RNAscope® LS Multiplex Fluorescent Assay Combined with Immunofluorescence Technical Note

Introduction

This Technical Note provides guidelines for performing automated *in situ* hybridization (ISH) using an RNAscope® LS Multiplex Fluorescent Reagent Kit (Cat. No. 322800) combined with immunofluorescence (IF) on the Leica BOND RX System. This procedure is based on the standard RNAscope® LS Multiplex Fluorescent Assay and requires the Leica BOND Detection Kit for immunofluorescent detection). Before starting the procedure, create a protocol for the RNAscope® assay combined with IF 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 proprietary protease that may not be compatible with all antibodies. Please validate your antibody for use with the RNAscope® Assay.

Materials Required

RNAscope® LS Multiplex Fluorescent Reagent Kit

The kit provides enough reagents to stain ~60 standard slides. The reagents are Ready-To-Use (RTU) except for the TSA® buffer, and are stored as indicated in the following table:

RNAscope® LS Multiplex Reagent Kit (Cat. No. 322440)			
☑	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® LS Multiplex AMP 1	21 mL x 1 bottle	2–8°C
	RNAscope® LS Multiplex AMP 2	21 mL x 1 bottle	2–8°C
	RNAscope® LS Multiplex AMP 3	21 mL x 1 bottle	2–8°C
	RNAscope® LS Multiplex HRP C1	21 mL x 1 bottle	2–8°C
	RNAscope® LS Multiplex HRP C2	21 mL x 1 bottle	2–8°C
	RNAscope® LS Multiplex HRP C3	21 mL x 1 bottle	2–8°C
	RNAscope® TSA Buffer	29 mL x 3 bottle	2–8°C
	RNAscope® LS Multiplex HRP Blocker	29 mL x 2 bottle	2–8°C
	RNAscope® LS Multiplex DAPI	21 mL x 1 bottle	2–8°C

Materials from Leica BOND RX

The RNAscope® LS Multiplex Fluorescent Assay 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 7 mL Open containers*	OP79193	Room temp (20–25°C)
	BOND Research Detection System	DS9455	Room temp (20–25°C)
	BOND Universal Covertiles 100 pack	S21.2001	Room temp (20–25°C)
	BOND Polymer Refine Detection	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)

*(Optional) Recommended for use with TSA® Plus fluorophores.

TSA® Plus Fluorophores or Opal™ Dyes

The assay requires TSA® Plus fluorophores or Opal™ dyes from PerkinElmer (see the following table). Dilute the fluorophores in TSA buffer provided by the RNAscope® LS Multiplex Reagent Kit. Choose a dilution factor for each fluorophore based on recommendations from ACD and your needs (for example, tissue quality or microscope setting). Materials are qualified using a 1:1500 dilution for all three fluorophores. We cannot guarantee assay results if you use other fluorescent dyes.

Fluorophores	Production number (PerkinElmer)	Recommended dilution range
PerkinElmer TSA® Plus fluorescein System	NEL741001KT*	1:750–1:3000
PerkinElmer TSA® Plus Cyanine 3 System	NEL744001KT*	1:750–1:3000
PerkinElmer TSA® Plus Cyanine 5 System	NEL745001KT*	1:750–1:3000
Opal 520	FP1487001KT: Opal 520 Reagent Pack†	1:750–1:3000
Opal 570	FP1488001KT: Opal 570 Reagent Pack†	1:750–1:3000
Opal 620	FP1495001KT: Opal 620 Reagent Pack†	1:750–1:3000
Opal 690	FP1497001KT: Opal 690 Reagent Pack†	1:750–1:3000

* Depending on the dilution factor used, this stock size (300 µl) is sufficient to run the assay on 750–3000 slides. More size options are available from the PerkinElmer product website.

† Depending on the dilution factor used, this stock size (150 µl) is sufficient to run the assay on 375–1500 slides.

Recommended fluorophore combinations

Use the TSA® Plus system or Opal™ dyes from PerkinElmer to develop the RNAscope® and IF signal. The following table lists examples of 3-plex fluorophore combinations using the TSA® Plus system or Opal™ dyes from PerkinElmer. Opal 520 and Opal 570 are interchangeable with TSA® Plus fluorescein and Cyanine 3, respectively (see Options 1 and 2 in the

table). Users may assign a certain fluorophore to any of the ACD multiplex TSA-F1, TSA-F2, and TSA-F3 channels (see Options 3 and 4). Do not assign the same fluorophore to more than one channel. For each assay, use only one fluorophore from each set of interchangeable fluorophores.

IMPORTANT! If Cyanine 5 is assigned to the TSA-F1 or TSA-F2, users may need to increase the concentration of TSA® Plus Cyanine 5.

Reagent registration name	Option1 (recommended)	Option 2	Option 3	Option 4
ACD Multiplex TSA-F1	TSA® Plus fluorescein	Opal 520	TSA® Plus Cyanine 3	Opal 570
ACD Multiplex TSA-F2	TSA® Plus Cyanine 3	Opal 570	TSA® Plus fluorescein	Opal 520
ACD Multiplex TSA-F3	TSA® Plus Cyanine 5	Opal 690	TSA® Plus Cyanine 5	Opal 690

If you are running a 3-plex RNAscope® *in situ* hybridization (ISH) plus immunofluorescent (IF) assay, see the following table for examples below of four fluorophore combinations. Use Opal 620 as the fourth color when using the TSA® Plus fluorophores (see Option 1 in the table). Alternatively, you may choose four colors from the Opal™ 7-color fIHC kit (see Options 2–4 in the table). Opal users may assign a certain fluorophore to any of the ACD multiplex TSA-F1, TSA-F2, and TSA-F3 channels for RNAscope® ISH, or the TSA-F4 channel for IF (for example, in Options 2–4).

Reagent registration name	Option 1 (recommended)	Option 2	Option 3	Option 4
ACD Multiplex TSA-F1	Opal 520	TSA® Plus Fluorescein	Opal620	Opal 520
ACD Multiplex TSA-F2	Opal 570	TSA® Plus Cyanine 3	Opal 520	Opal 570
ACD Multiplex TSA-F3	Opal 620	Opal 620	Opal 690	Opal 690
TSA-F4	Opal 690	TSA® Plus Cyanine 5	Opal 570	Opal 620

IMPORTANT! Use a fluorescent multispectral imaging system, such as the Nuance® EX, Mantra™, or Vectra® Systems, to successfully analyze your multiplex fluorescent staining. Always check the viewing capacity of your imaging system before setting up experiments.

Workflow

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

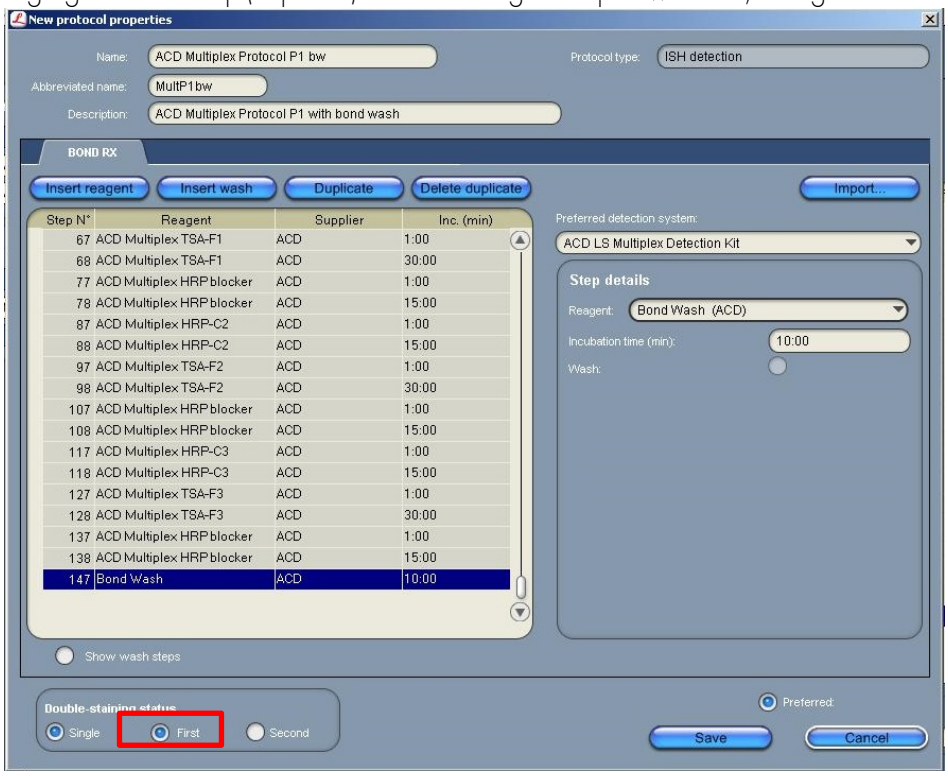
This section provides instructions for creating two *in situ* hybridization (ISH) software protocols on the Leica BOND RX System. The protocols are compatible with performing immunofluorescence on the same samples after ISH is completed. To detect three targets using ISH, follow the instructions in **Create a 3-plex ISH protocol** on page 3. To detect two targets using ISH, follow the instructions in **Create a duplex ISH protocol**.

Create a 3-plex ISH protocol

IMPORTANT! After combining the 3-plex ISH protocol with IF, you will need four filters on your microscope to visualize the results.

1. In the Protocol setup screen, select **Staining** under the Protocol group menu.
2. Highlight the protocol for the standard RNAscope® LS Multiplex Fluorescent Assay set up by your ACD FAS (for example, **ACD Multiplex Protocol P1**). Select **Copy**.
3. Change the protocol name for your first probe to **ACD Multiplex Protocol P1 bw** in the Name text box, **MultP1bw** in the Abbreviated name text box, and **ACD Multiplex Protocol P1 with Bond Wash** in the Description text box.

4. Highlight the **DAPI** step (step 147). From the Reagent drop down menu, change **DAPI** to **Bond Wash**.



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

   Note: The **Single** button is optional unless you would like to run a single stain assay with this protocol.

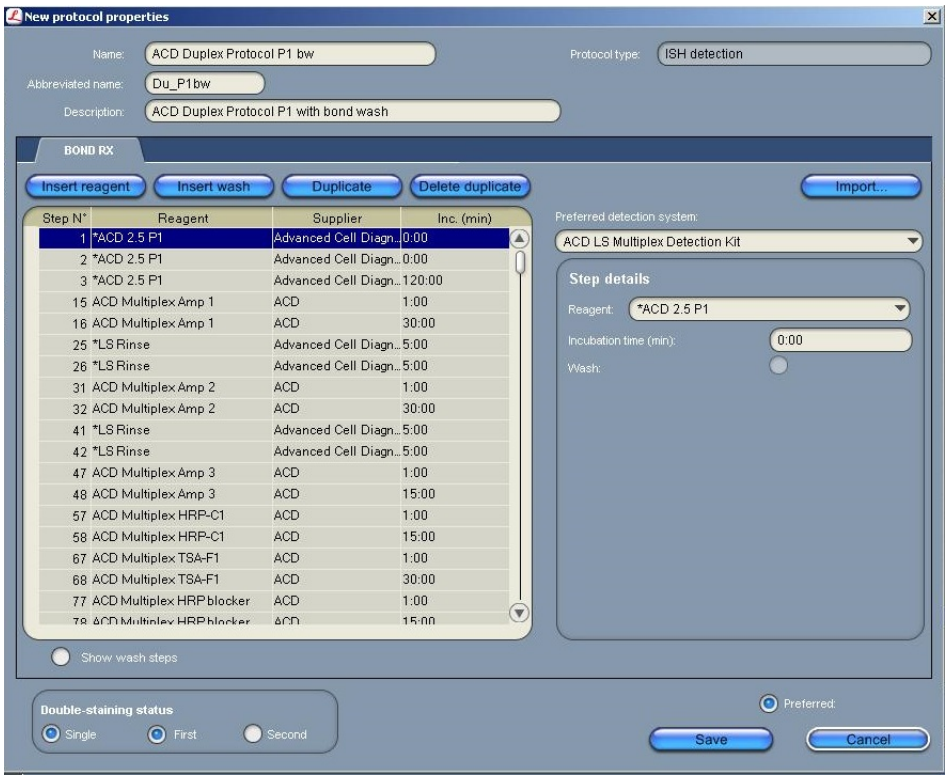
6. Select **Save**.
7. Click **Next** to proceed. Ignore any pop ups that may appear on the screen.
8. To create a protocol for each additional probe, follow steps 1–7.

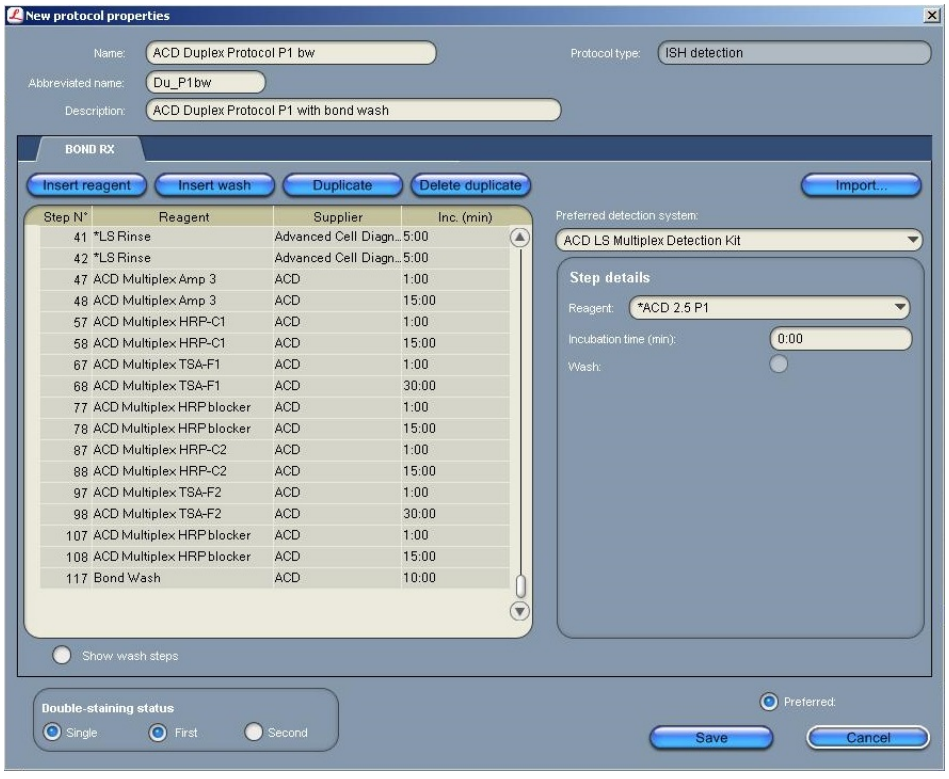
Create a duplex ISH protocol

1. In the Protocol setup screen, select **Staining** under the Protocol group menu.
2. Highlight the protocol for the standard RNAscope® LS Multiplex Fluorescent Assay set up by your ACD FAS (for example, **ACD Multiplex Protocol P1**). Select **Copy**.
3. Change the protocol name for your first probe to **ACD Duplex Protocol P1 bw** in the Name text box, **Du_P1bw** in the Abbreviated name text box, and **ACD Duplex Protocol P1 with Bond Wash** in the Description text box.
4. Highlight the **DAPI** step (step 147). From the Reagent drop down menu, change **DAPI** to **Bond Wash**.
5. To perform a sequential dual stain (ds stain), make sure that **First** is selected under Double-staining status.

   Note: The **Single** button is optional unless you would like to run a single stain assay with this protocol.

6. Click on **Show wash steps** to view all of the wash steps.
7. Select **Delete duplicate** or **Delete wash** to delete steps 117–146.
8. Select **Save**.
9. Click **Next** to proceed. Ignore any pop ups that may appear on the screen.
10. To create a protocol for each additional probe, follow steps 1–7.





Note: The preceding two figures display all reagent steps.

Part 2: Create a software protocol to perform immunofluorescence (IF)

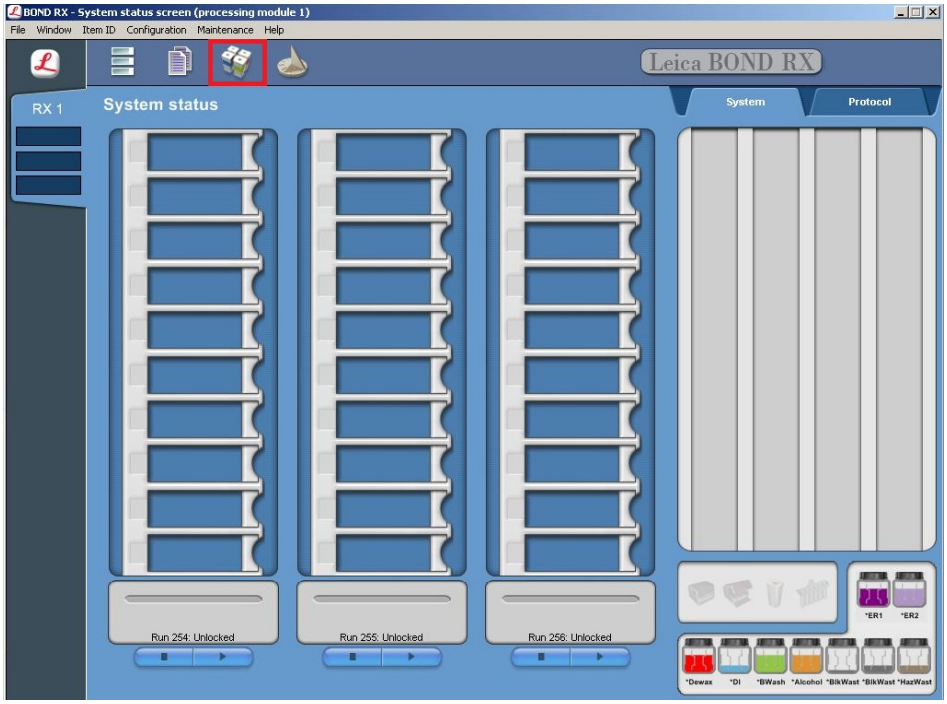
To perform immunofluorescence on the instrument with your chosen antibody, you must create an IF protocol in the RX software that uses the Leica BOND Refine Detection Kit.

Register the reagents

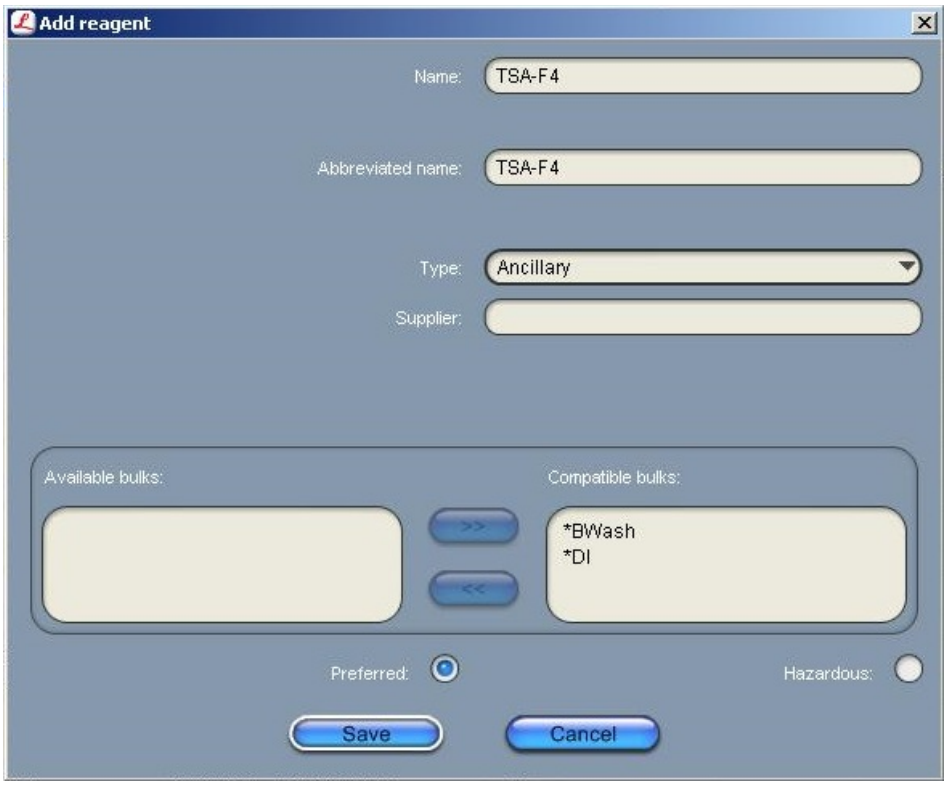
1. To add the fourth fluorophore to the assay, select the **Reagent Setup** icon at the top of the screen.

   Note: If performing duplex ISH followed by IHC, you do not need to add a fourth fluorophore to the assay. You may use ACD Multiplex TSA-F3 for the IF protocol.

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



3. Enter a name for the fluorophore (for example, **TSA-F4**) in the Name text box.
4. Enter **TSA-F4** (for example) in the Abbreviated name text box.
5. Select **Ancillary** in the Type drop-down menu.

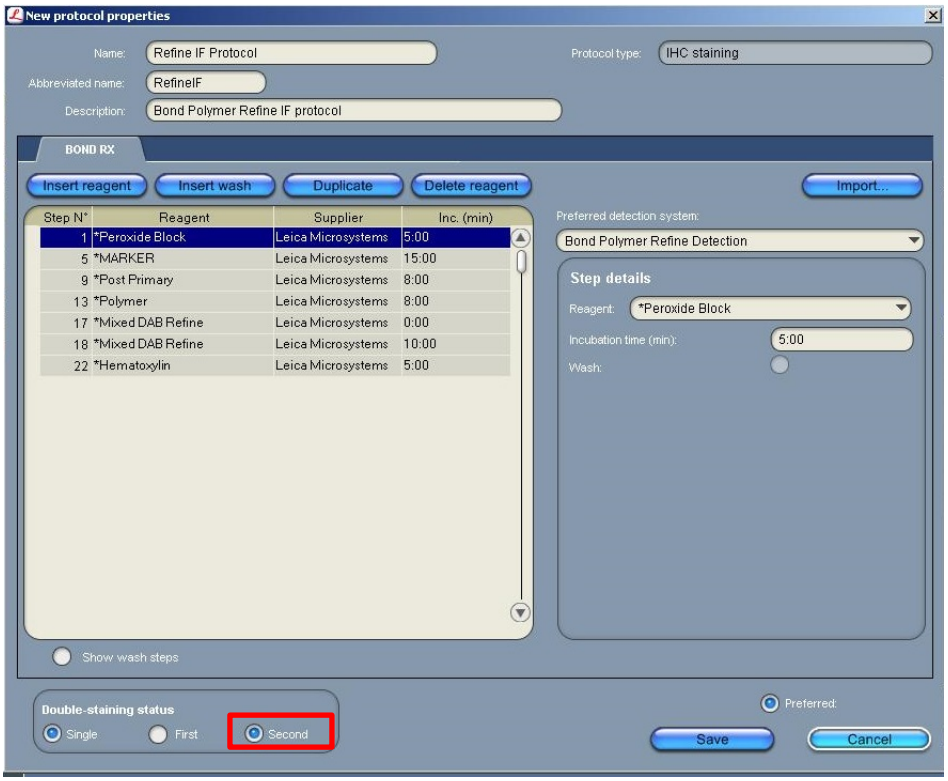


Note: You may leave the Supplier text box empty.

6. Select **Save**.

Create an immunofluorescent (IF) protocol using the Leica BOND Refine Detection Kit

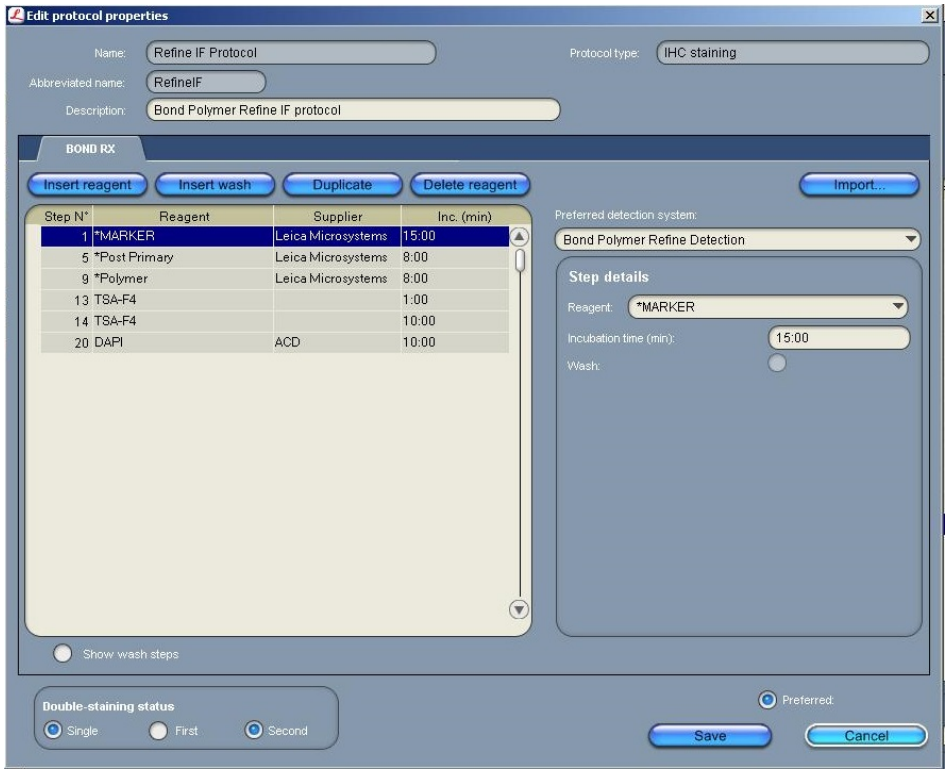
1. To create an IF protocol, highlight the***IHC Protocol F** protocol. Select **Copy.**
2. Name the protocol (for example, **Refine IF Protocol)** in the Name text box, **RefineIF** in the Abbreviated name text box, and **Bond Polymer Refine IF Protocol** in the Description text box.
3. Select **Second** in the Double-staining status menu. Other buttons are optional.



4. Modify the protocol according to the following table. Delete ***Peroxide Block**, change ***Mixed DAB Refine** to **TSA-F4** (or **TSA-F3** if running a duplex ISH assay followed by IF), and change ***Hematoxylin** to **DAPI**. Adjust the incubation time for each step.

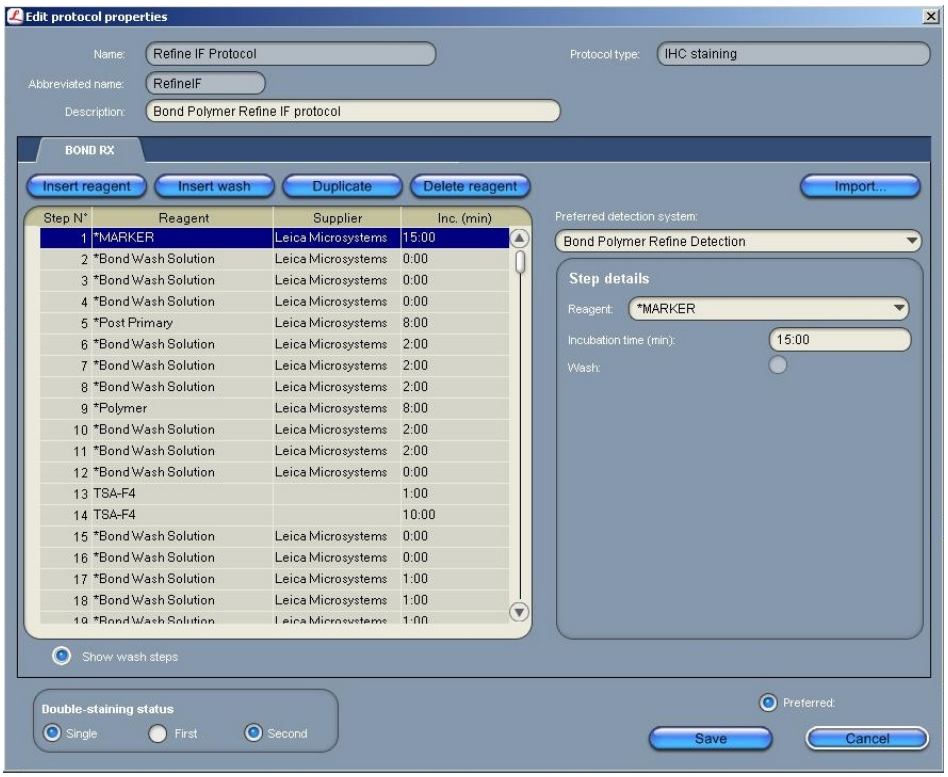
Step No.	Reagent	Step Type	Incubation Time	Temperature
1	*MARKER	Reagent	15 MIN	Ambient
2	*Bond Wash Solution	Wash	0 MIN	Ambient
3	*Bond Wash Solution	Wash	0 MIN	Ambient
4	*Bond Wash Solution	Wash	0 MIN	Ambient
5	*Post Primary	Reagent	8 MIN	Ambient
6	*Bond Wash Solution	Wash	2 MIN	Ambient
7	*Bond Wash Solution	Wash	2 MIN	Ambient
8	*Bond Wash Solution	Wash	2 MIN	Ambient
9	*Polymer	Reagent	8 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	TSA-F4/F3	Reagent	1 MIN	Ambient
14	TSA-F4/F3	Reagent	10 MIN	Ambient
15	*Bond Wash Solution	Wash	0 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature
16	*Bond Wash Solution	Wash	0 MIN	Ambient
17	*Bond Wash Solution	Wash	1 MIN	Ambient
18	*Bond Wash Solution	Wash	1 MIN	Ambient
19	*Bond Wash Solution	Wash	1 MIN	Ambient
20	DAPI	Reagent	10 min	Ambient
21	*De-ionized Water	Wash	0 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



Note: To perform DAPI on the instrument for the IF protocol, you will need to register a separate DAPI container. The software cannot use the DAPI container from the Bond Detection system. The software will display an error message if you do not include an additional container of DAPI on the instrument.

5. Click **Show wash steps** to display the wash steps.
6. Select **Insert wash** to add BOND Washes. Match each of the protocol steps shown.



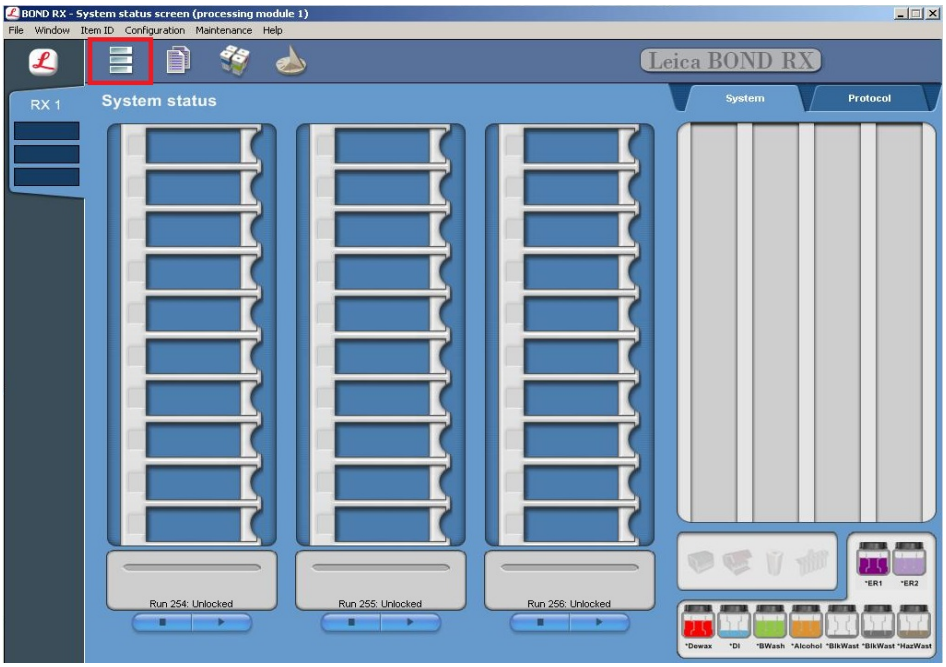
7. Select **Save**.

Part 3: Set up a sequential dual stain (ds) study for duplex or multiplex ISH followed by IF

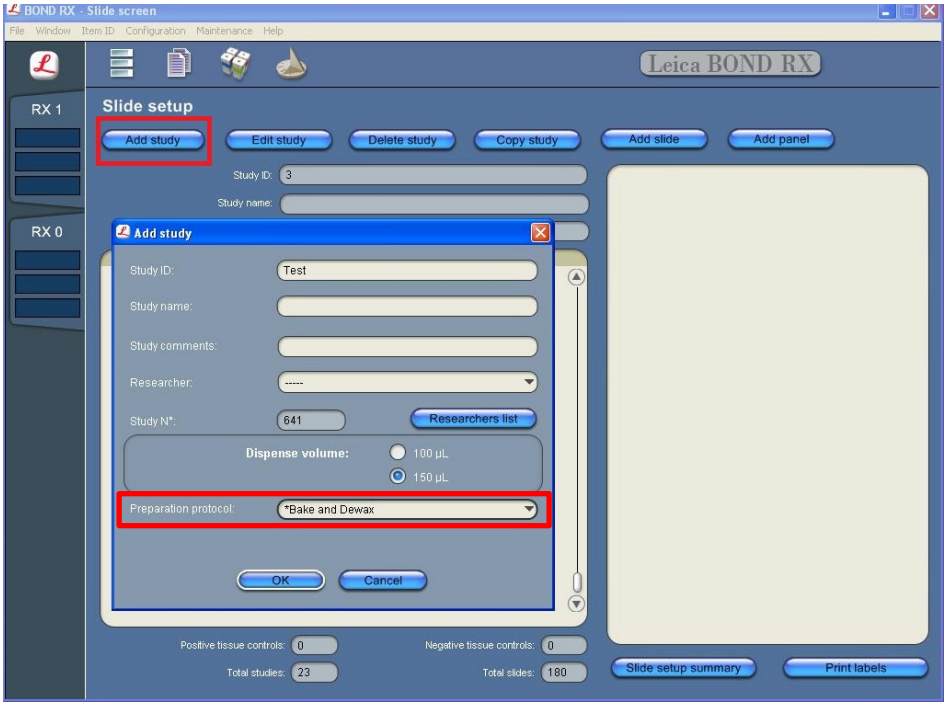
IMPORTANT! Only run a maximum of two trays. Running three trays will result in significant instrument errors including loss of dispensers.

Build a study

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



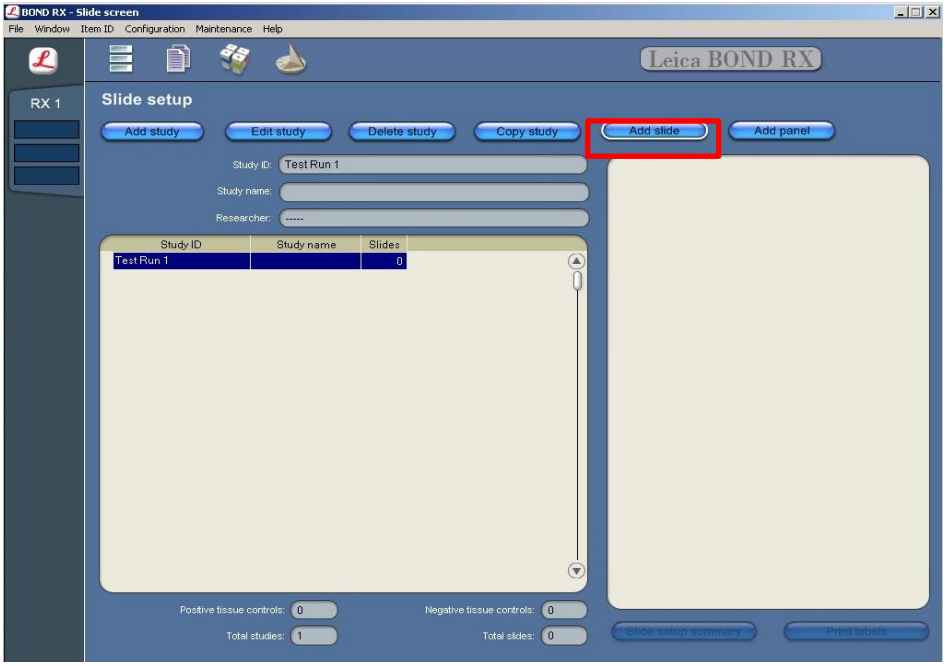
2. Select **Add study** and enter a name in the Study ID field (keep the Dispense volume at **150** µL as shown).



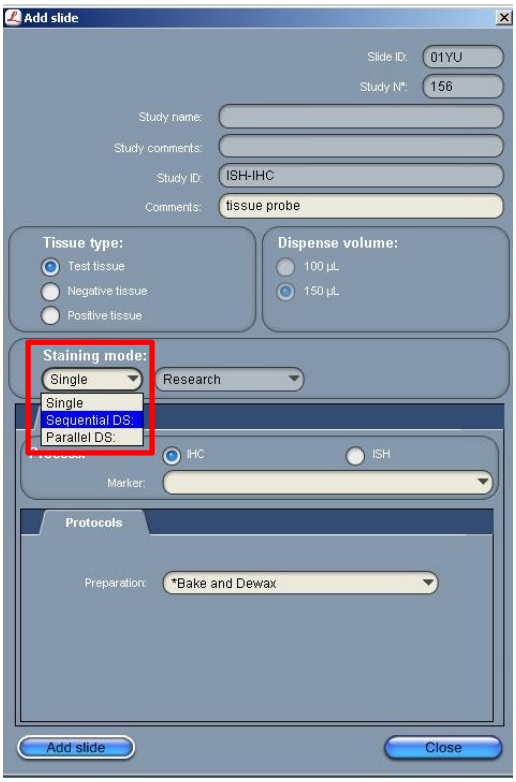
3. For FFPE tissues, select ***Bake and Dewax** as the Preparation protocol (otherwise, leave blank).
4. Select **OK**.

Add an ISH and IHC protocol to each slide

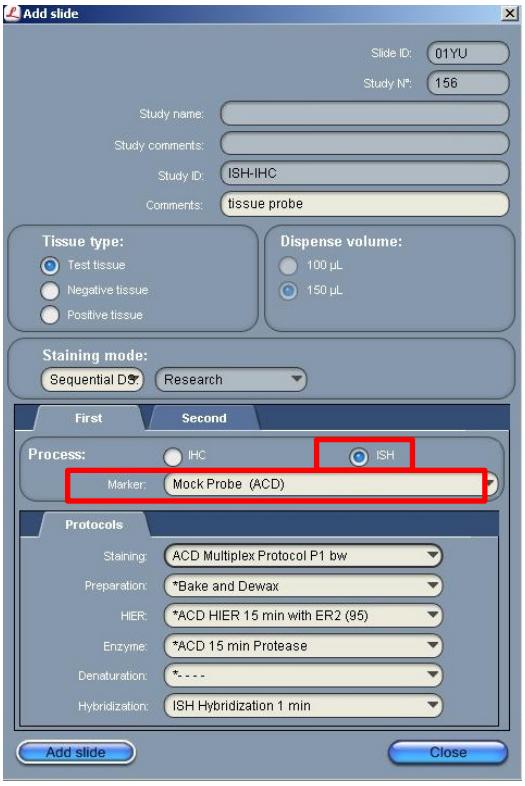
1. Select **Add slide**.



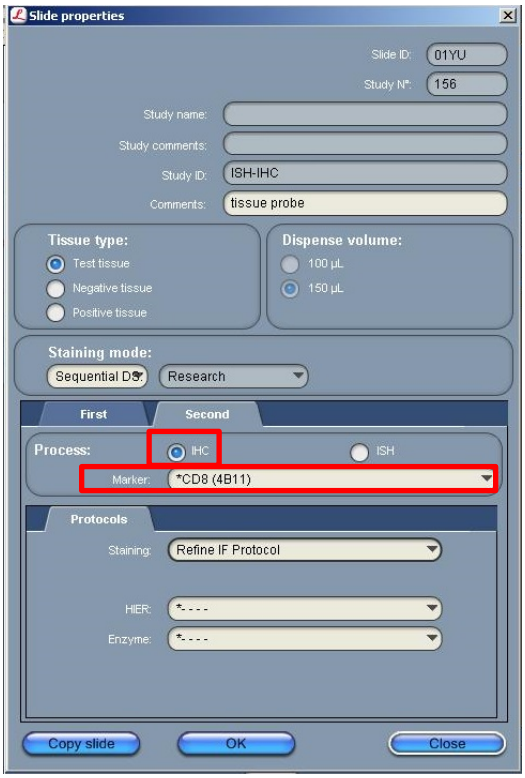
2. Enter the tissue type and probe name under the Comments field.
3. Select **Sequential DS** from the Staining mode drop down menu



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.



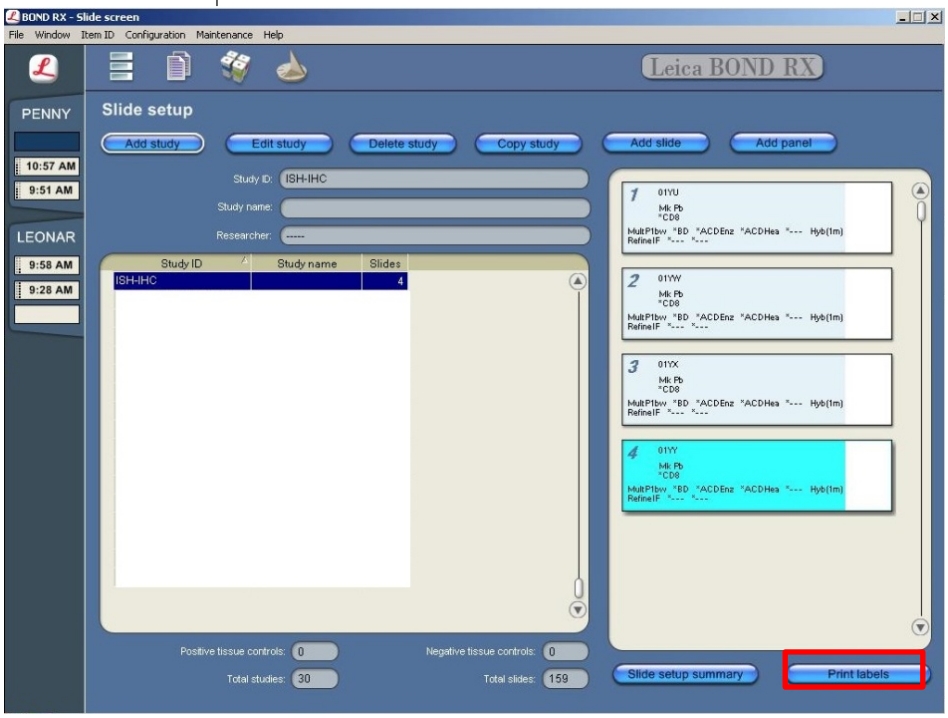
6. Under **Protocols**:
   a. Select a protocol from the Staining drop down menu for each probe. Make sure that each probe is associated with a different protocol (for example, **ACD Multiplex Protocol P1 bw** for multiplex ISH or **ACD Duplex Protocol P1 bw** for duplex ISH).
   b. , Select the protocol ***Bake and Dewax** from the Preparation drop down menu for standard FFPE tissues (otherwise, leave blank).
   c. Select ***ACD HIER 15 min with ER2 (95)** as the HIER protocol or the appropriate HIER protocol for your tissue.
   d. Select ***ACD 15 min Protease** for Enzyme or the appropriate HIER protocol for your tissue.
   e. 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, ***CD8**) 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. Under **Protocols**, select **Refine IF Protocol** from the Staining menu. Leave HIER and Enzyme blank.
   Note: Including additional HIER or Enzyme steps following ISH staining may decrease the intensity of 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.



Part 4: Imaging

To image fourplex fluorescent staining, use a fluorescent multispectral imaging system, such as the Nuance® EX, Mantra™, or Vectra® Systems. The following table lists the corresponding filter setting for each dye.

TSA® Plus System	Opal™ system	Filter setting
TSA® Plus fluorescein	Opal 520	FITC
TSA® Plus Cyanine 3	Opal 570	Cy3
	Opal 620	Texas Red
TSA® Plus Cyanine 5	Opal 690	Cy5

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