RNAscope® 2.5 LS Assay- BROWN Combined with Immunohistochemistry (IHC)

Introduction

This Technical Note provides guidelines for performing automated *in situ* hybridization (ISH) using an RNAscope® 2.5 LS Reagent Kit-BROWN (Cat. No. 322100) combined with immunohistochemistry (IHC) on the Leica BOND RX System. This procedure is based on the standard RNAscope® 2.5 LS BROWN Assay and requires the Leica BOND Refine Detection Kit or Leica BOND Red Refine Detection Kit for immunohistochemistry. Before starting the procedure, create a protocol for the RNAscope® assay combined with IHC on the RX controller with the help of your ACD FAS. For every chemical, read the Material Safety Data Sheet (MSDS) and follow handling instructions. Wear appropriate protective eyewear, clothing, and gloves. For the latest service and support information, go to **www.acdbio.com/support**.

Consult **www.leicabiosystems.com/ihc-ish-fish/immunohistochemistry-ihc-antibodies-novocastra-reagents/primary-antibodies/** for Ready-To-Use (RTU) compatible antibodies with the BOND RX.

Note: RNAscope® uses a proprietary protease that may not be compatible with all antibodies. Please validate your antibody for use with the RNAscope® Assay.

ISH - IHC Chromogen Combinations

For optimal results using ISH – IHC chromogen combinations, see the following table:

RNAscope® ISH	Sequential IHC	Automated RNAscope® Detection Kit	IHC Detection System/Reagents
Brown (DAB)	Red	RNAscope® 2.5 LS Reagent Kit - BROWN or RNAscope® LSx Reagent Kit	Leica BOND Red Refine Detection Kit
Brown (DAB)	Green	RNAscope® 2.5 LS Reagent Kit - BROWN or RNAscope® LSx Reagent Kit	Leica BOND Refine Detection Kit, RNAscope® 2.5 LS Green Accessory Pack
Red*	Green	RNAscope® 2.5 LS Reagent Kit - RED	Leica BOND Refine Detection Kit, RNAscope® 2.5 LS Green Accessory Pack

* To perform RED ISH - GREEN IHC, refer to the *RNAscope® 2.5 LS Assay – RED Combined with Immunohistochemistry (IHC) Technical Note* at **www.acdbio.com/support**.

Note: We do not recommend the RED ISH - BROWN IHC combination because of low contrast.

Note: We do not recommend using the green chromogen to perform ISH because of its instability.

Materials Required

RNAscope® 2.5 LS Reagent Kit- BROWN

The RNAscope® 2.5 LS Reagent kit - BROWN (Cat. No. 322100) provides enough reagents to stain ~60 standard slides on the Leica Biosystems' BOND RX System. The RNAscope® 2.5 LS Probes are available separately. The reagents are Ready-To-Use (RTU) and are stored as indicated in the following table:

RNAscope® 2.5 LS Reagent Kit - BROWN (Cat. No. 322100)			
☑	Reagent	Quantity	Storage
	RNAscope® 2.5 LS Hydrogen Peroxide	21 mL x 1 bottle	2–8°C
	RNAscope® 2.5 LS Protease III	21 mL x 1 bottle	2–8°C
	RNAscope® 2.5 LS AMP 1	21 mL x 1 bottle	2–8°C
	RNAscope® 2.5 LS AMP 2	21 mL x 1 bottle	2–8°C
	RNAscope® 2.5 LS AMP 3	21 mL x 1 bottle	2–8°C
	RNAscope® 2.5 LS AMP 3	21 mL x 1 bottle	2–8°C
	RNAscope® 2.5 LS AMP 4	21 mL x 1 bottle	2–8°C
	RNAscope® 2.5 LS AMP 5 – BROWN	21 mL x 1 bottle	2–8°C
	RNAscope® 2.5 LS AMP 6 – BROWN	21 mL x 1 bottle	2–8°C
	RNAscope® 2.5 LS Rinse	30 mL x 2 bottles	2–8°C
	RNAscope® 2.5 LS Bluing Reagent	21 mL x 1 bottle	2–8°C

IMPORTANT! Use only RNAscope® 2.5 LS Probes. Do not substitute the reagent components of the RNAscope® 2.5 LS Reagent Kit with those of other RNAscope® Reagent Kits, including the RNAscope® 2.0 LS Reagent Kit.

RNAscope® 2.5 LS Green Accessory Pack (Cat. No. 322550)*		
Reagent	Quantity	Storage
RNAscope® 2.5 LS Duplex Green A	12 mL x 1 bottle	2–8°C
RNAscope® 2.5 LS Duplex Green A	240 µL x 1 tube	2–8°C
RNAscope® 50X Wash Buffer	60 mL x 1 bottle	Room temp (20–25°C)

* This kit is only required for semi-automated Green IHC. To perform automated Red IHC, use the BOND Polymer Refine Red Detection and Hematoxylin Kit.

Required Materials from Leica BOND RX

The RNAscope® 2.5 LS Brown Assay combined with IHC requires specific materials and equipment available *only* from Leica Biosystems.

☑	Component	Cat. No.	Storage
	BOND 30 mL Open containers	OP309700	Room temp (20–25°C)
	BOND Universal Covertiles 100 pack	S21.2001	Room temp (20–25°C)
	BOND Polymer Refine Detection (DAB) and Hematoxylin*	DS9800	2–8°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 Aspirating Probe Cleaning System	CS9100	2–8°C
	BOND Mixing Stations	S21.1971	Room temp (20–25°C)
	BOND Polymer Refine Red Detection and Hematoxylin*†	CS9390	2–8°C

* Do not substitute with any other chromogen kit.

† This kit is only required for Red IHC. To perform semi-automated Green IHC, use the materials in the RNAscope® 2.5 LS Green Accessory Pack.

Workflow

Part 1: Create software protocols to perform in situ hybridization (ISH)

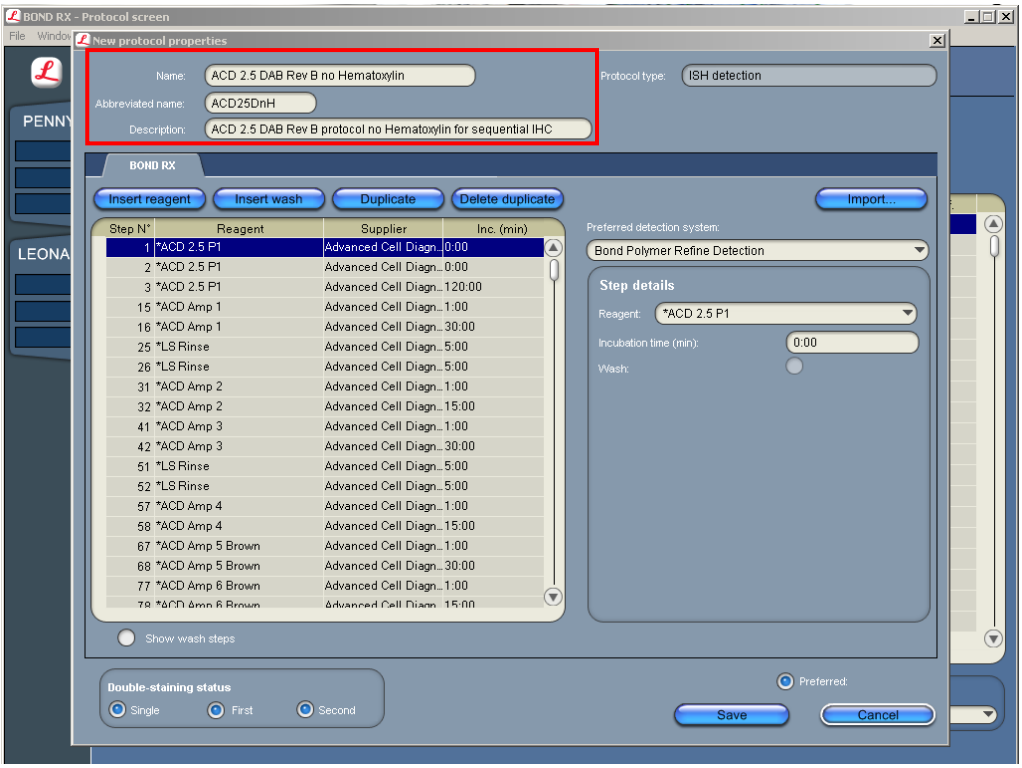
This section provides instructions for creating *in situ* hybridization (ISH) software protocols compatible with sequential immunohistochemistry (IHC) on the Leica BOND RX System.

Create a ISH protocol for sequential IHC

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**.

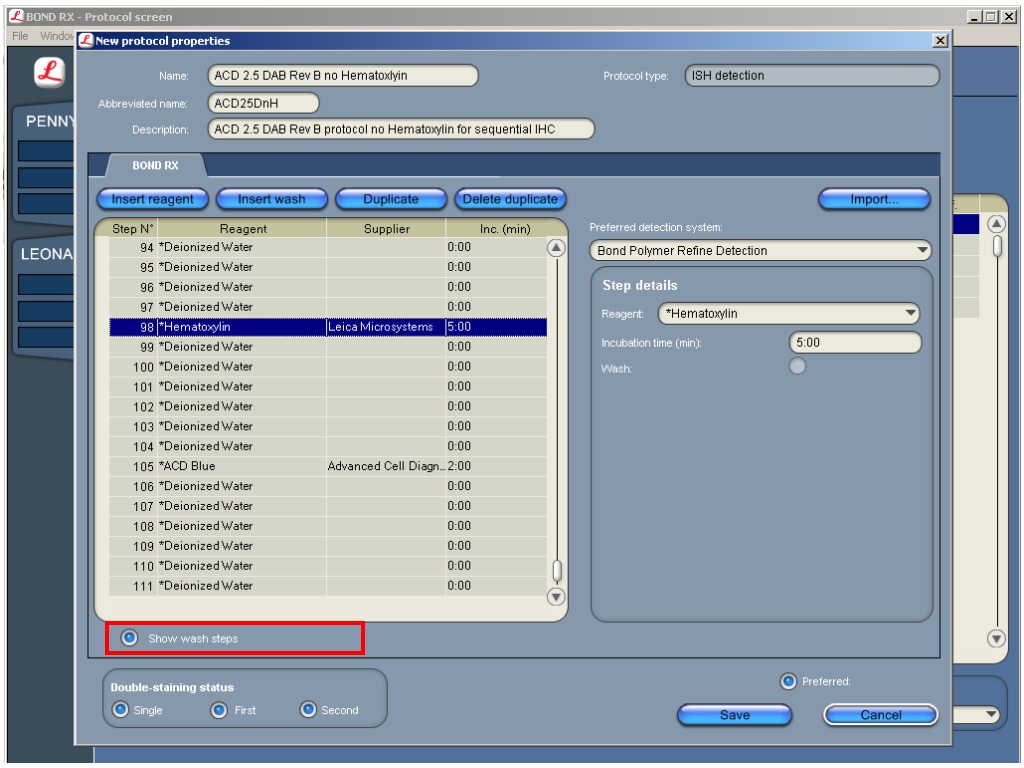
   Note: If you are using software version BDZ 9, select the standard RNAscope® 2.5 LS BROWN Assay protocol set up by your ACD FAS.

3. Change the protocol name for your first probe to **ACD 2.5 DAB Rev B no Hematoxylin** in the Name text box, **ACD25DnH** in the Abbreviated name text box, and **ACD 2.5 DAB Rev B protocol no Hematoxylin** in the Description text box.

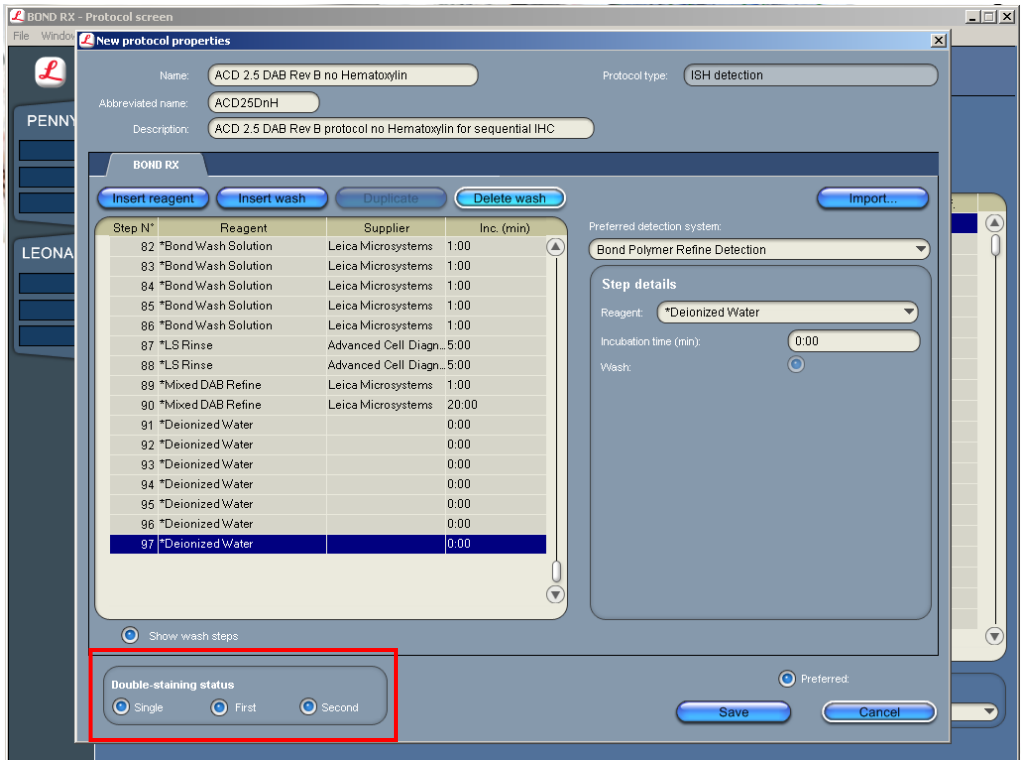


4. Select **Show wash steps**. Delete the **Hematoxylin** step (step 98) and all the following steps including the **ACD Blue** step (step 105).

   Note: You may not be able to delete some wash steps in software version BDZ 9. Change these steps to Bond wash for 0 minutes.



5. To perform a sequential dual stain (ISH-IHC), make sure that **First** is selected under Double-staining status.

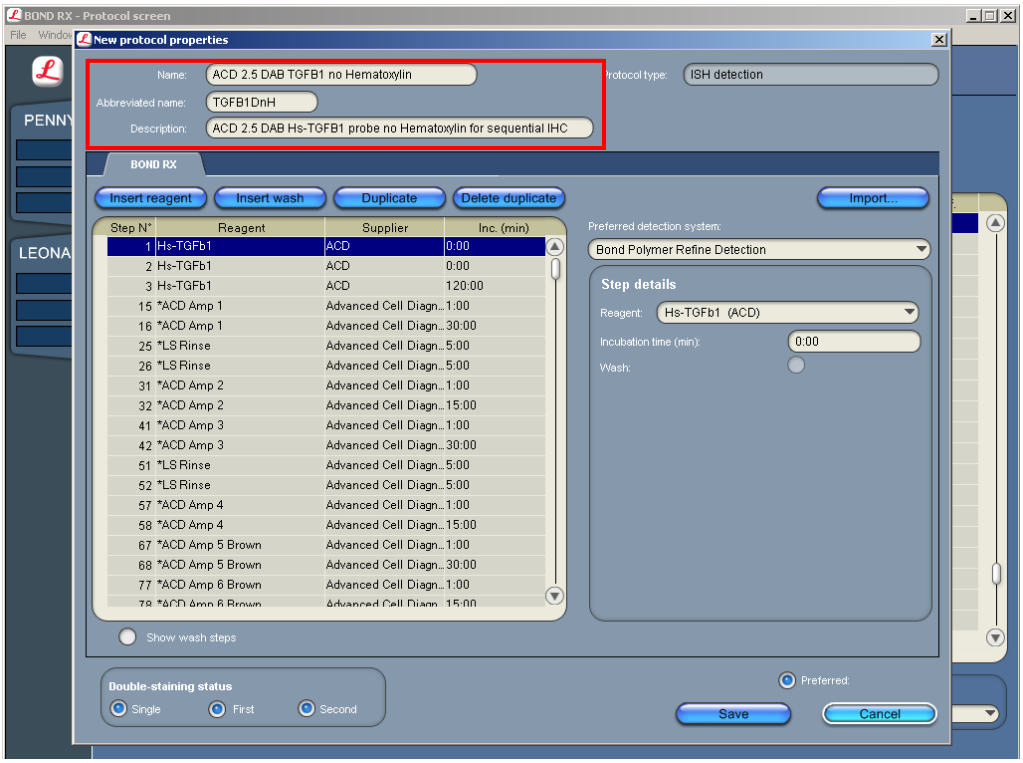


Note: (Optional) Select the **Single** button if you are running a single stain assay with no Hematoxylin counterstaining.

6. Select **Save**.

7. Click **Next** to proceed. Ignore any pop ups that may appear on the screen.
8. To create a protocol for each additional probe, follow these steps:
   a. Highlight the **ACD 2.5 DAB Rev B no Hematoxylin** protocol. Select **Copy**.
   b. Change the protocol name by adding your probe name (for example, **ACD 2.5 DAB TGFB1 no Hematoxylin**) in the Name text box. Change the Abbreviated name text and Description text box accordingly.
   c. Select ***ACD 2.5 P1**. Change the **Reagent** to your registered probe (for example, **TGFB1**).

IMPORTANT! Make sure to change all three probe steps.



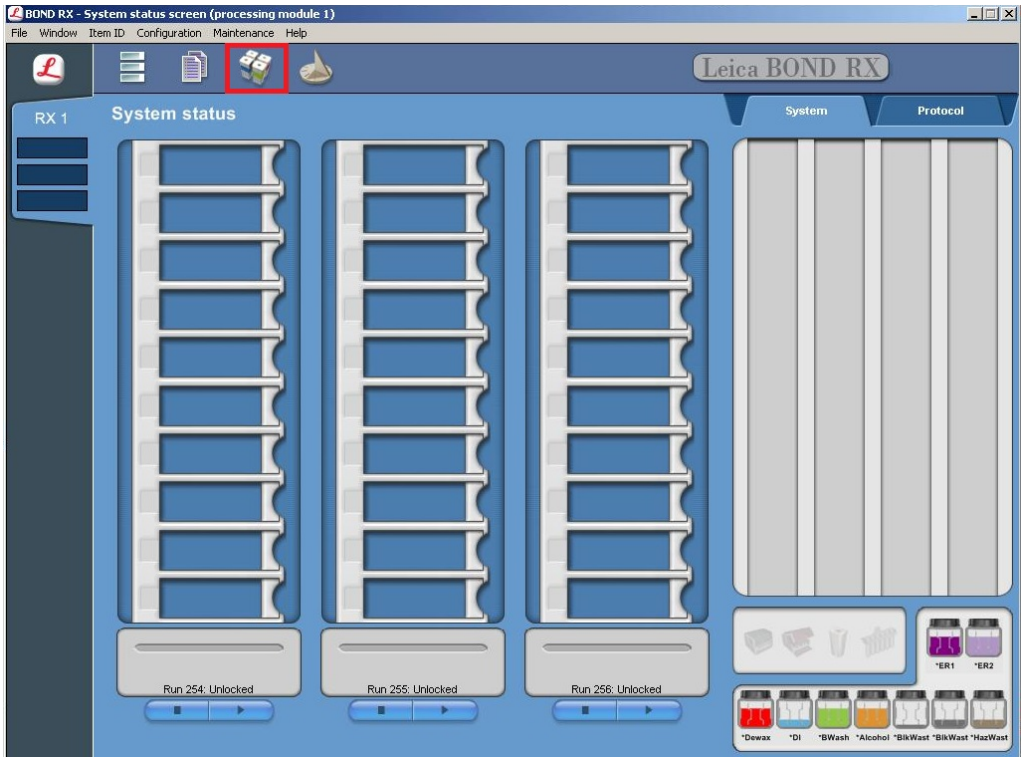
Part 2: Register the reagents for immunohistochemistry (IHC)

To perform IHC using a Leica Ready-to-Use (RTU) primary antibody on the Leica BOND RX instrument, you must set up the antibody as a **Preferred** reagent. Follow the steps in ***Change the Preferred status of existing reagents*** on page 6.

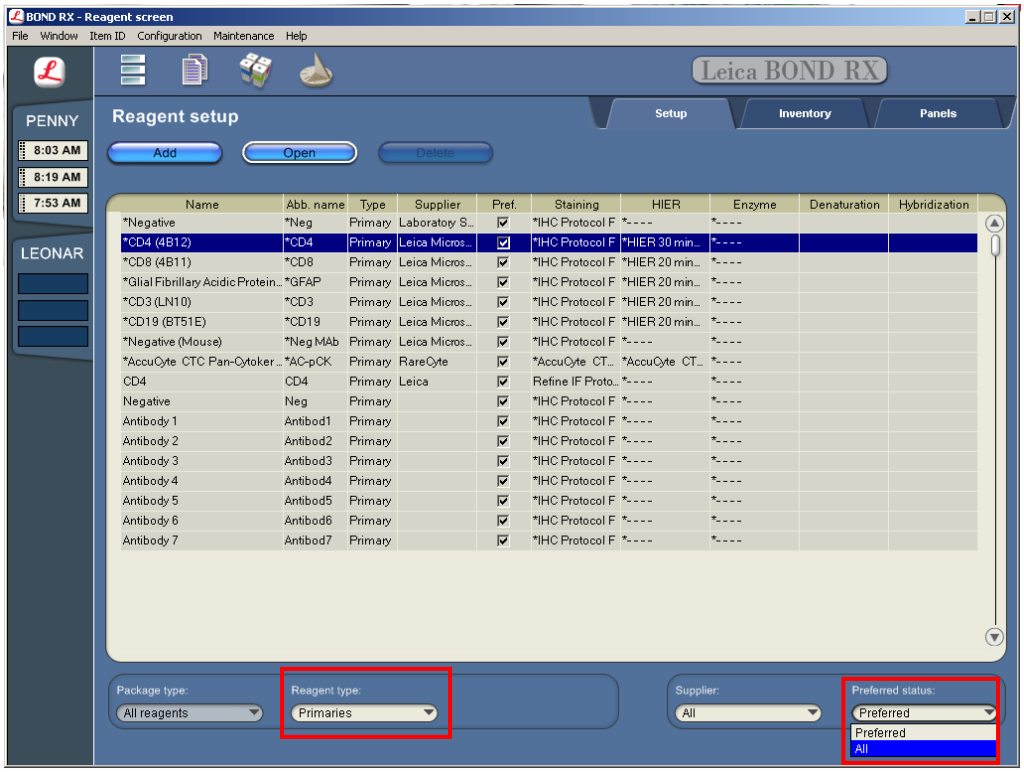
To perform IHC using a diluted antibody or an antibody from a third vendor, you must add the antibody to an open container and register it as a new reagent. Follow the steps in ***Register the reagents*** on page 7.

Change the Preferred status of existing reagents

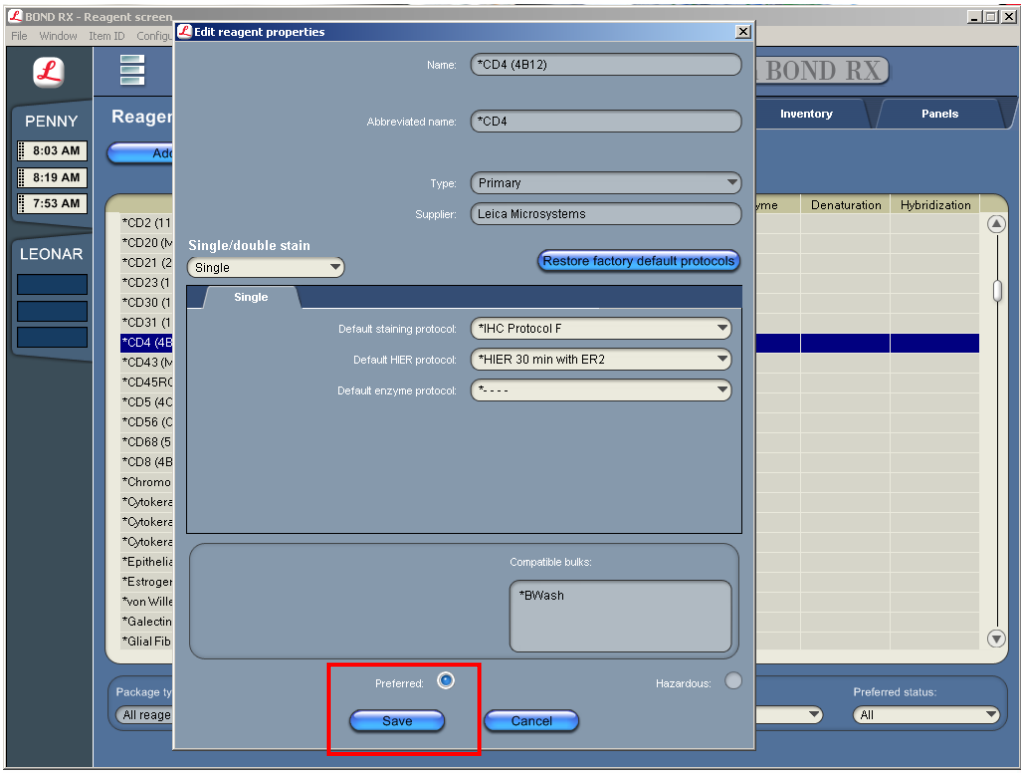
1. Select the **Reagent Setup** icon at the top of the screen.



2. Select **Primaries** for **Reagent type** and **All** for the **Preferred status**. All Leica's primary antibody will be displayed.

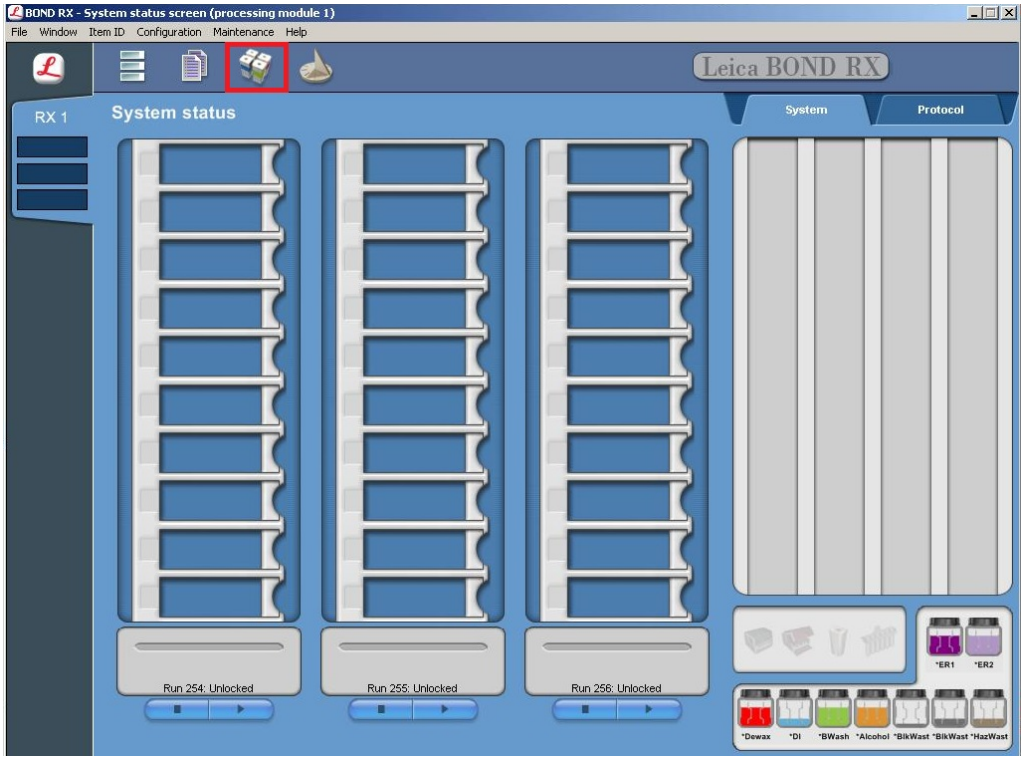


3. Select the antibody to be used (for example, *CD4). Double click to edit reagent properties
4. Select **Preferred** then **Save**.

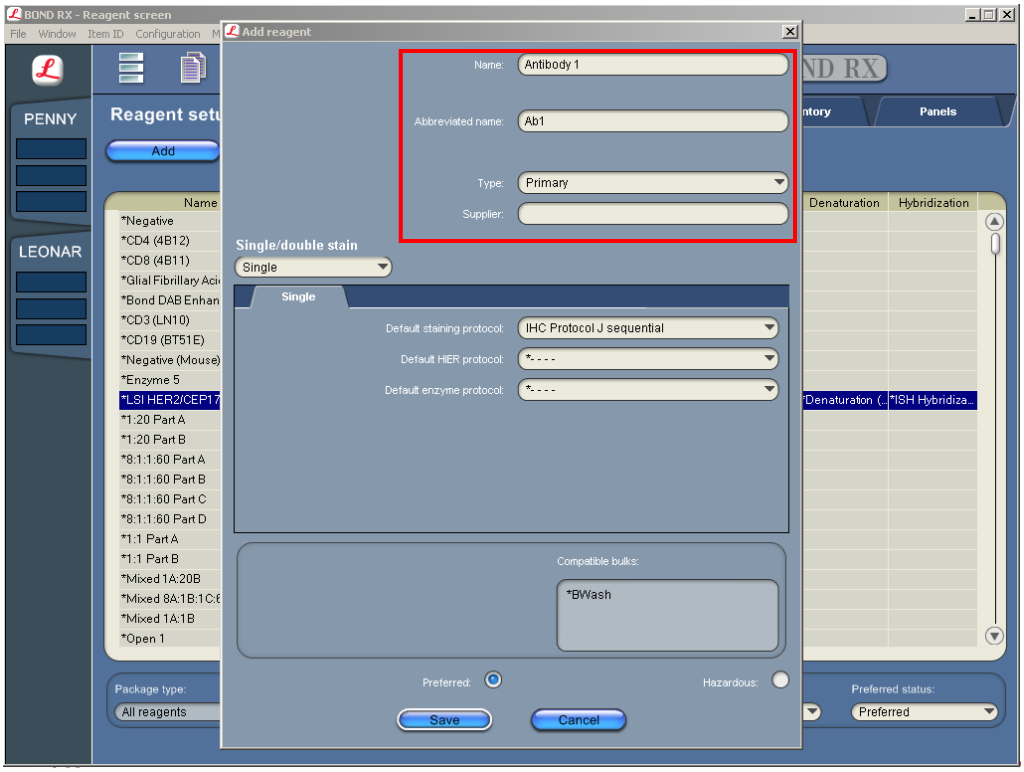


Register the reagents

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



2. Select **Add** to register a new primary antibody.
3. Enter a name for the antibody (for example, **Antibody 1**) in the Name text box. Enter the Abbreviated name text box (for example, **Ab1**).



4. Select **Primary** in the Type drop-down menu. Add the Supplier name.

   Note: You may leave the Supplier text box empty.

5. Select **Save**.

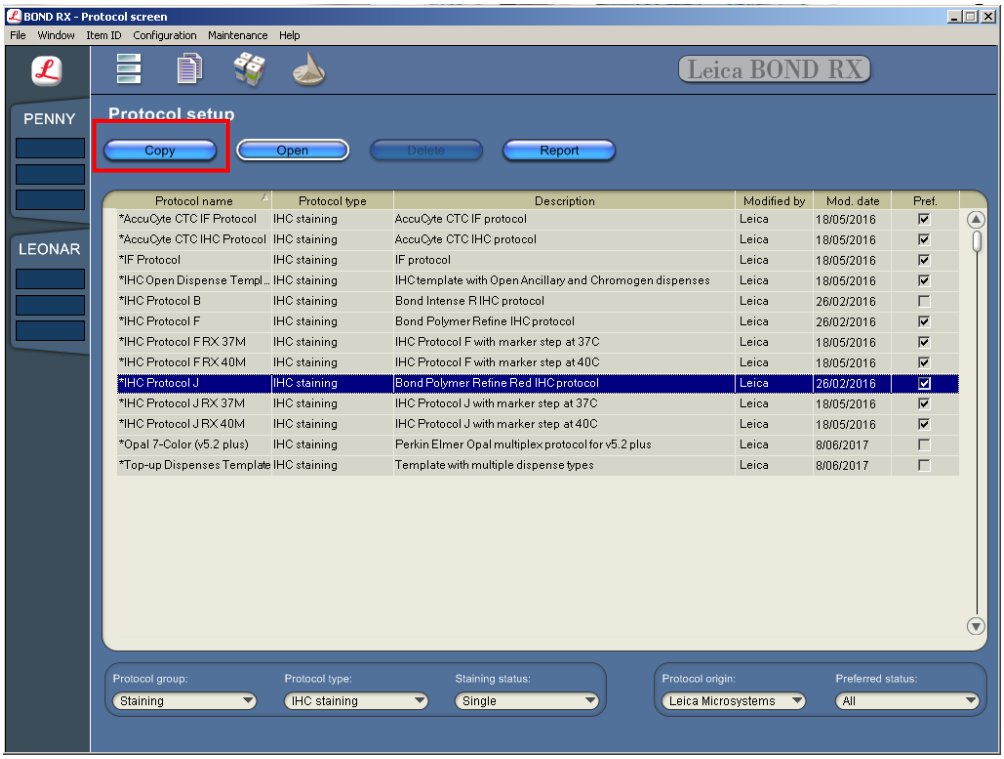
Part 3: Create the protocol for sequential immunohistochemistry (IHC)

You can combine the RNAscope® Brown ISH assay with fully automated Red IHC or semi-automated Green IHC. To combine the RNAscope® Brown ISH assay with fully automated Red IHC, follow the steps in ***Create a sequential Red immnunohistochemistry (IHC) protocol using the Leica BOND Red Refine Detection kit*** on page 9.

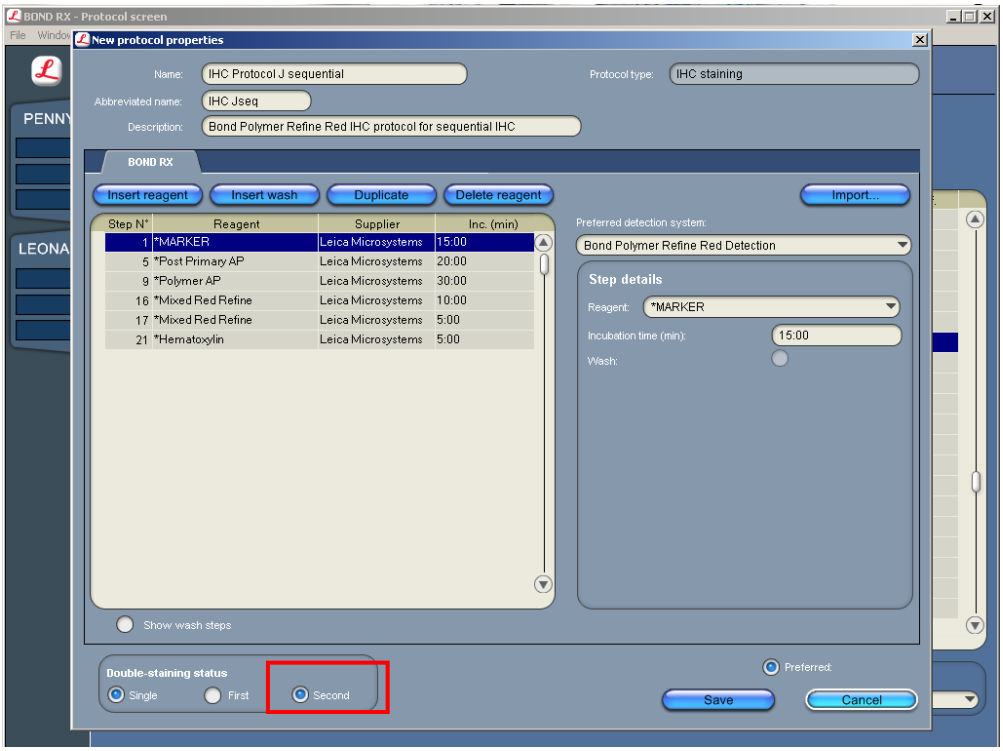
To combine the RNAscope® Brown ISH assay with semi-automated Green IHC, follow the steps in ***Create a semi-automated Green IHC protocol using the Leica BOND Refine Detection kit*** on page 10.

Create a sequential Red immnunohistochemistry (IHC) protocol using the Leica BOND Red Refine Detection Kit

1. To create a sequential Red IHC protocol, highlight ***IHC Protocol J** in the protocol set up page and select **Copy.**



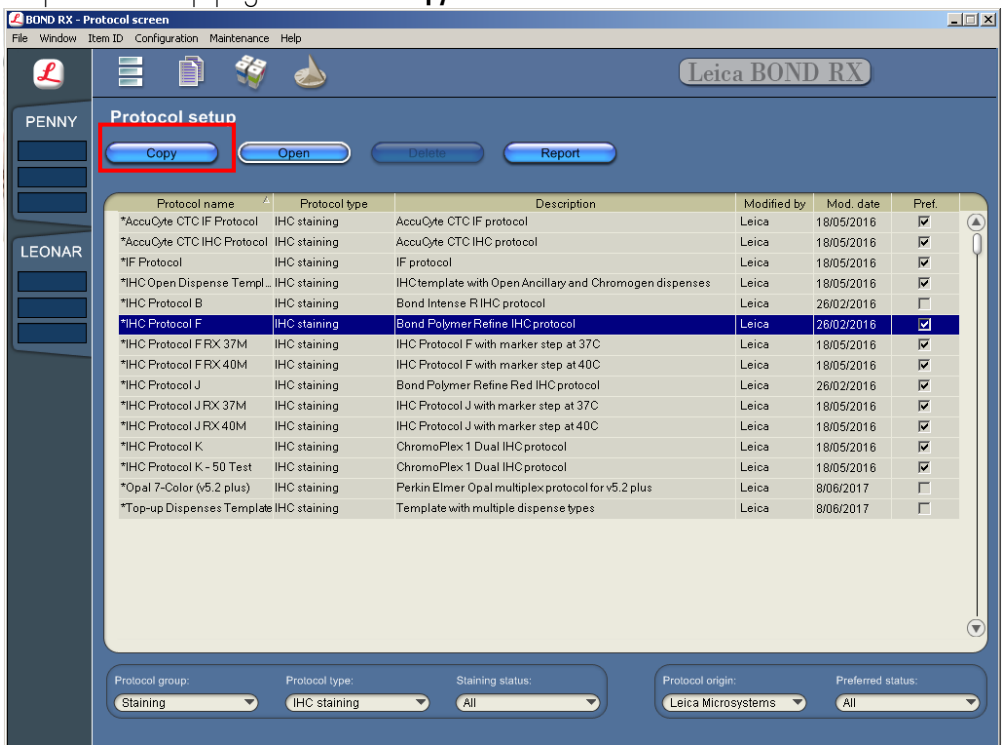
2. Enter **IHC Protocol J sequential** in the Name text box, **IHC Jseq** in the Abbreviated name text box, and **Bond Polymer Red Refine IHC Protocol for sequential IHC** in the Description text box.
3. Select **Second** in the Double-staining status menu. Other buttons are optional.



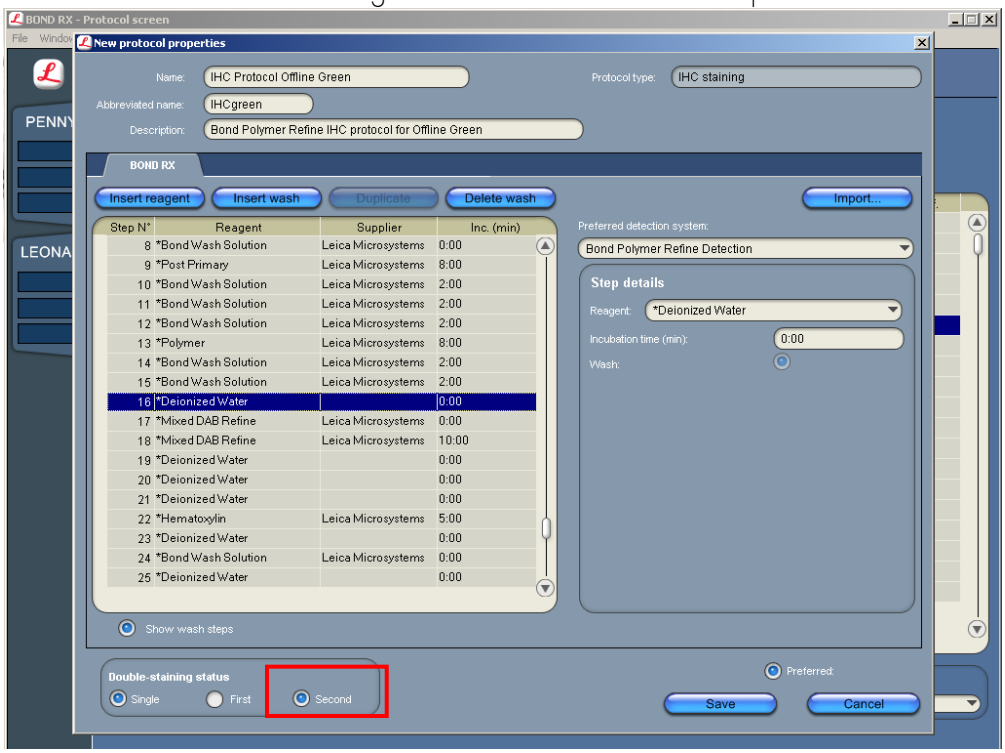
4. Select **Save**.

Create a semi-automated Green IHC protocol using the Leica BOND Refine Detection Kit

1. To create a semi-automated IHC protocol using Green Chromogen, highlight the***IHC Protocol F** protocol in the protocol set up page and select **Copy**.



2. Enter **IHC Protocol Offline Green** in the Name text box, **IHCgreen** in the Abbreviated name text box, and **Bond Polymer Refine IHC Protocol for offline Green** in the Description text box.
3. Select **Second** in the Double-staining status menu. Other buttons are optional.



4. Modify the protocol according to the following table. Delete the steps from ***Mixed DAB Refine** to ***Hematoxylin**. Select **Show wash steps** to display the wash steps, and delete the ***** **Deionized Water** wash steps.

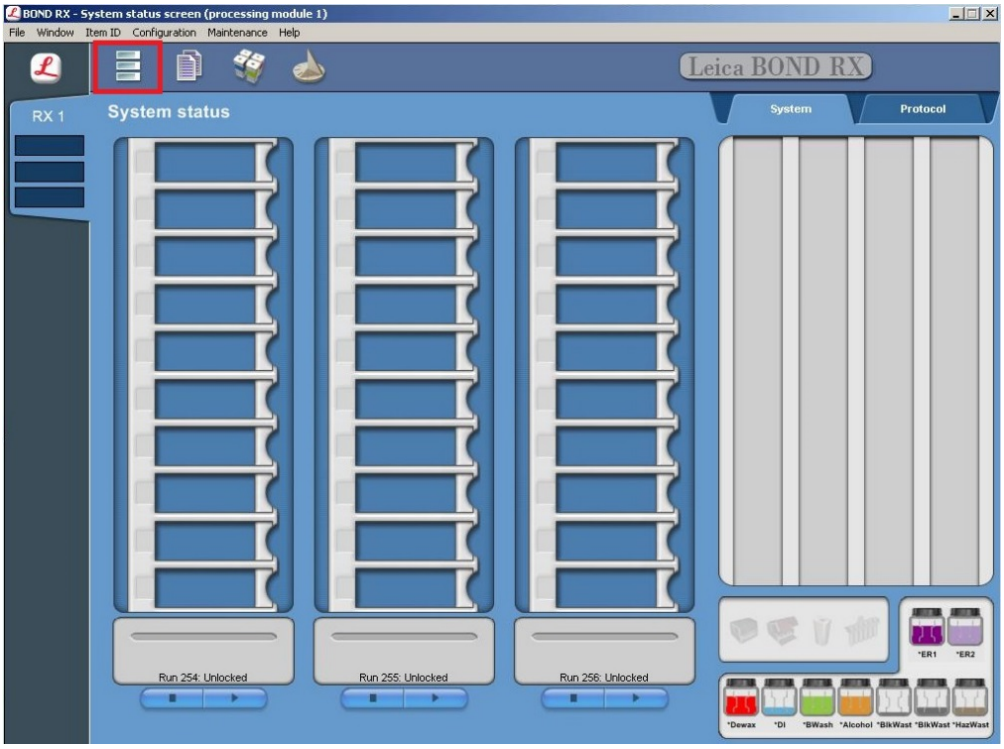
Step No.	Reagent	Step Type	Incubation Time	Temperature
1	*Peroxide Block	Reagent	5 MIN	Ambient
2	*Bond Wash Solution	Wash	0 MIN	Ambient
3	*Bond Wash Solution	Wash	0 MIN	Ambient
4	*Bond Wash Solution	Wash	0 MIN	Ambient
5	*MARKER	Reagent	15 MIN/30 MIN	Ambient
6	*Bond Wash Solution	Wash	0 MIN	Ambient
7	*Bond Wash Solution	Wash	0 MIN	Ambient
8	*Bond Wash Solution	Wash	0 MIN	Ambient
9	*Post Primary	Reagent	16 MIN	Ambient
10	*Bond Wash Solution	Wash	2 MIN	Ambient
11	*Bond Wash Solution	Wash	2 MIN	Ambient
12	*Bond Wash Solution	Wash	2 MIN	Ambient
13	*Polymer	Reagent	16 MIN	Ambient
14	*Bond Wash Solution	Wash	2 MIN	Ambient
15	*Bond Wash Solution	Wash	2 MIN	Ambient
16	*Bond Wash Solution	Wash	2 MIN	Ambient

5. Adjust the incubation times for ***Post primary** and ***Polymer** to **16 MIN**.
6. **(Optional)** Change the incubation time for ***MARKER** from **15 MIN** to **30 MIN**.
   Note: Increasing the primary antibody (*MARKER) incubation time can increase sensitivity.
7. Select **Save**.

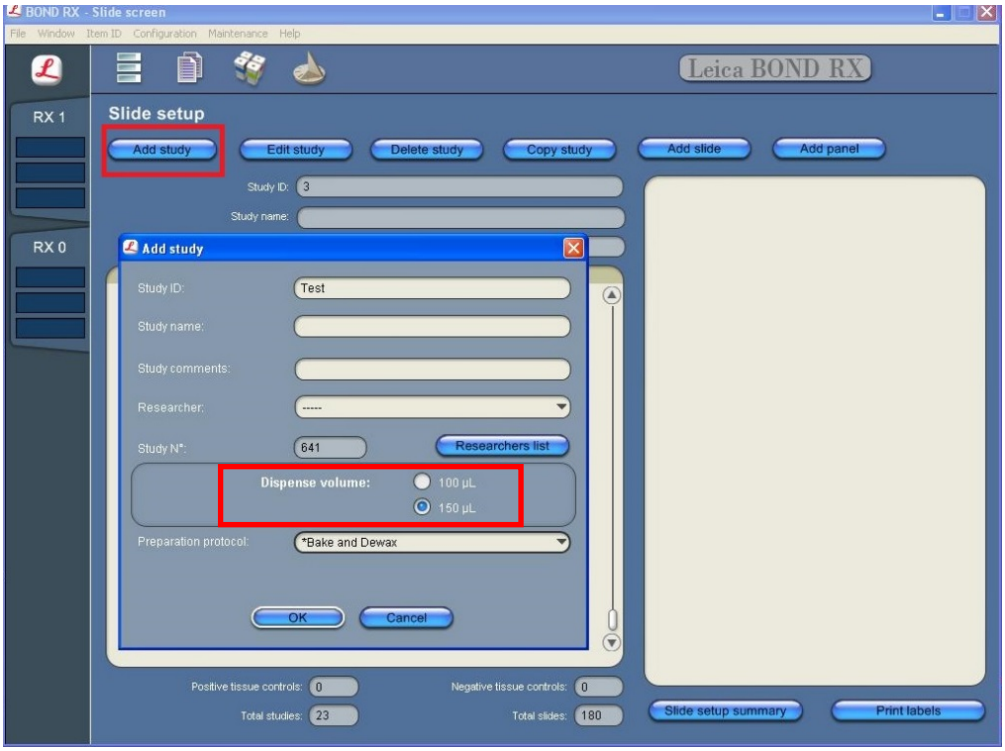
Part 4: Set up a study for sequential ISH-IHC

Build a study

1. Select the **Slide setup** icon at the top of the screen.



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

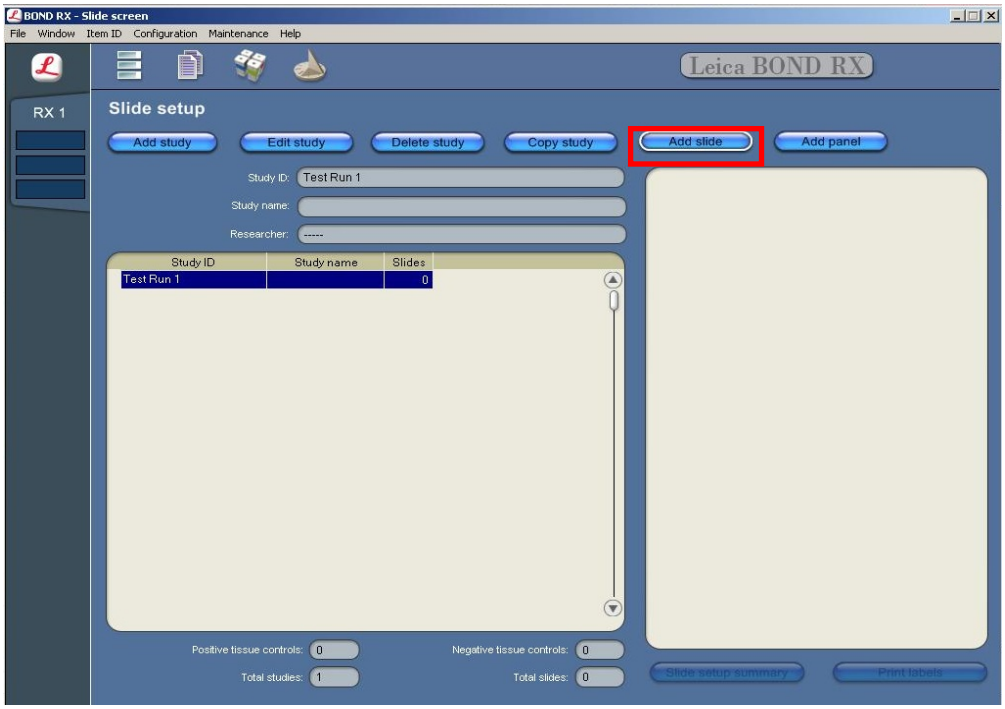


3. For FFPE tissues, select ***Bake and Dewax** as the Preparation protocol (leave blank for other tissue types).

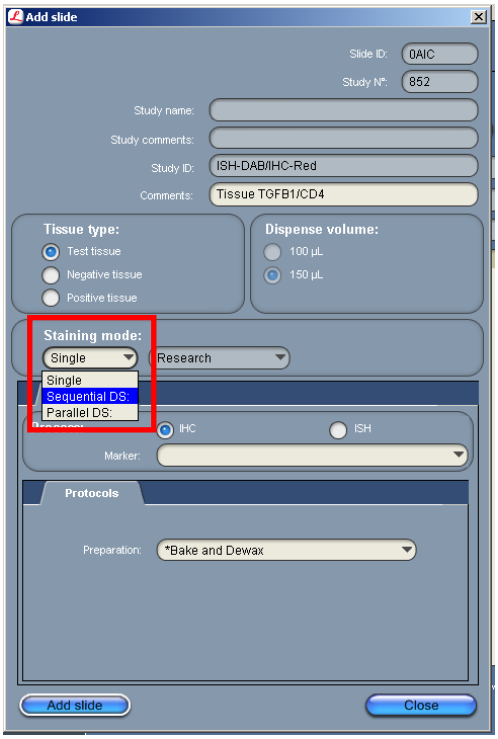
4. Select **OK**.

Add an ISH and IHC protocol to each slide

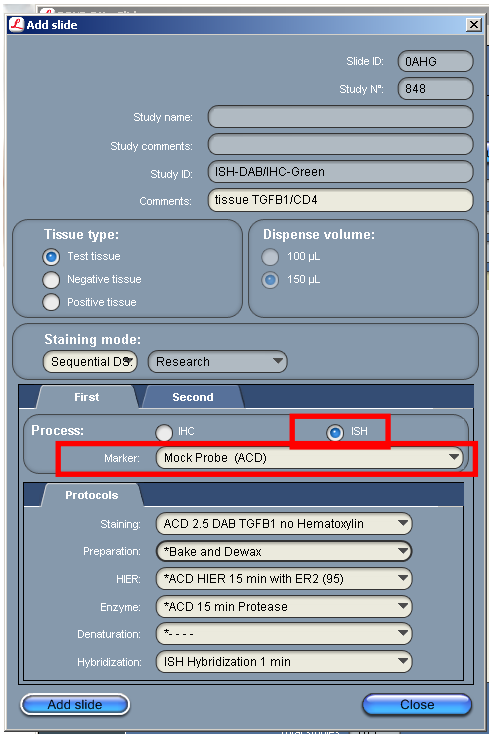
1. Select **Add slide**.



2. Enter the tissue type and probe name under the Comments field.
3. Select **Sequential DS** from the Staining mode drop down menu.



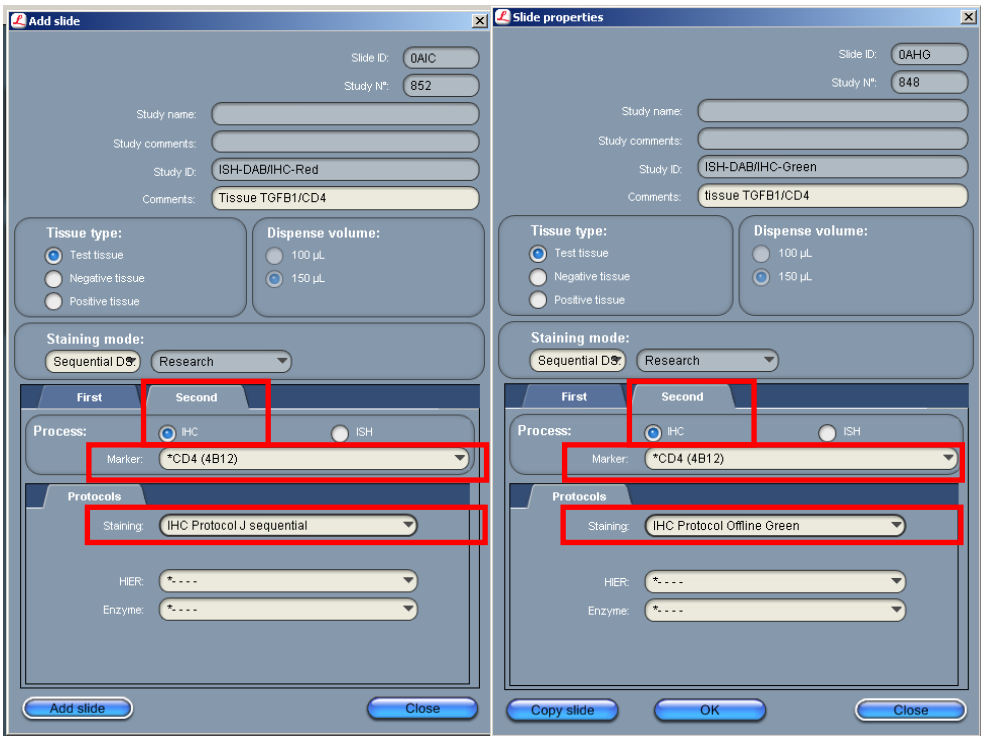
4. Add the ISH staining protocol by selecting the **First** tab.
5. Select **ISH** under Process, and **mock probe (ACD)** from the Marker drop down menu.



6. Under **Protocols**:
   a. Select a protocol from the Staining drop down menu for each probe. Make sure that each probe is associated with a different protocol (for example, **ACD 2.5 DAB TGFB1 no Hematoxylin** for the RNAscope® ISH Brown Assay).
   b. Select ***ACD HIER 15 min with ER2 (95)** as the HIER protocol, or the appropriate HIER protocol for your tissue.
   c. Select ***ACD 15 min Protease** for Enzyme, or the appropriate protease protocol for your tissue.
   d. Select **ACD 1 min Hybridization** for Hybridization.
7. Add the IHC protocol by selecting the **Second** tab.
8. Select **IHC** under Process and the antibody of interest (for example, ***CD4**) from the Marker menu.

   Note: For antibodies to be available from the menu, you must first register any antibodies not already available through Leica.
9. Select an IHC staining under **Protocols**:
   a. For Red IHC, select **IHC Protocol J sequential** from the Staining menu. Leave HIER and Enzyme blank.
   b. For semi-automated Green IHC, select **IHC Protocol Offline Green** from the Staining menu. Leave HIER and Enzyme blank.

IMPORTANT! Including additional HIER or Enzyme steps following the ISH staining may decrease the intensity of the ISH markers.



10. Repeat steps 1–9 for each slide.

    Note: To use a different probe on the new slide, change the staining protocol in the **First** tab. To use a different antibody on the new slide, change the marker selection in the **Second** tab.

Complete the study

1. After adding all the slides to the study, select **Close** to return to the Slide setup screen.
2. Select **Print labels** to print barcodes to attach to the slides.
3. Place the tray in the Leica BOND RX, and press the button to load the tray onto the machine.

IMPORTANT! Slides for RNAscope® Brown ISH - Red IHC and RNAscope® Brown ISH - Green IHC cannot be placed on the same tray.

4. 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 the scanned label images and select **Delayed Start** to start the run at a future time.

IMPORTANT! For semi-automated Green IHC, follow the instructions in Part 5.

Part 5: Detect green IHC staining off the instrument

Prepare reagents and equipment

1. Before the run completes, remove the Green A and Green B reagents from the refrigerator and warm to ambient temperature.

IMPORTANT! View the wash step video at **www.acdbio.com/technical-support/learn-more** before proceeding.

Detect Green staining off the instrument

1. As soon as the run is complete, press the button on the front of the instrument and unload the slides immediately.

IMPORTANT! If you do not perform the Green assay immediately, store the slides in 4X SSC at 4°C. 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.

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

2. 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.
3. Repeat Step 2 with fresh 1X Wash Buffer.
4. 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.
5. Prepare 200 µL 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.

6. Take each slide one at a time from the Tissue-Tek® Slide Rack and tap and/or flick to remove the excess liquid.
7. Pipette ~200 µL Green solution onto each tissue section. Ensure sections are covered.
8. Incubate the slides for **15 MIN** at **RT**.
9. 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 distilled water.
10. Quickly rinse the slides with fresh distilled water for less than 30 seconds.

IMPORTANT! Proceed quickly to the next step. Green substrate may fade if stored in water for too long.

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.
3. Repeat Step 2 once or twice.

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 a 24 mm x 50 mm coverslip over the tissue section. Avoid trapping air bubbles.
5. Repeat steps 2 and 3 for each slide.
6. Air dry the slides for **5 MIN**.

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