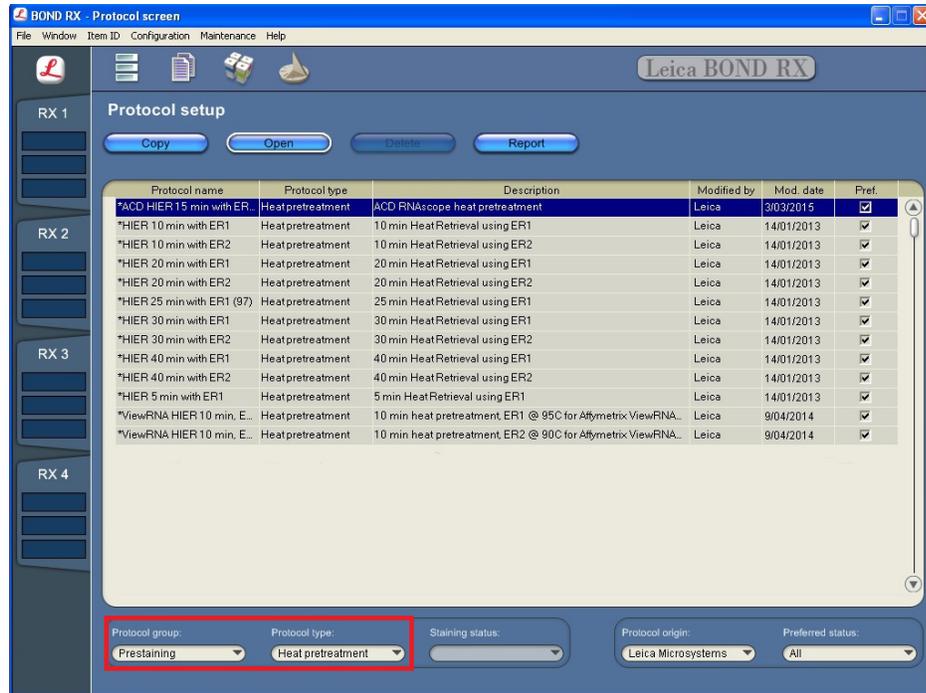
The following example shows how to edit the Epitope Retrieval procedure from within the **software**. The same pretreatment condition can be used for both LS single-plex and duplex.

## Create a prestaining protocol

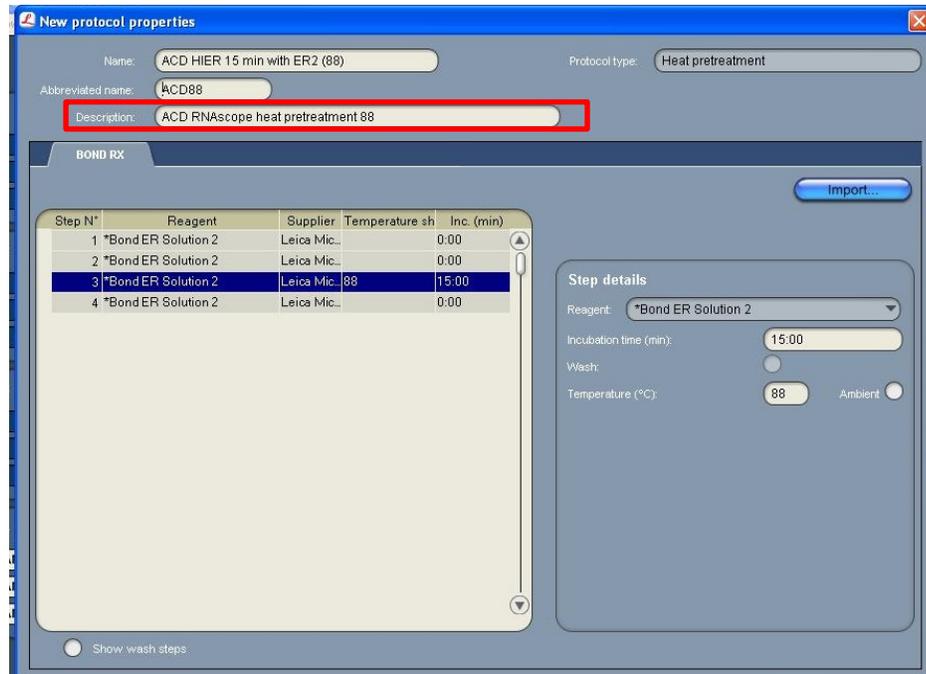
1. Open the Leica BOND software and click on the Protocol setup icon as shown.



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



- Highlight the **\*ACD HIER 15 min with ER2 (95)** protocol. Select **Copy**.
- Note:** ER2 = Epitope Retrieval 2.
- Rename the protocol as **ACD HIER 15 min with ER2 (88)**.
  - Rename the Abbreviated name as **ACD88**.
  - Rename the Description to **ACD RNAscope heat pretreatment 88** (see the following figure).



- 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

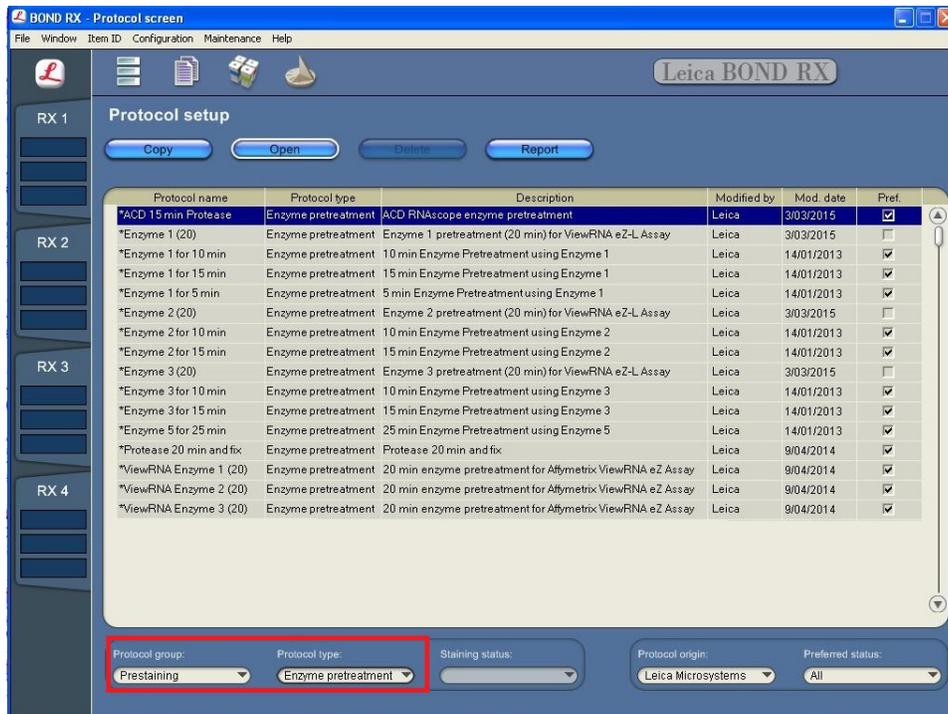
\*Human tissue unless otherwise noted.

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).

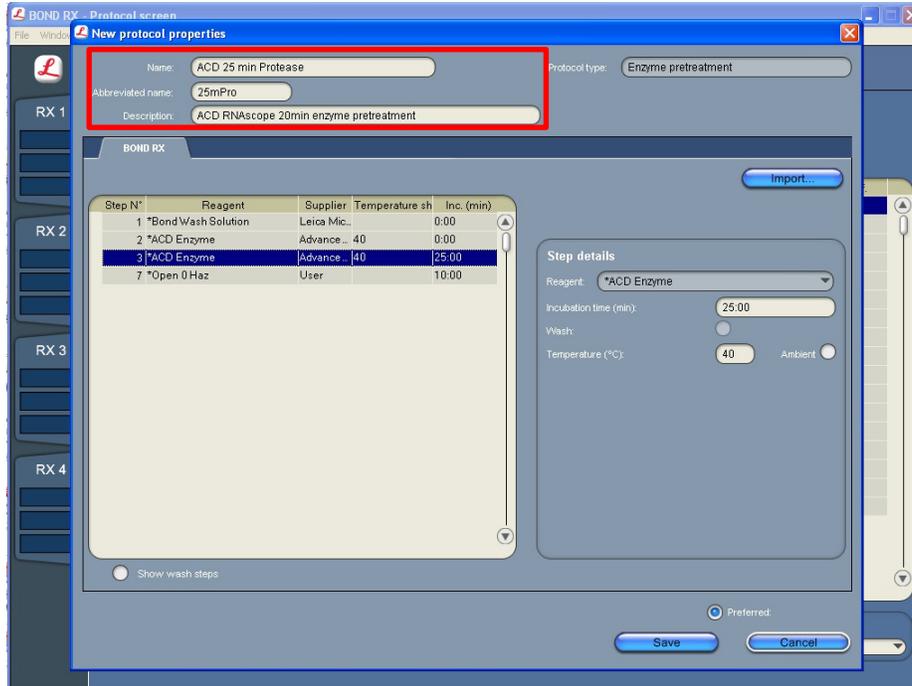
# Appendix E. Edit the Protease Protocol

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** (see the following figure).



- 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

- Select **Save**.
- 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).



# Appendix F. Safety

## Chemical safety



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

- Read and understand the Safety Data Sheets (SDSs) before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see <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](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/](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: <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](mailto:info.acd@bio-techne.com)  
**Orders:** [order.acd@bio-techne.com](mailto:order.acd@bio-techne.com)  
**Support Email:** [support.acd@bio-techne.com](mailto: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**

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For support, email [support.acd@bio-techne.com](mailto:support.acd@bio-techne.com)

[www.acdbio.com](http://www.acdbio.com)

