

# RNAscope® LS Multiplex Fluorescent Assay combined with Immunofluorescence - Integrated Co-Detection Workflow (ICW) on Leica Bond RX

## Contents

<b>Introduction</b> .....	<b>2</b>
<b>Materials Required</b> .....	<b>2</b>
RNAscope® LS Multiplex Fluorescent Reagent Kit .....	2
Ancillary materials for RNA-Protein Co-Detection .....	3
Materials from Leica BOND RX .....	3
TSA® Plus Fluorophores or Opal™ Dyes .....	3
Recommended fluorophore combinations .....	4
<b>Workflow 1: Fluorescent Multiplex Co-Detection Using TSA-based RNA and Protein Detection</b>	<b>6</b>
Workflow steps .....	6
Add new Co-Detection Reagents .....	7
Create Part A of the LS Multiplex RNA-Protein Co-Detection Sequential Dual Stain protocol .....	8
Create Part B of the Co-Detection Sequential Dual Stain protocol to perform IF with TSA-based secondary antibody detection .....	13
Build a Sequential Dual Stain study for LS Multiplex RNA-Protein Co-Detection .....	15
<b>Workflow 2: Fluorescent Multiplex Co-Detection Using a Fluorophore-Conjugated Secondary Antibody for Protein Detection.</b> .....	<b>20</b>
Workflow steps .....	20
Add new Co-Detection Reagents .....	21
Create a Co-Detection Single Stain protocol using a fluorophore-conjugated secondary antibody for IF detection. ....	22
Build a single stain study for Multiplex RNA-Protein Co-Detection .....	28
<b>Imaging</b> .....	<b>31</b>
<b>Appendix A. Creating a new HIER Protocol</b> .....	<b>31</b>
<b>Appendix B. Multiplex Co-Detection Using Opal™ Polaris 780 for RNA Detection and TSA fluorophore for Protein Detection.</b> .....	<b>33</b>
<b>Appendix C. Multiplex Co-Detection Using TSA Fluorophores for RNA Detection and Opal™ Polaris 780 for Protein Detection.</b> .....	<b>41</b>
<b>Appendix D. Multiplex Co-Detection</b> ..... <b>Using Opal™ Polaris 780 for RNA Detection and Fluorophore-conjugated Secondary for Protein Detection</b> .....	<b>48</b>

## Introduction

This Technical Note provides guidelines for performing automated fluorescent Co-Detection of RNA and protein by combining *in situ* hybridization (ISH) using an RNAscope® LS Multiplex Fluorescent Reagent Kit (Cat. No. 322800) and immunofluorescence (IF) on the Leica BOND RX System. You can use these protocols with a wide range of protein targets, including those that are protease sensitive. On the Leica BOND RX, the samples undergo deparaffinization and heat-induced epitope retrieval, followed by primary antibody incubation and post-primary fixation. Samples are then treated with protease and undergo probe hybridization and full LS Multiplex RNAscope® detection, followed by secondary antibody incubation, detection, and counterstaining. Workflow 1 describes how to perform Co-Detection using TSA-based fluorescent detection of both RNA and protein. Workflow 2 describes how to perform Co-Detection using TSA-based fluorescent detection of RNA together with a fluorophore-conjugated secondary antibody.

If you are using a Cy7 filter, you can use Opal™ Polaris 780 for either RNA or protein detection. Opal™ Polaris 780 uses two-tier detection, requiring both the TSA-DIG and Polaris 780 reagents. Because the 780 fluorophore is extremely sensitive to cleavage by HRP activity, we recommend following TSA-DIG incubation with detection of the remaining targets, then applying Polaris 780 as the last step of the workflow before counterstaining and mounting. To use Opal™ Polaris 780 for ISH detection with TSA-based protein detection, see **Appendix B**. To use Opal™ Polaris 780 for immunofluorescence with TSA-based ISH detection, see **Appendix C**. If you are using Opal™ Polaris 780 for ISH detection with a fluorophore-conjugated secondary antibody, see **Appendix D**.

Before starting the procedure, you will need to create a protocol for multiplex Co-Detection on the BOND RX controller with the help of your ACD FAS that fits your detection preferences. For every chemical, read the Safety Data Sheet (SDS) and follow handling instructions. Wear appropriate protective eyewear, clothing, and gloves. For the latest service and support information, go to [www.acdbio.com/support](http://www.acdbio.com/support).

## 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)			
<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® 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

## Ancillary materials for RNA-Protein Co-Detection

Quantities provide enough reagents to stain ~60 standard slides. The reagents are Ready-To-Use (RTU) except for the TSA<sup>®</sup> buffer and Co-Detection Antibody Diluent, and are stored as indicated in the following table:

Ancillary Materials for Co-Detection				
<input checked="" type="checkbox"/>	Reagent	Source	Quantity	Storage
	Co-Detection Antibody Diluent (Cat. No. 323160)	ACD	120 mL x 1 bottle	2–8°C
	Primary Antibody Concentrate*	User	As needed	Per manufacturer's recommendation
	10% Neutral Buffered Formalin (NBF)	User	5–10 mL	Per manufacturer's recommendation

**Note:** Ready-to-use antibodies are not recommended for use with this workflow. Using primary antibody concentrate diluted in Co-Detection Antibody Diluent is recommended for optimal RNA signal.

## Materials from Leica BOND RX

The RNAscope<sup>®</sup> LS Multiplex Fluorescent Assay requires specific materials and equipment available *only* from Leica Biosystems.

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

## TSA<sup>®</sup> Plus Fluorophores or Opal<sup>™</sup> Dyes

The RNAscope<sup>®</sup> LS Multiplex Fluorescent Reagent Kit (Cat. No. 322800) requires purchase of Opal<sup>™</sup> dyes or TSA<sup>®</sup> Plus fluorophores from Akoya Biosciences. Dilute the fluorophores in TSA buffer provided in the kit. The Opal<sup>™</sup> Polaris 780 Reagent Pack contains two reagents: Opal<sup>™</sup> TSA-DIG and Opal<sup>™</sup> Polaris 780. We recommend diluting Opal<sup>™</sup> TSA-DIG in TSA buffer, and diluting Opal<sup>™</sup> Polaris 780 in either Primary Antibody Diluent BOND from Leica (PN: AR9352) or Antibody Diluent/Block from Akoya Biosciences (PN: ARD1001EA). Choose a dilution factor for each fluorophore based on recommendations from ACD and your specific experimental conditions such as target expression levels, tissue quality, or microscope setting. Materials are qualified with 1:1500 dilution for all three fluorophores. We cannot guarantee assay results if you use other fluorescent dyes.

Fluorophore	Production number	Recommended dilution range
Opal <sup>™</sup> 520	FP1487001KT: Opal <sup>™</sup> 520 Reagent Pack†	1:750–1:3000
Opal <sup>™</sup> 570	FP1488001KT: Opal <sup>™</sup> 570 Reagent Pack†	1:750–1:3000
Opal <sup>™</sup> 620	FP1495001KT: Opal <sup>™</sup> 620 Reagent Pack†	1:750–1:3000

Fluorophore	Production number	Recommended dilution range
Opal™ 690	FP1497001KT: Opal™ 690 Reagent Pack†	1:750–1:3000
Opal™ Polaris 780	FP1501001KT: Opal™ Polaris 780 Reagent Pack†	TSA-DIG: 1:750–1:3000 Polaris 780: 1:187.5–1:750
TSA® Plus fluorescein	NEL741001KT*	1:750–1:3000
TSA® Plus Cyanine 3	NEL744001KT*	1:750–1:3000
TSA® Plus Cyanine 5	NEL745001KT*	1:750–1:3000

\* Depending on the dilution factor used, this stock size (150 µl) is sufficient to run the assay on 375–1500 slides. More size options are available from the Akoya Biosciences product website.

† Depending on the dilution factor used, this stock size (75 µl) is sufficient to run the assay on 187–750 slides.

## Recommended fluorophore combinations

Use the Opal™ dyes or TSA® Plus system from Akoya Biosciences to develop the RNAscope® and IF signal. The following table lists examples of three-plex fluorophore combinations using the Opal™ dyes or TSA® Plus system. Opal™ 520 and Opal™ 570 are interchangeable with TSA® Plus fluorescein and Cyanine 3, respectively (see Options 1 and 2 in the table). You can assign a 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, you may need to increase the concentration of TSA® Plus Cyanine 5.

	Reagent registration name	Option 1 (recommended)	Option 2	Option 3	Option 4
RNAscope® Multiplex Assay – C1	ACD Multiplex TSA-F1	TSA® Plus fluorescein	Opal™ 520	TSA® Plus Cyanine 3	Opal™ 570
RNAscope® Multiplex Assay – C2	ACD Multiplex TSA-F2	TSA® Plus Cyanine 3	Opal™ 570	TSA® Plus fluorescein	Opal™ 520
Immunofluorescence	ACD Multiplex TSA-F3	TSA® Plus Cyanine 5	Opal™ 690	TSA® Plus Cyanine 5	Opal™ 690

If you are running a three-plex RNAscope® *in situ* hybridization (ISH) plus immunofluorescent (IF) assay, see the following table for examples 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 can choose four colors from the Opal™ 7-color fIHC kit (see Options 2–4 in the table). You may assign a 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 (see Options 2–4).

	Reagent registration name	Option 1 (recommended)	Option 2	Option 3	Option 4
RNAscope® Multiplex Assay – C1	ACD Multiplex TSA-F1	Opal™ 520	TSA® Plus Fluorescein	Opal™ 620	Opal™ 520
RNAscope® Multiplex Assay – C2	ACD Multiplex TSA-F2	Opal™ 570	TSA® Plus Cyanine 3	Opal™ 520	Opal™ 570
RNAscope® Multiplex Assay – C3	ACD Multiplex TSA-F3	Opal™ 620	Opal 620	Opal™ 690	Opal™ 690
Fluorescent IHC	TSA-F4	Opal™ 690	TSA® Plus Cyanine 5	Opal™ 570	Opal™ 620

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

### 2-plex ISH combined with immunofluorescence using Opal™ Polaris 780

The Opal™ Polaris 780 Reagent Pack is a two-part reaction, which consists of Opal™ TSA-DIG and Opal™ Polaris 780. When autofluorescence in the FITC channel is a concern, use Opal™ Polaris 780 instead of TSA® Plus fluorescein or Opal™ 520.

	Reagent registration name	Option 1	Option 2
RNAscope® Multiplex Assay – C1	ACD Multiplex TSA-F1	Opal™ 570	TSA® Plus Cyanine 3
RNAscope® Multiplex Assay – C2	ACD Multiplex TSA-F2	Opal™ 690	TSA® Plus Cyanine 5
Fluorescent IHC	TSA-DIG	Opal™ Polaris 780	Opal™ Polaris 780
	Polaris 780		

**IMPORTANT!** To successfully develop the Opal™ Polaris 780 signal, you must use both the TSA-Dig and Polaris 780 reagents.

### 3-plex ISH combined with immunofluorescence using Opal™ Polaris 780

If the Cy7 filter is available, you can use Opal™ Polaris 780 as the fourth color in addition to FITC, Cy3, and Cy5. See the following table for fluorophore combinations:

	Reagent registration name	Option 1 (recommended)	Option 2
RNAscope® Multiplex Assay – C1	ACD Multiplex TSA-F1	Opal™ 520	TSA® Plus Fluorescein
RNAscope® Multiplex Assay – C2	ACD Multiplex TSA-F2	Opal™ 570	TSA® Plus Cyanine 3
RNAscope® Multiplex Assay – C3	ACD Multiplex TSA-F3	Opal™ 690	TSA® Plus Cyanine 5
Fluorescent IHC	TSA-DIG	Opal™ Polaris 780	Opal™ Polaris 780
	Polaris 780		

Many users prefer to use TSA® Plus Fluorescein or Opal™ 520 for immunofluorescent staining. You may use Opal™ 780 for ISH staining in any of the three channels. The following table displays options for assigning Opal™ 780 to the third channel.

### 3-plex ISH using Opal™ Polaris 780

	Reagent registration name	Option 3	Option 4
RNAscope® Multiplex Assay – C1	ACD Multiplex TSA-F1	Opal™ 570	TSA® Plus Cyanine 3
RNAscope® Multiplex Assay – C2	ACD Multiplex TSA-F2	Opal™ 690	TSA® Plus Cyanine 5
RNAscope® Multiplex Assay – C3	TSA-DIG	Opal™ Polaris 780	Opal™ Polaris 780
	Polaris 780		

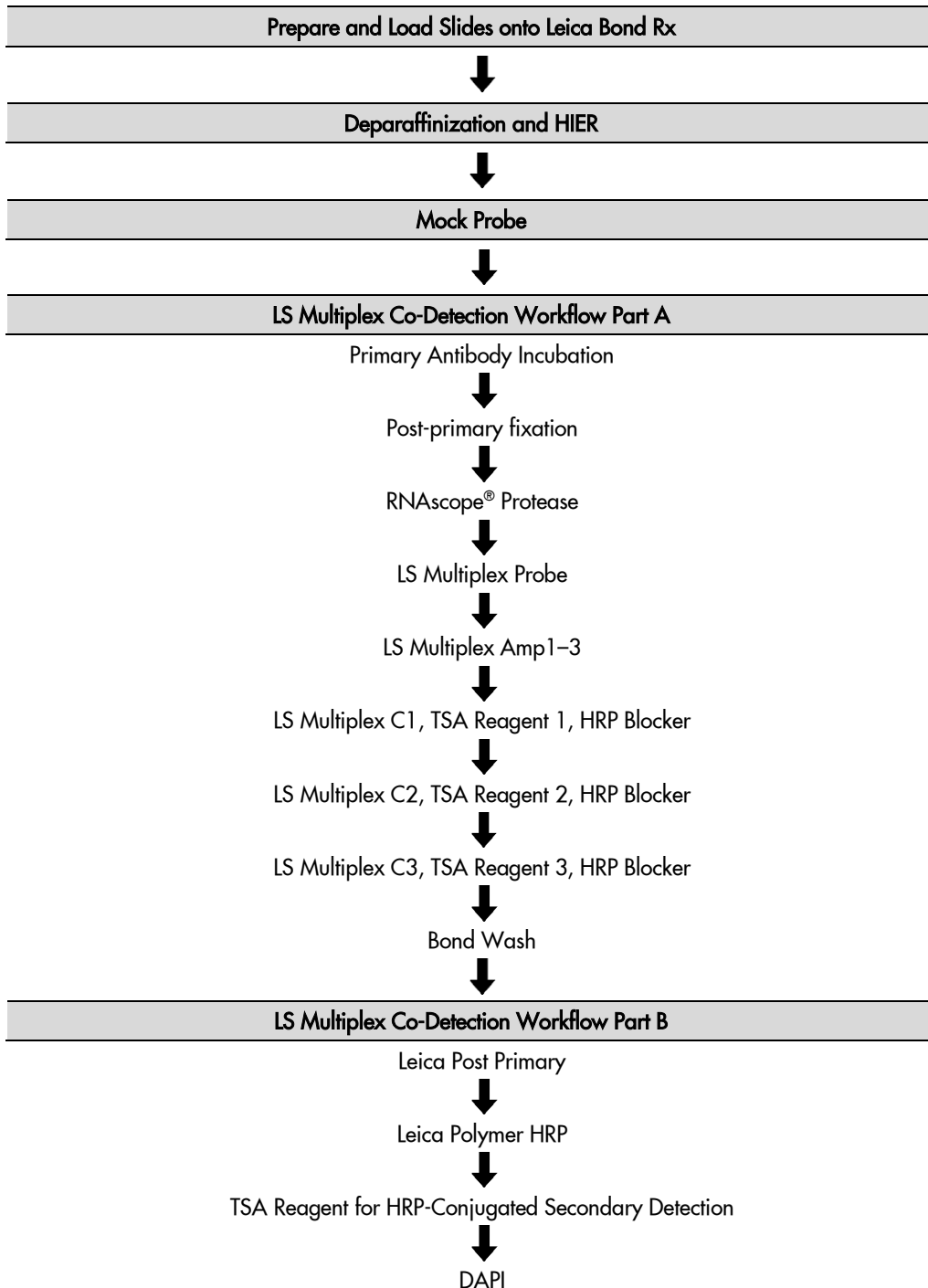
**IMPORTANT!** If Opal™ Polaris 780 is assigned to an ISH marker, do not follow it with any other ISH markers. The 780 fluorophore is extremely sensitive to cleavage by HRP activity and must be developed last. Apply Opal™ Polaris 780 as the last step of the Co-Detection workflow before counter staining and mounting. For detailed steps on using Polaris ISH detection with TSA-based protein detection, see **Appendix B**. For detailed steps on using Polaris ISH detection with fluorophore-conjugated secondary antibody for protein detection, see **Appendix D**.

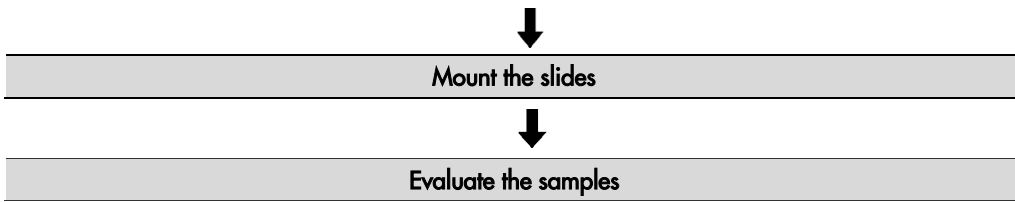
## Workflow 1: Fluorescent Multiplex Co-Detection Using TSA-based RNA and Protein Detection

This section provides instructions for creating LS Multiplex RNA-Protein Co-Detection Part A and Part B software protocols on the Leica BOND RX System using the 6.0 software version. Use these protocols with TSA fluorophores.

**Note:** To use Opal™ Polaris 780 for RNA detection, please refer to **Appendix B**. To use Opal™ Polaris 780 for protein detection, please refer to **Appendix C**.

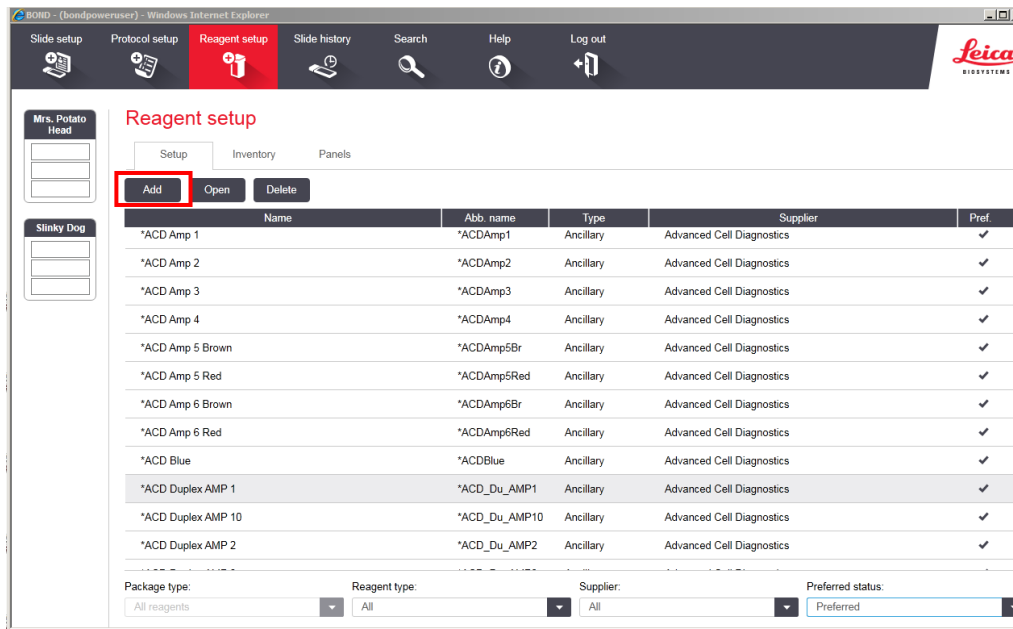
### Workflow steps





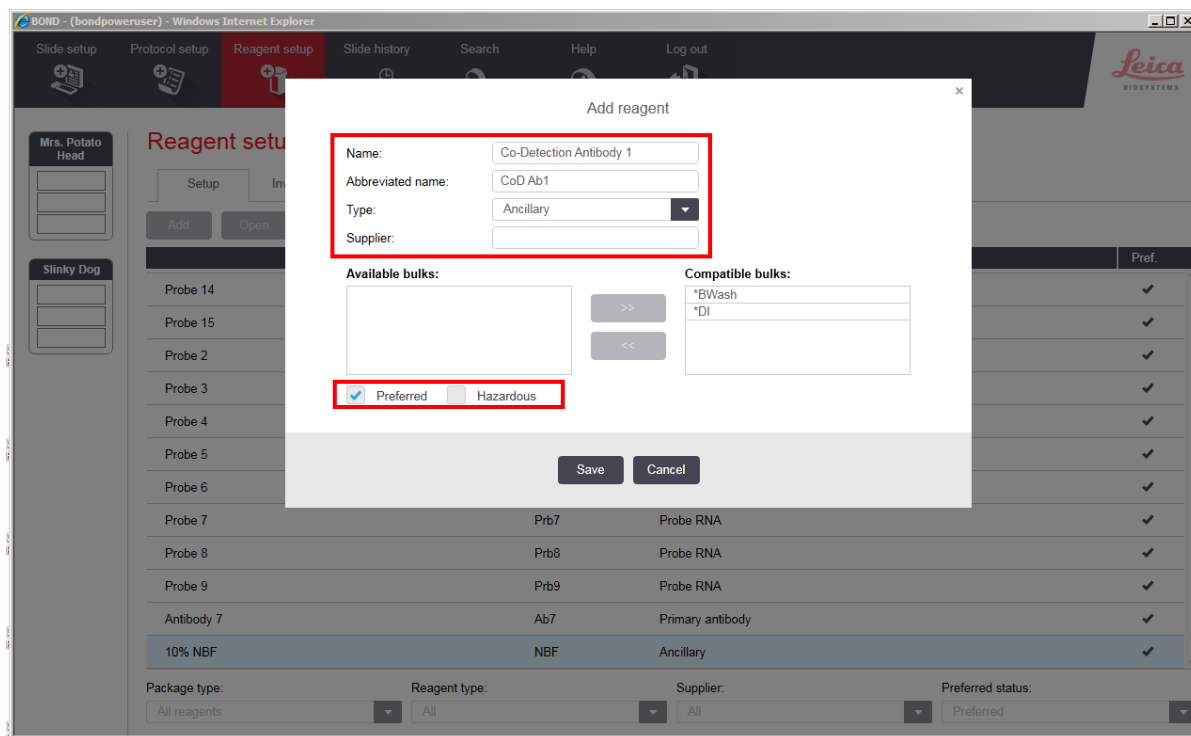
## Add new Co-Detection Reagents

1. Select the **Reagent Setup** icon at the top of the screen.
2. Select **Add** to create a new reagent.



3. To create a generic Co-Detection antibody reagent, enter **Co-Detection Antibody 1** in the Name text box.
4. Enter **CoD Ab1** in the Abbreviated name text box.
5. Select **Ancillary** in the Type drop-down menu. You can leave the Supplier field empty.

**IMPORTANT!** For Co-Detection antibodies, you must select **Ancillary** as the reagent type. Reagents registered as **Antibody** are not compatible with this protocol. To avoid confusion, include "Co-Detection" in the name (for example, **Co-Detection CD3**).



6. Select **Preferred**, then **Save**.

7. The reagents in the following table are required for this Co-Detection workflow. Repeat Steps 2–8 to register all additional reagents that do not currently exist on your controller.

Reagent	Reagent Registration Name	Reagent Type	Reagent Status
Primary Antibody	Co-Detection Antibody 1 (2, etc)*	Ancillary	Preferred
10% NBF	10% NBF	Ancillary	Preferred, Hazardous†
RNAscope® Probe	Probe 1 (2, etc)*	Ancillary	Preferred, Hazardous†
Opal™ TSA Fluorophore	TSA-F4	Ancillary	Preferred
DAPI	Co-Detection DAPI	Ancillary	Preferred, Hazardous†

\* To use RNAscope® probes and antibodies with the Co-Detection workflow, you must register them as **Ancillary** reagent types with unique names not shared by any probes or antibodies registered as different reagent types.

† Please refer to the reagent SDS and local guidelines for the proper collection and disposal of all generated waste.

## Create Part A of the LS Multiplex RNA-Protein Co-Detection Sequential Dual Stain protocol

This section provides instructions for creating the first part of the Sequential Dual Stain protocol for Multiplex RNA-Protein Co-Detection on the Leica BOND RX System using TSA-based detection for both RNA and protein. In Part A, apply primary antibody, followed by post-primary fixation, RNAscope® pretreatment, and Multiplex ISH staining. The following steps describe how to create a Part A protocol that is compatible with Multiplex ISH detection, using TSA® Plus dyes or Opal™ 520, 570, 620 or 690. If you use Opal™ Polaris 780 for ISH detection, please refer to **Appendix B**.

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**.

**Note:** For instructions on how to create ACD Multiplex Protocol P1, please refer to the *RNAscope® LS Multiplex Reagent Kit User Manual for BDZ11* (322800-USM).



3. Change the protocol name of your first probe to **ACD Multiplex Co-Detection Part A** in the Name text box, enter **Mx CoD A** in the Abbreviated name text box, and enter **ACD Multiplex RNA-Protein Co-Detection Part A** in the Description text box.

New protocol properties

Name: ACD Multiplex Co-Detection Part A  
 Abbreviated name: Mx CoD A  
 Description: ACD Multiplex RNA-Protein Co-Detection Part A

Staining method:  Single  First  Second  Preferred

BOND RX [Import protocol](#) Protocol type: ISH detection

Preferred detection system: ACD LS Multiplex Detection Kit

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
1		*ACD 2.5 P1	Advanced Cell Diagnostics	✓		0:00	150 µL
2		*ACD 2.5 P1	Advanced Cell Diagnostics	✓		0:00	150 µL
3		*ACD 2.5 P1	Advanced Cell Diagnostics		42	120:00	150 µL
15		ACD Multiplex Amp1	ACD		42	1:00	150 µL
16		ACD Multiplex Amp1	ACD		42	30:00	150 µL
25		*LS Rinse	Advanced Cell Diagnostics	✓		5:00	150 µL
26		*LS Rinse	Advanced Cell Diagnostics	✓		5:00	150 µL
31		ACD Multiplex Amp 2	ACD		42	1:00	150 µL
32		ACD Multiplex Amp 2	ACD		42	30:00	150 µL

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

[Save](#) [Cancel](#)

4. For Staining method, select **First**.

New protocol properties

Name: ACD Multiplex Co-Detection Part A  
 Abbreviated name: Mx CoD A  
 Description: ACD Multiplex RNA-Protein Co-Detection Part A

Staining method:  Single  First  Second  Preferred

BOND RX [Import protocol](#) Protocol type: ISH detection

Preferred detection system: ACD LS Multiplex Detection Kit

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
1		*ACD 2.5 P1	Advanced Cell Diagnostics	✓		0:00	150 µL
2		*ACD 2.5 P1	Advanced Cell Diagnostics	✓		0:00	150 µL
3		*ACD 2.5 P1	Advanced Cell Diagnostics		42	120:00	150 µL
15		ACD Multiplex Amp1	ACD		42	1:00	150 µL
16		ACD Multiplex Amp1	ACD		42	30:00	150 µL
25		*LS Rinse	Advanced Cell Diagnostics	✓		5:00	150 µL
26		*LS Rinse	Advanced Cell Diagnostics	✓		5:00	150 µL
31		ACD Multiplex Amp 2	ACD		42	1:00	150 µL
32		ACD Multiplex Amp 2	ACD		42	30:00	150 µL

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

[Save](#) [Cancel](#)

5. If using 5.2 software, select the appropriate tab for your instrument (BOND RXm or BOND RX).
6. Select **Show wash steps**. Add steps 1–35 from the following table before the probe step. Once additional steps have been added, verify that probe application **\*ACD 2.5 P1** begins at step 36.

**IMPORTANT!** Ensure that the temperature is set correctly. For heated steps, you must deselect **Ambient** before inputting the heated temperature.

Step No.	Reagent	Step Type	Incubation Time	Temperature
1	*Bond Wash Solution	Wash	0 MIN	Ambient
2	*Bond Wash Solution	Wash	0 MIN	Ambient
3	*Bond Wash Solution	Wash	0 MIN	Ambient
4	Co-Detection Antibody 1	Reagent	15 MIN	Ambient
5	*Bond Wash Solution	Wash	0 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	*Bond Wash Solution	Wash	0 MIN	Ambient
10	10% NBF	Reagent	30 MIN	Ambient
11	*Bond Wash Solution	Wash	0 MIN	Ambient
12	*Bond Wash Solution	Wash	0 MIN	Ambient
13	*Bond Wash Solution	Wash	0 MIN	Ambient
14	*Bond Wash Solution	Wash	2 MIN	Ambient
15	*Bond Wash Solution	Wash	2 MIN	Ambient
16	*Bond Wash Solution	Wash	0 MIN	Ambient
17	*ACD Enzyme	Reagent	0 MIN	40°C
18	*ACD Enzyme	Reagent	30 MIN	40°C
19	*Bond Wash Solution	Wash	0 MIN	Ambient
20	*Bond Wash Solution	Wash	0 MIN	Ambient
21	*Bond Wash Solution	Wash	0 MIN	Ambient
22	*Open 0 Haz	Reagent	10 MIN	Ambient
23	*Bond Wash Solution	Wash	0 MIN	Ambient
24	*Bond Wash Solution	Wash	0 MIN	Ambient
25	*Bond Wash Solution	Wash	0 MIN	Ambient
26	*Bond Wash Solution	Wash	0 MIN	Ambient
27	*Bond Wash Solution	Wash	0 MIN	Ambient
28	*Bond Wash Solution	Wash	0 MIN	Ambient
29	*Bond Wash Solution	Wash	0 MIN	Ambient
30	*Bond Wash Solution	Wash	0 MIN	Ambient
31	*Bond Wash Solution	Wash	0 MIN	Ambient
32	*Bond Wash Solution	Wash	0 MIN	Ambient
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	*ACD 2.5 P1	Reagent	0 MIN	Ambient
37	*ACD 2.5 P1	Reagent	0 MIN	Ambient
38	*ACD 2.5 P1	Reagent	120 MIN	42°C

7. Highlight the **DAPI** step at the end of the protocol (step 168).

8. From the Reagent drop down menu, change **DAPI** to **Bond Wash**.

Edit protocol properties

Name:

Abbreviated name:

Description:

Staining method:  Single  First  Second  Preferred

[Import protocol](#) Protocol type: ISH detection

Preferred detection system:

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
164	<input checked="" type="checkbox"/>	*Bond Wash Solution	Leica Microsystems	<input checked="" type="checkbox"/>		0:00	150 µL
165	<input checked="" type="checkbox"/>	*Bond Wash Solution	Leica Microsystems	<input checked="" type="checkbox"/>		1:00	150 µL
166	<input checked="" type="checkbox"/>	*Bond Wash Solution	Leica Microsystems	<input checked="" type="checkbox"/>		1:00	150 µL
167	<input checked="" type="checkbox"/>	*Bond Wash Solution	Leica Microsystems	<input checked="" type="checkbox"/>		10:00	150 µL
168	<input checked="" type="checkbox"/>	Bond Wash		<input checked="" type="checkbox"/>		10:00	150 µL
169	<input checked="" type="checkbox"/>	*Deionized Water		<input checked="" type="checkbox"/>		0:00	150 µL
170	<input checked="" type="checkbox"/>	*Deionized Water		<input checked="" type="checkbox"/>		0:00	150 µL
171	<input checked="" type="checkbox"/>	*Deionized Water		<input checked="" type="checkbox"/>		0:00	150 µL

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

9. Select **Save**.

10. Click **Yes** to proceed.

11. To create a protocol for each probe/primary antibody combination, follow these steps:

- a. Highlight the ACD Multiplex Co-Detection Part A protocol. Select **Copy**.
- b. Change the protocol name by adding your probe and antibody name in the Name text box (for example, **ACD Multiplex Co-Detection Part A – P1 CD3**). Change the Abbreviated name text and Description text box accordingly.
- c. For Staining method, select **First**.
- d. If using 5.2 software, select the appropriate tab for your instrument (BOND RXm or BOND RX).
- e. Select **Co-Detection Antibody 1**. Change the Reagent to your registered ancillary antibody (for example, **Co-Detection CD3**).

New protocol properties

Name:

Abbreviated name:

Description:

Staining method:  Single  First  Second  Preferred

BOND RX [Import protocol](#) Protocol type: ISH detection

Preferred detection system:

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
4		Co-Detection CD3		✓		15:00	150 µL
10		10% NBF		✓		30:00	150 µL
17		*ACD Enzyme	Advanced Cell Diagnostics		40	0:00	150 µL
18		*ACD Enzyme	Advanced Cell Diagnostics		40	30:00	150 µL
22		*Open 0 Haz	User	✓		10:00	150 µL
36		*Open 1	User	✓		0:00	150 µL
37		*Open 1	User	✓		0:00	150 µL
38		*Open 1	User		42	120:00	150 µL

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

f. Select \*ACD 2.5 P1. Change the Reagent to your registered ancillary probe (for example, Probe 1 or \*Open 1).

**IMPORTANT!** Make sure to change all three probe steps.

New protocol properties

Name:

Abbreviated name:

Description:

Staining method:  Single  First  Second  Preferred

BOND RX [Import protocol](#) Protocol type: ISH detection

Preferred detection system:

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
4		Co-Detection CD3		✓		15:00	150 µL
10		10% NBF		✓		30:00	150 µL
17		*ACD Enzyme	Advanced Cell Diagnostics		40	0:00	150 µL
18		*ACD Enzyme	Advanced Cell Diagnostics		40	30:00	150 µL
22		*Open 0 Haz	User	✓		10:00	150 µL
36		*Open 1	User	✓		0:00	150 µL
37		*Open 1	User	✓		0:00	150 µL
38		*Open 1	User		42	120:00	150 µL

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

g. Select Save.

## Create Part B of the Co-Detection Sequential Dual Stain protocol to perform IF with TSA-based secondary antibody detection

This section provides instructions for creating the second part of the Sequential Dual Stain protocol for Multiplex RNA-Protein Co-Detection on the Leica BOND RX System using TSA-based detection for both RNA and protein. In Part B, apply an HRP-based secondary antibody using the Leica BOND Polymer Refine Detection Kit, followed by detection with a TSA fluorophore. The following steps describe how to create an IF protocol using any of the TSA® Plus dyes or Opal™ 520, 570, 620 or 690. To use Opal™ Polaris 780 for RNA detection, see **Appendix B** for a modified Part B protocol. To use Opal™ Polaris 780 for protein detection, see **Appendix C** for a modified Part B protocol.

1. To create a Part B protocol with TSA-based secondary antibody detection, highlight the **\*IHC Protocol F** protocol. Select **Copy**.
2. Enter the protocol name in the Name text box (for example, **ACD Multiplex Co-Detection Part B TSA**), **Mx CoD B** in the Abbreviated name text box, and **ACD Multiplex Co-Detection Part B TSA** in the Description text box.

New protocol properties x

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](#)

3. For Staining method, select **Second**.

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](#)

4. Select **Show wash steps**.
5. Modify the protocol to match the following table:
  - a. Delete all steps before **\*Post Primary**.
  - b. Change **\*Mixed DAB Refine** to **TSA-F4**.
  - c. Change **\*Hematoxylin** to **DAPI**.
  - d. Adjust reagent incubation times and wash steps.

Step No.	Reagent	Step type	Incubation time	Temperature
1	*Post Primary	Reagent	8 MIN	Ambient
2	*Bond Wash Solution	Wash	2 MIN	Ambient
3	*Bond Wash Solution	Wash	2 MIN	Ambient
4	*Bond Wash Solution	Wash	2 MIN	Ambient
5	*Polymer	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	TSA-F4	Reagent	1 MIN	Ambient
10	TSA-F4	Reagent	10 MIN	Ambient
11	*Bond Wash Solution	Wash	0 MIN	Ambient
12	*Bond Wash Solution	Wash	0 MIN	Ambient
13	*Bond Wash Solution	Wash	1 MIN	Ambient
14	*Bond Wash Solution	Wash	1 MIN	Ambient
15	*Bond Wash Solution	Wash	1 MIN	Ambient

Step No.	Reagent	Step type	Incubation time	Temperature
16	Co-Detection DAPI	Reagent	10 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

**Note:** To perform DAPI on the instrument for Part B, register a Co-Detection DAPI Ancillary container that is separate from the ACD LS Multiplex Detection Kit. The software cannot use the DAPI container from the ACD LS Multiplex Detection Kit for the Part B protocol.

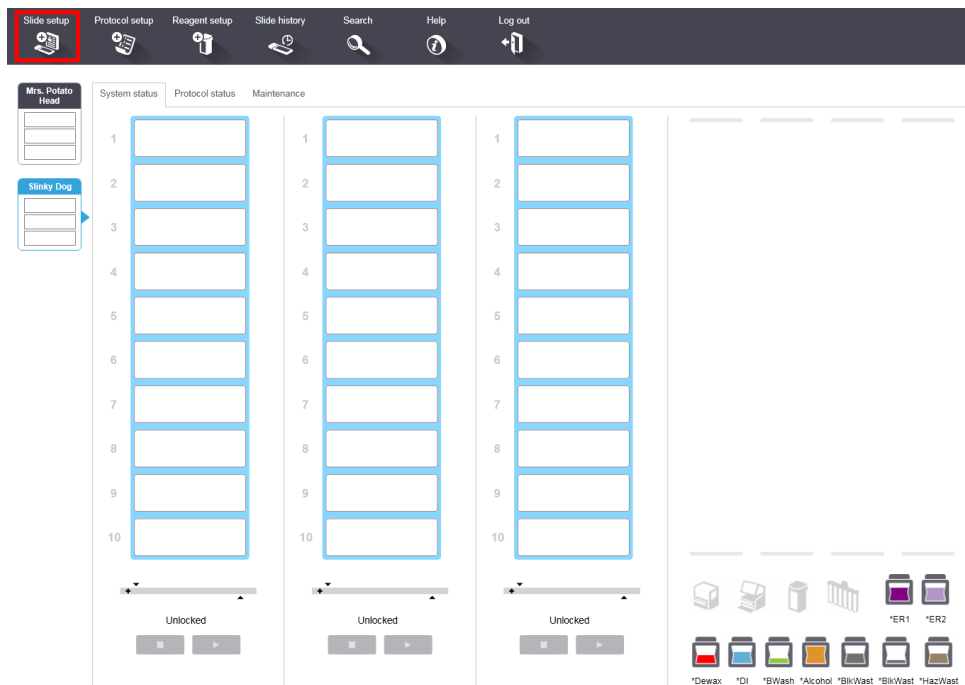
6. Select **Save**.

## Build a Sequential Dual Stain study for LS Multiplex RNA-Protein Co-Detection

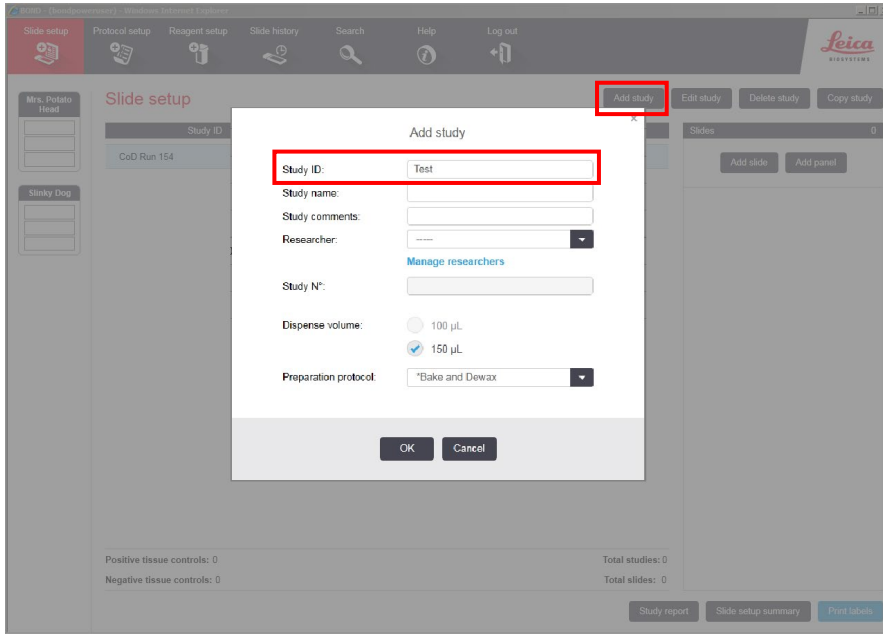
**IMPORTANT!** You can run a maximum of two slide trays/SSAs simultaneously to co-detect three RNA targets with one protein target.

**IMPORTANT!** We recommend using an extended heat-induced epitope retrieval (HIER) incubation for optimal RNA and protein Co-Detection. Before proceeding, refer to **Appendix A** for instructions on how to create an **ACD HIER 30 min with ER2 (95)** protocol.

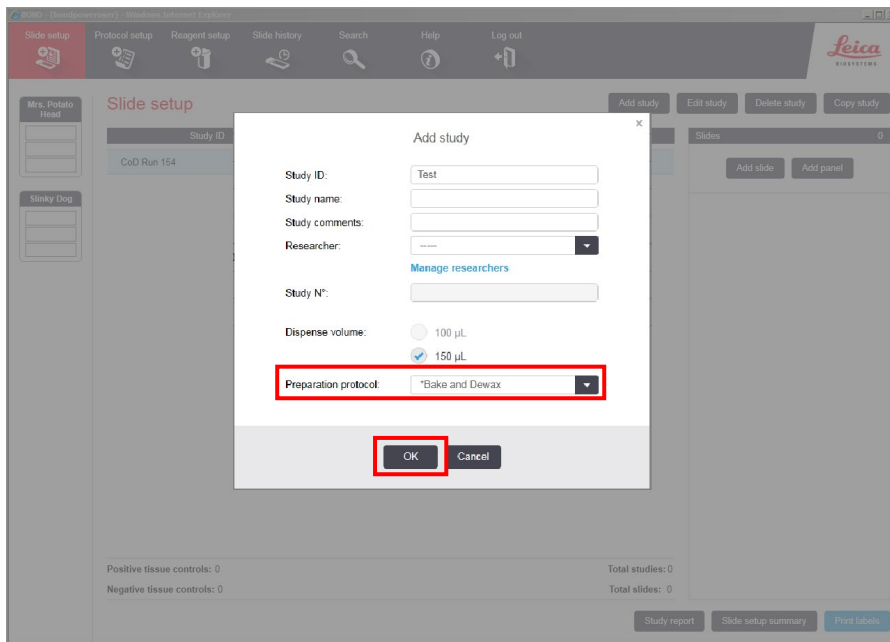
1. Select the **Slide setup** icon at the top of the screen.



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



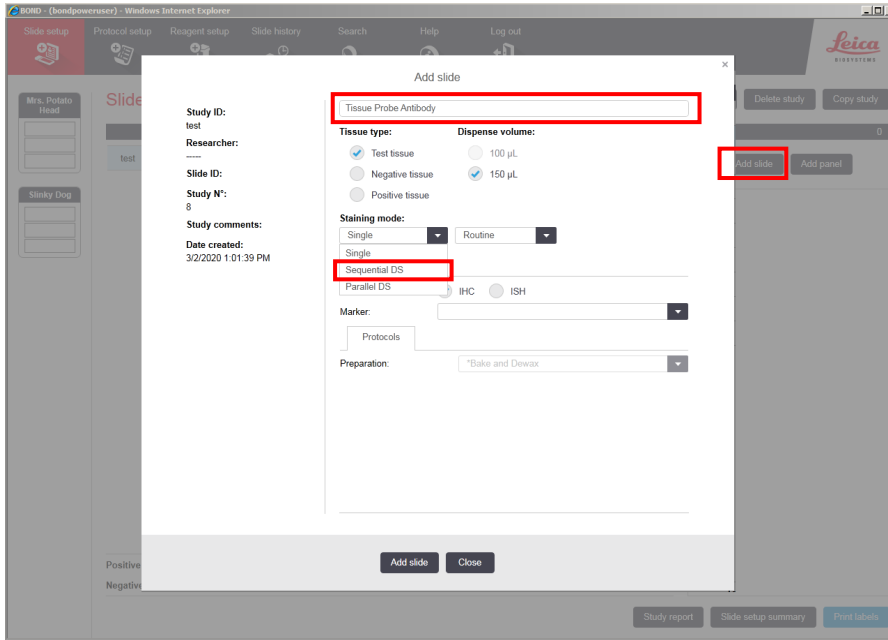
3. For FFPE tissues, select **\*Bake and Dewax** as the Preparation protocol. Otherwise, select **\*----** instead of **\*Bake and Dewax**.



4. Select **OK**.
5. Select **Add slide**.
6. In the Comments field, enter the tissue type, probe name, and antibody information.

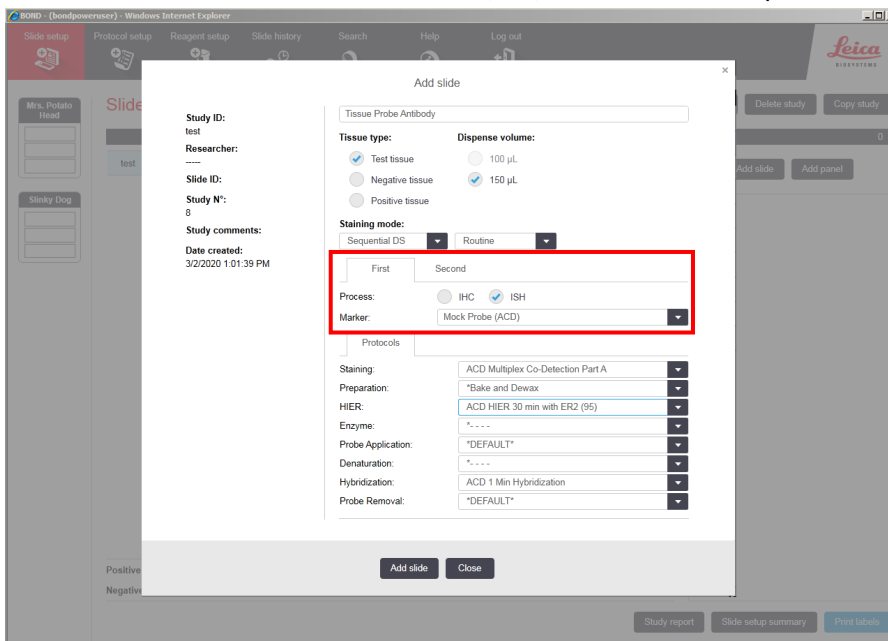


7. Select **Sequential DS** from the Staining mode drop down menu



8. Select the **First** tab to add the Part A protocol.

9. Select **ISH** under Process, and **Mock Probe (ACD)** from the Marker drop down menu.



10. In the Protocols tab, do the following:

- Select a Part A protocol from the Staining drop down menu (for example, ACD Multiplex Co-Detection Part A)

**Note:** Make sure that each probe and primary antibody selection is associated with a different protocol.

- Verify the **\*Bake and Dewax** protocol is selected from the Preparation drop down menu for standard FFPE tissues (otherwise, leave blank).
- Select **ACD HIER 30 min with ER2 (95)** as the HIER protocol or the appropriate HIER protocol for your tissue.
- For Enzyme, select **\*- - -**
- Select **\*DEFAULT\*** for Probe Application and Probe Removal.

f. Select \*--- for Denaturation and **ACD 1 Min Hybridization** for Hybridization.

The screenshot shows the 'Add slide' dialog box with the following settings:

- Study ID: test
- Researcher: ---
- Slide ID: ---
- Study N°: 8
- Study comments:
- Date created: 3/2/2020 1:01:39 PM
- Tissue Probe Antibody: (empty)
- Tissue type:
  - Test tissue
  - Negative tissue
  - Positive tissue
- Dispense volume:
  - 100 µL
  - 150 µL
- Staining mode:
  - Sequential DS
  - Routine
- Process:
  - IHC
  - ISH
- Marker: Mock Probe (ACD)
- Protocols:
  - Staining: ACD Multiplex Co-Detection Part A
  - Preparation: \*Bake and Dewax
  - HIER: ACD HIER 30 min with ER2 (95)
  - Enzyme: \*---
  - Probe Application: \*DEFAULT\*
  - Denaturation: \*---
  - Hybridization: ACD 1 Min Hybridization
  - Probe Removal: \*DEFAULT\*

11. To add the Part B protocol, select the **Second** tab.

12. For Process, select **IHC**.

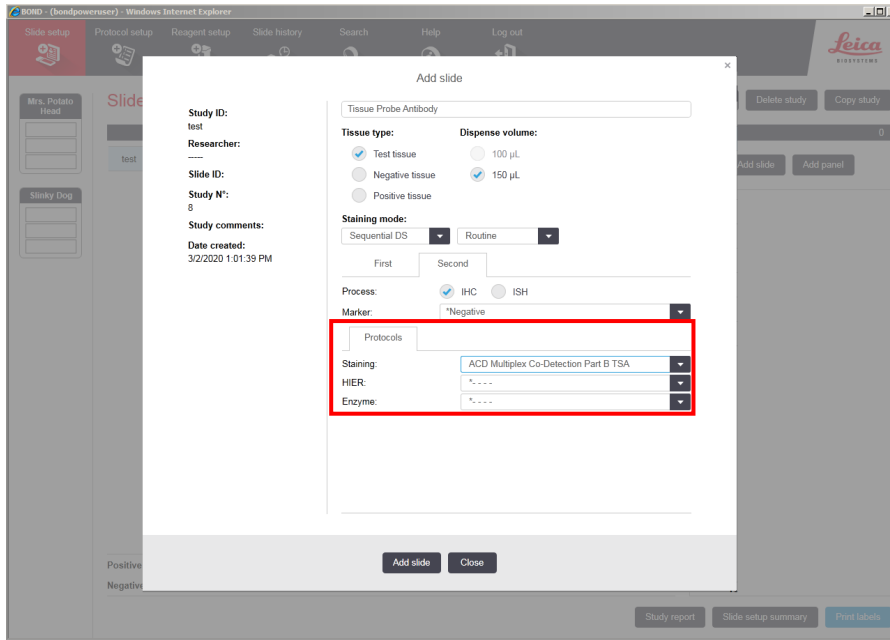
13. Since primary antibody is added to the Part A protocol, select **\*Negative** for Marker.

The screenshot shows the 'Add slide' dialog box with the following settings:

- Study ID: test
- Researcher: ---
- Slide ID: ---
- Study N°: 8
- Study comments:
- Date created: 3/2/2020 1:01:39 PM
- Tissue Probe Antibody: (empty)
- 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 Multiplex Co-Detection Part B TSA
  - HIER: \*---
  - Enzyme: \*---

14. In the Protocols tab, select **ACD Multiplex Co-Detection Part B TSA** from the Staining menu. For HIER and Enzyme, select **\*---**.

**Note:** Including additional HIER or Enzyme steps in Part B can decrease ISH and IHC staining.

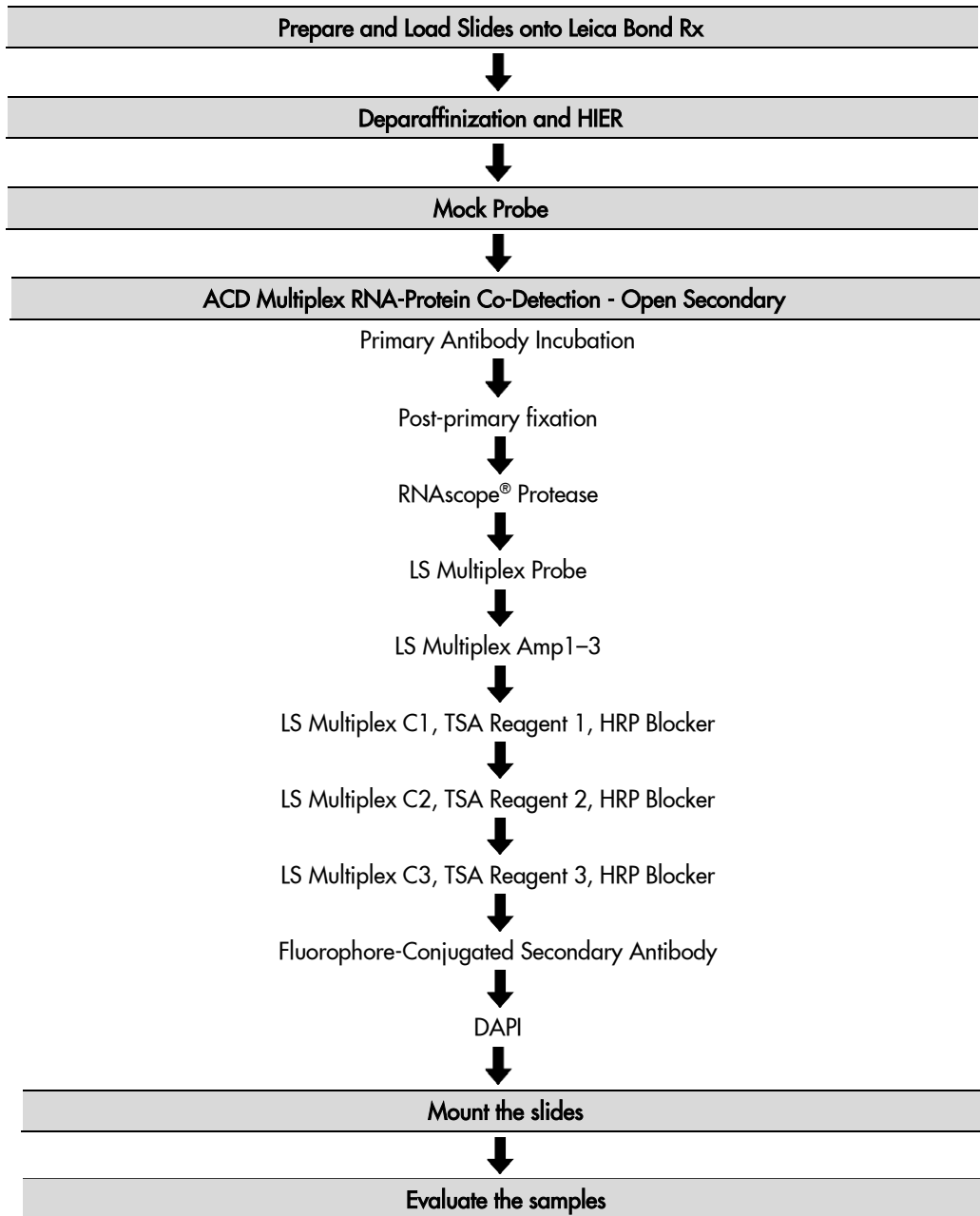


15. Repeat steps 5–14 for each slide.
16. After adding all the slides to the study, select **Close** to return to the Slide setup screen.
17. Select **Print labels** to print barcodes to attach to the slides.

## Workflow 2: Fluorescent Multiplex Co-Detection Using a Fluorophore-Conjugated Secondary Antibody for Protein Detection.

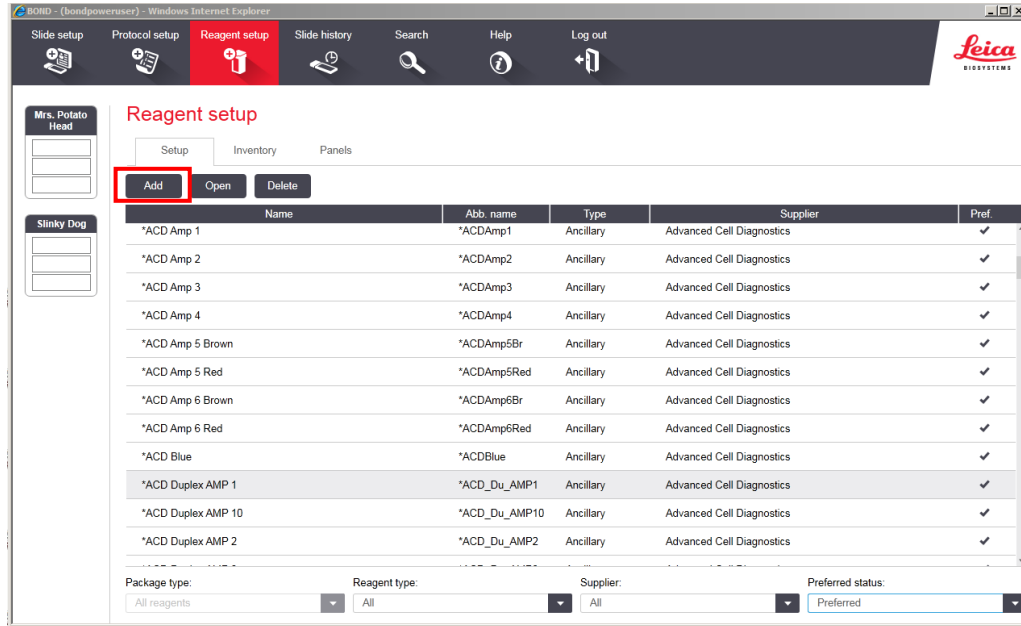
This section provides instructions for performing Multiplex RNA-Protein Co-Detection on the Leica BOND RX System using a fluorophore-conjugated secondary antibody and TSA-based RNA detection. You will need to set up a single stain protocol configuration on the Leica BOND RX. To use Opal™ Polaris 780 for ISH detection, please refer to **Appendix D**.

### Workflow steps



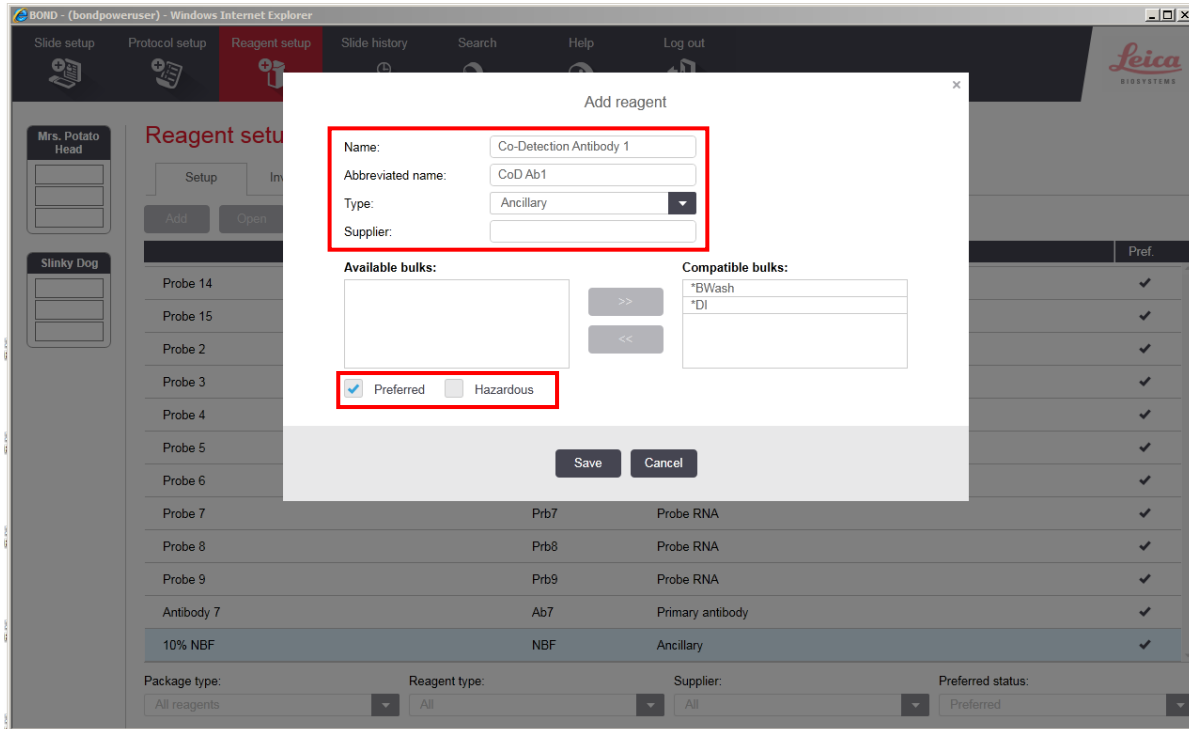
## Add new Co-Detection Reagents

1. Select the **Reagent Setup** icon at the top of the screen.
2. Select **Add** to create a new reagent.



3. To create a generic Co-Detection antibody reagent, enter **Co-Detection Antibody 1** in the Name text box.
4. Enter **CoD Ab1** in the Abbreviated name text box.
5. Select **Ancillary** in the Type drop-down menu. You can leave the Supplier field empty.

**IMPORTANT!** For Co-Detection antibodies, you must select **Ancillary** as the reagent type. Reagents registered as **Antibody** are not compatible with this protocol. To avoid confusion, include “Co-Detection” in the name (for example, **Co-Detection CD3**).



6. Select **Preferred**, then **Save**.
7. The reagents in the following table are required for this Co-Detection workflow. Repeat Steps 2–8 to register all additional reagents that do not currently exist on your controller.

Reagent	Reagent Registration Name	Reagent Type	Reagent Status
Primary Antibody	Co-Detection Antibody 1 (2, etc)*	Ancillary	Preferred
10% NBF	10% NBF	Ancillary	Preferred, Hazardous†
RNAscope® Probe	Probe 1 (2, etc)*	Ancillary	Preferred, Hazardous†
Opal™ TSA Fluorophore	TSA-F4	Ancillary	Preferred, Hazardous†
DAPI	Co-Detection DAPI	Ancillary	Preferred

\* To use RNAscope® probes and antibodies with the Co-Detection workflow, you must register them as **Ancillary** reagent types with unique names not shared by any probes or antibodies registered as different reagent types.

† Please refer to the reagent SDS and local guidelines for the proper collection and disposal of all generated waste.

## Create a Co-Detection Single Stain protocol using a fluorophore-conjugated secondary antibody for IF detection.

The following steps describe how to create a Single Stain Multiplex Co-Detection protocol using a fluorophore-conjugated secondary antibody of your choice.

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**.

**Note:** For instructions on how to create ACD Multiplex Protocol P1, please refer to the *RNAscope® LS Multiplex Reagent Kit User Manual for BDZ11* (322800-USM).

3. Change the protocol name to **ACD Multiplex Co-Detection Open 2nd** in the Name text box, **Mx CoD 2** in the Abbreviated name text box, and **ACD Multiplex RNA-Protein Co-Detection Open Secondary** in the Description text box.

New protocol properties

Name:

Abbreviated name:

Description:

Staining method:  Single  First  Second  Preferred

BOND RX  [Import protocol](#) Protocol type: ISH detection

Preferred detection system:

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
1		*ACD 2.5 P1	Advanced Cell Diagnostics	✓		0:00	150 µL
2		*ACD 2.5 P1	Advanced Cell Diagnostics	✓		0:00	150 µL
3		*ACD 2.5 P1	Advanced Cell Diagnostics		42	120:00	150 µL
15		ACD Multiplex Amp1	ACD		42	1:00	150 µL
16		ACD Multiplex Amp1	ACD		42	30:00	150 µL
25		*LS Rinse	Advanced Cell Diagnostics	✓		5:00	150 µL
26		*LS Rinse	Advanced Cell Diagnostics	✓		5:00	150 µL
31		ACD Multiplex Amp 2	ACD		42	1:00	150 µL
32		ACD Multiplex Amp 2	ACD		42	30:00	150 µL

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

4. For Staining method, select **Single**.

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
1		*ACD 2.5 P1	Advanced Cell Diagnostics	✓		0:00	150 µL
2		*ACD 2.5 P1	Advanced Cell Diagnostics	✓		0:00	150 µL
3		*ACD 2.5 P1	Advanced Cell Diagnostics		42	120:00	150 µL
15		ACD Multiplex Amp1	ACD		42	1:00	150 µL
16		ACD Multiplex Amp1	ACD		42	30:00	150 µL
25		*LS Rinse	Advanced Cell Diagnostics	✓		5:00	150 µL
26		*LS Rinse	Advanced Cell Diagnostics	✓		5:00	150 µL
31		ACD Multiplex Amp 2	ACD		42	1:00	150 µL
32		ACD Multiplex Amp 3	ACD		42	30:00	150 µL

- If using 5.2 software, select the appropriate tab for your instrument (BOND RXm or BOND RX).
- Select **Show wash steps**. Add steps 1–35 from the following table before the probe step. Once additional steps have been added, verify that probe application **\*ACD 2.5 P1** begins at step 36.

**IMPORTANT!** Ensure that the temperature is set correctly. For heated steps, you must deselect **Ambient** before inputting the heated temperature.

Step No.	Reagent	Step Type	Incubation Time	Temperature
1	*Bond Wash Solution	Wash	0 MIN	Ambient
2	*Bond Wash Solution	Wash	0 MIN	Ambient
3	*Bond Wash Solution	Wash	0 MIN	Ambient
4	Co-Detection Antibody 1	Reagent	15 MIN	Ambient
5	*Bond Wash Solution	Wash	0 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	*Bond Wash Solution	Wash	0 MIN	Ambient
10	10% NBF	Reagent	30 MIN	Ambient
11	*Bond Wash Solution	Wash	0 MIN	Ambient
12	*Bond Wash Solution	Wash	0 MIN	Ambient
13	*Bond Wash Solution	Wash	0 MIN	Ambient
14	*Bond Wash Solution	Wash	2 MIN	Ambient
15	*Bond Wash Solution	Wash	2 MIN	Ambient
16	*Bond Wash Solution	Wash	0 MIN	Ambient
17	*ACD Enzyme	Reagent	0 MIN	40 °C

18	*ACD Enzyme	Reagent	30 MIN	40 °C
19	*Bond Wash Solution	Wash	0 MIN	Ambient
20	*Bond Wash Solution	Wash	0 MIN	Ambient
21	*Bond Wash Solution	Wash	0 MIN	Ambient
22	*Open 0 Haz	Reagent	10 MIN	Ambient
23	*Bond Wash Solution	Wash	0 MIN	Ambient
24	*Bond Wash Solution	Wash	0 MIN	Ambient
25	*Bond Wash Solution	Wash	0 MIN	Ambient
26	*Bond Wash Solution	Wash	0 MIN	Ambient
27	*Bond Wash Solution	Wash	0 MIN	Ambient
28	*Bond Wash Solution	Wash	0 MIN	Ambient
29	*Bond Wash Solution	Wash	0 MIN	Ambient
30	*Bond Wash Solution	Wash	0 MIN	Ambient
31	*Bond Wash Solution	Wash	0 MIN	Ambient
32	*Bond Wash Solution	Wash	0 MIN	Ambient
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	*ACD 2.5 P1	Reagent	0 MIN	Ambient
37	*ACD 2.5 P1	Reagent	0 MIN	Ambient
38	*ACD 2.5 P1	Reagent	120 MIN	42°C

7. Above the **DAPI** Reagent, add five additional Reagent and Wash steps according to the following table:

Step No.	Reagent	Step Type	Incubation Time	Temperature
164	Co-Detection Secondary	Reagent	60 MIN	Ambient
165	*Bond Wash Solution	Wash	0 MIN	Ambient
166	*Bond Wash Solution	Wash	1 MIN	Ambient
167	*Bond Wash Solution	Wash	1 MIN	Ambient
168	*Bond Wash Solution	Wash	1 MIN	Ambient
168	DAPI	Reagent	10 MIN	Ambient



New protocol properties

Name: ACD Multiplex Co-Detection Open 2nd  
 Abbreviated name: Mx CoD 2  
 Description: ACD Multiplex RNA-Protein Co-Detection Open Secondary  
 Staining method:  Single  First  Second  Preferred

BOND RX [Import protocol](#) Protocol type: ISH detection

Preferred detection system: ACD LS Multiplex Detection Kit

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
166	<input checked="" type="checkbox"/>	*Bond Wash Solution	Leica Microsystems	<input checked="" type="checkbox"/>		1:00	150 µL
167	<input checked="" type="checkbox"/>	*Bond Wash Solution	Leica Microsystems	<input checked="" type="checkbox"/>		10:00	150 µL
168		Co-Detection Secondary 1		<input checked="" type="checkbox"/>		60:00	150 µL
169	<input checked="" type="checkbox"/>	*Bond Wash Solution	Leica Microsystems	<input checked="" type="checkbox"/>		0:00	150 µL
170	<input checked="" type="checkbox"/>	*Bond Wash Solution	Leica Microsystems	<input checked="" type="checkbox"/>		1:00	150 µL
171	<input checked="" type="checkbox"/>	*Bond Wash Solution	Leica Microsystems	<input checked="" type="checkbox"/>		1:00	150 µL
172	<input checked="" type="checkbox"/>	*Bond Wash Solution	Leica Microsystems	<input checked="" type="checkbox"/>		1:00	150 µL
173		DAPI		<input checked="" type="checkbox"/>		10:00	150 µL
174	<input checked="" type="checkbox"/>	*Deionized Water		<input checked="" type="checkbox"/>		0:00	150 µL

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

[Save](#) [Cancel](#)

8. Select **Save**.
9. Click **Yes** to proceed.
10. To create a protocol for each additional probe/primary antibody/secondary antibody combination, follow these steps:
  - a. Highlight the ACD Multiplex Co-Detection Open 2nd protocol. Select **Copy**.
  - b. Change the protocol name by adding your probe and antibody name in the Name text box (for example, **ACD Multiplex Co-Detection Open 2nd – P1 CD3**). Change the Abbreviated name text and Description text box accordingly.

New protocol properties

Name: ACD Multiplex Co-Detection Open 2nd - P1 CD3  
 Abbreviated name: MxCoD2-1  
 Description: ACD Multiplex RNA-Protein Co-Detection Open Secondary  
 Staining method:  Single  First  Second  Preferred

BOND RX [Import protocol](#) Protocol type: ISH detection

Preferred detection system: ACD LS Multiplex Detection Kit

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
4		Co-Detection Antibody 1		<input checked="" type="checkbox"/>		15:00	150 µL
10		10% NBF		<input checked="" type="checkbox"/>		30:00	150 µL
17		*ACD Enzyme	Advanced Cell Diagnostics		40	0:00	150 µL
18		*ACD Enzyme	Advanced Cell Diagnostics		40	30:00	150 µL
22		*Open 0 Haz.	User	<input checked="" type="checkbox"/>		10:00	150 µL
36		*ACD 2.5 P1	Advanced Cell Diagnostics	<input checked="" type="checkbox"/>		0:00	150 µL
37		*ACD 2.5 P1	Advanced Cell Diagnostics	<input checked="" type="checkbox"/>		0:00	150 µL
38		*ACD 2.5 P1	Advanced Cell Diagnostics		42	120:00	150 µL

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

[Save](#) [Cancel](#)

c. For Staining method, select **Single**.

New protocol properties

Name: ACD Multiplex Co-Detection Open 2nd - P1 CD3  
 Abbreviated name: MxCoD2-1  
 Description: ACD Multiplex RNA-Protein Co-Detection Open Secondary

Staining method:  **Single**  First  Second  Preferred

BOND RX [Import protocol](#) Protocol type: ISH detection

Preferred detection system: ACD LS Multiplex Detection Kit

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
4		Co-Detection Antibody 1		✓		15:00	150 µL
10		10% NBF		✓		30:00	150 µL
17		*ACD Enzyme	Advanced Cell Diagnostics		40	0:00	150 µL
18		*ACD Enzyme	Advanced Cell Diagnostics		40	30:00	150 µL
22		*Open 0 Haz	User	✓		10:00	150 µL
36		*ACD 2.5 P1	Advanced Cell Diagnostics	✓		0:00	150 µL
37		*ACD 2.5 P1	Advanced Cell Diagnostics	✓		0:00	150 µL
38		*ACD 2.5 P1	Advanced Cell Diagnostics		42	120:00	150 µL

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

[Save](#) [Cancel](#)

- d. If using 5.2 software, select the appropriate tab for your instrument (BOND RXm or BOND RX).
- e. To change the primary antibody reagent, select **Co-Detection Antibody 1**, and change the name to your registered ancillary antibody (for example, **Co-Detection CD3**)

New protocol properties

Name: ACD Multiplex Co-Detection Open 2nd - P1 CD3  
 Abbreviated name: MxCoD2-1  
 Description: ACD Multiplex RNA-Protein Co-Detection Open Secondary

Staining method:  Single  First  Second  Preferred

BOND RX [Import protocol](#) Protocol type: ISH detection

Preferred detection system: ACD LS Multiplex Detection Kit

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
4		Co-Detection CD3		✓		15:00	150 µL
10		10% NBF		✓		30:00	150 µL
17		*ACD Enzyme	Advanced Cell Diagnostics		40	0:00	150 µL
18		*ACD Enzyme	Advanced Cell Diagnostics		40	30:00	150 µL
22		*Open 0 Haz	User	✓		10:00	150 µL
36		*Open 1	User	✓		0:00	150 µL
37		*Open 1	User	✓		0:00	150 µL
38		*Open 1	User		42	120:00	150 µL

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

[Save](#) [Cancel](#)

- f. To change the probe reagent, select **\*ACD 2.5 P1**, and change the name to your registered ancillary probe (for example, **\*Open 1**).

New protocol properties

Name:

Abbreviated name:

Description:

Staining method:  Single  First  Second  Preferred

BOND RX Import protocol Protocol type: ISH detection

Preferred detection system:

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
4		Co-Detection CD3		✓		15.00	150 µL
10		10% NBF		✓		30.00	150 µL
17		*ACD Enzyme	Advanced Cell Diagnostics		40	0.00	150 µL
18		*ACD Enzyme	Advanced Cell Diagnostics		40	30.00	150 µL
22		*Open 0 Haz	User	✓		10.00	150 µL
36		*Open 1	User	✓		0.00	150 µL
37		*Open 1	User	✓		0.00	150 µL
38		*Open 1	User		42	120.00	150 µL

Show wash steps Insert wash | Insert reagent | Delete step

Save Cancel

**IMPORTANT!** Make sure to change all three probe Reagent steps.

- g. To change the secondary antibody reagent, select **Co-Detection Secondary**, and change the name to your registered ancillary secondary antibody (for example, **Co-Detection Secondary 2** or **Co-Detection Anti-Ms 488**)

New protocol properties

Name:

Abbreviated name:

Description:

Staining method:  Single  First  Second  Preferred

BOND RX Import protocol Protocol type: ISH detection

Preferred detection system:

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
166	✓	*Bond Wash Solution	Leica Microsystems	✓		1.00	150 µL
167	✓	*Bond Wash Solution	Leica Microsystems	✓		10.00	150 µL
168		Co-Detection Secondary 2		✓		60.00	150 µL
169	✓	*Bond Wash Solution	Leica Microsystems	✓		0.00	150 µL
170	✓	*Bond Wash Solution	Leica Microsystems	✓		1.00	150 µL
171	✓	*Bond Wash Solution	Leica Microsystems	✓		1.00	150 µL
172	✓	*Bond Wash Solution	Leica Microsystems	✓		1.00	150 µL
173		DAPI		✓		10.00	150 µL
174	✓	*Deionized Water		✓		0.00	150 µL

Show wash steps Insert wash | Insert reagent | Delete step

Save Cancel

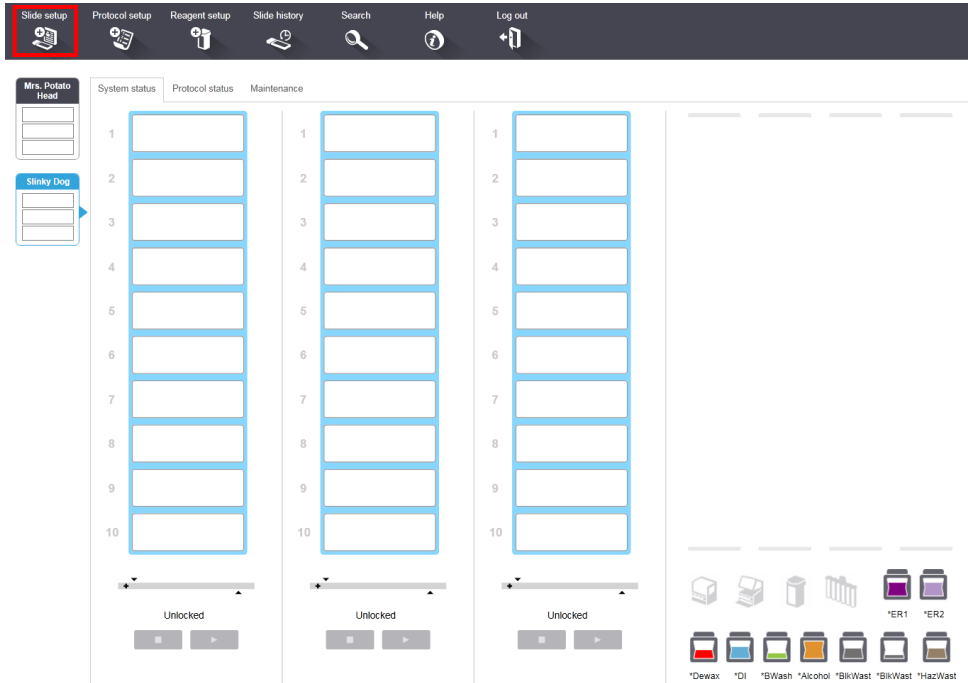
- h. Select **Save**.

## Build a single stain study for Multiplex RNA-Protein Co-Detection

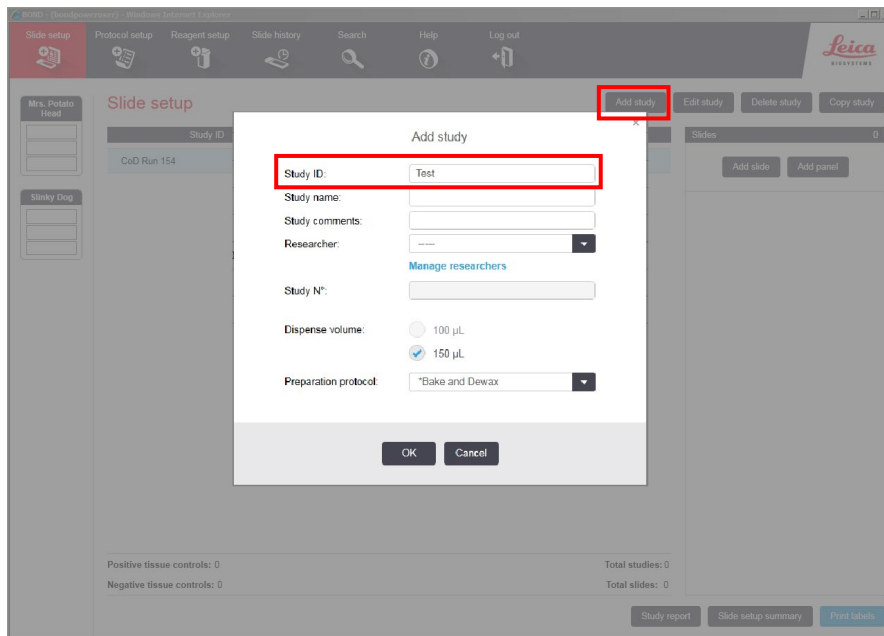
**IMPORTANT!** You can run a maximum of two slide trays/SSAs simultaneously to co-detect three RNA targets with one protein target.

**IMPORTANT!** We recommend using an extended heat-induced epitope retrieval (HIER) incubation for optimal RNA and protein Co-Detection. Before proceeding to slide setup, refer to **Appendix A** for instructions on how to create an **ACD HIER 30 min with ER2 (95)** protocol.

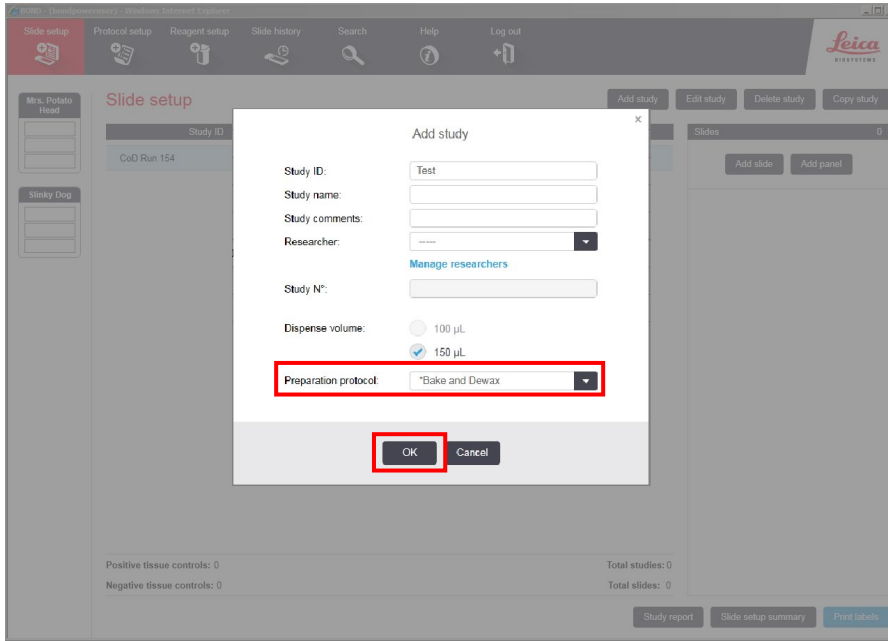
1. Select the **Slide setup** icon at the top of the screen.



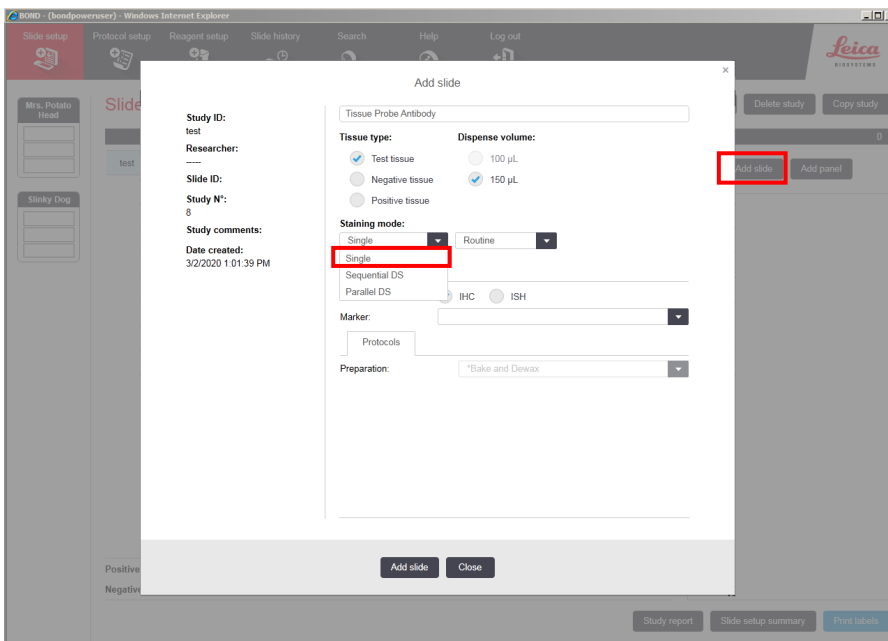
2. Select **Add study** and enter a name in the Study ID field (keep the Dispense volume at 150  $\mu$ L as shown).



- For FFPE tissues, select **\*Bake and Dewax** as the Preparation protocol. Otherwise, select **\*----** instead of **\*Bake and Dewax**.
- Select **OK**.



- Select **Add slide**.
- In the Comments field, enter the tissue type, probe name, and antibody information.
- Select **Single** from the Staining mode drop down menu.



- Select **ISH** under Process, and **mock probe (ACD)** from the Marker drop down menu.

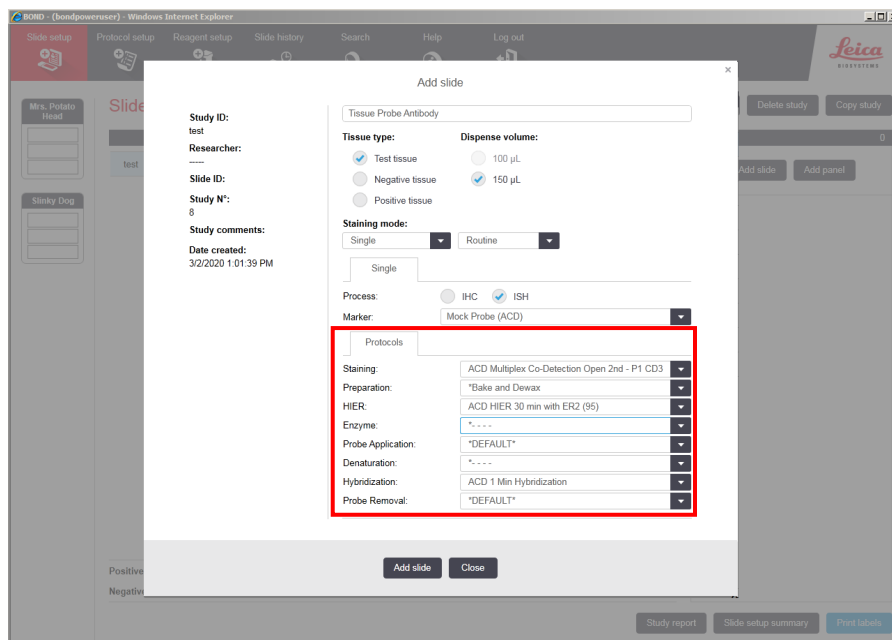
9. Under the Protocols tab, do the following:

- a. Select your desired Co-Detection protocol from the Staining drop down menu (for example, **ACD Multiplex Co-Detection Open 2nd**)

**Note:** Make sure that each probe, primary, and secondary antibody selection is associated with a different protocol.

- b. Select the protocol **\*Bake and Dewax** from the Preparation drop down menu for standard FFPE tissues (otherwise, leave blank).
- c. Select **ACD HIER 30 min with ER2 (95)** as the HIER protocol or the appropriate HIER protocol for your tissue.
- d. For Enzyme, select **\*- - -**
- e. Select **\*DEFAULT\*** for Probe Application and Probe Removal.

f. Select \*--- for Denaturation and **ACD 1 Min Hybridization** for Hybridization



10. Repeat steps 5–9 for each slide.
11. After adding all the slides to the study, select **Close** to return to the Slide setup screen.
12. Select **Print labels** to print barcodes to attach to the slides.

## Imaging

To image four-plex fluorescent staining, use a fluorescent multispectral imaging system, such as the Nuance<sup>®</sup> EX, Mantra<sup>™</sup>, Vectra<sup>®</sup>, or Polaris<sup>®</sup> Systems. The following table lists the corresponding filter setting for each dye.

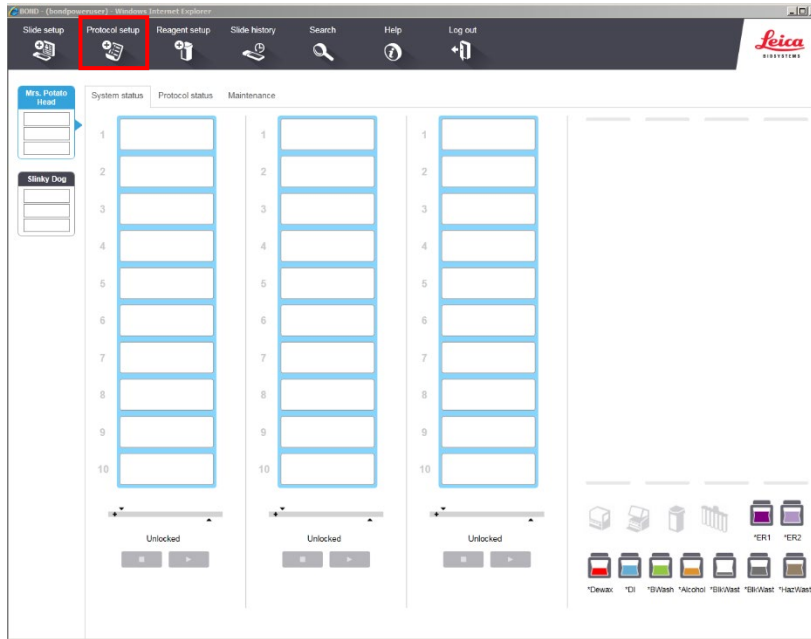
Opal <sup>™</sup> system	TSA <sup>®</sup> Plus System	Filter setting
Opal <sup>™</sup> 520	TSA <sup>®</sup> Plus fluorescein	FITC
Opal <sup>™</sup> 570	TSA <sup>®</sup> Plus Cyanine 3	Cy3
Opal <sup>™</sup> 620		Texas Red
Opal <sup>™</sup> 690	TSA <sup>®</sup> Plus Cyanine 5	Cy5/Cy5.5
Opal <sup>™</sup> 780		Cy7

## Appendix A. Creating a new HIER Protocol

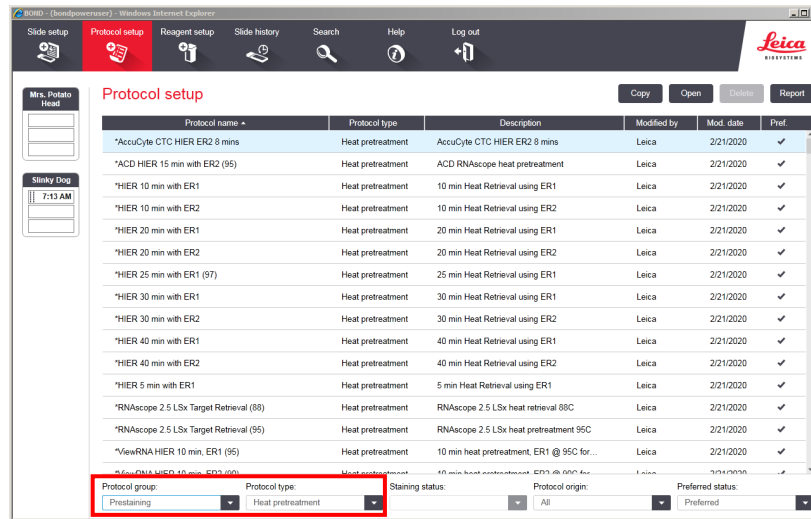
For optimal combined RNA and protein detection, an extended heat-induced epitope retrieval (HIER) is recommended for use with the RNA-Protein Co-Detection workflow. The following example shows how to edit the Epitope Retrieval procedure from within the software.

## Create a prestaining protocol

1. Open the Leica BOND software and click on the Protocol Setup icon on the home screen.



2. Under the Protocol group menu, select **Prestaining**. Under the Protocol type menu, select **Heat Pretreatment**.
3. Highlight the **\*ACD HIER 15min with ER2 (95)** protocol and select **Copy**.



4. Rename the protocol as **ACD HIER 30min with ER2 (95)** and the abbreviated name as **ACDHet30**



New protocol properties

Name: ACD HIER 30 min with ER2 (95)  
 Abbreviated name: ACDHet30  
 Description: ACD RNAscope heat pretreatment

Preferred

BOND RX Import protocol Protocol type: Heat pretreatment

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
1		*Bond ER Solution 2	Leica Microsystems	✓		0.00	150 µL
2		*Bond ER Solution 2	Leica Microsystems	✓		0.00	150 µL
3		*Bond ER Solution 2	Leica Microsystems		95	15.00	Intermediate
4		*Bond ER Solution 2	Leica Microsystems	✓		0.00	150 µL

Show wash steps

Save Cancel

5. Highlight the third **\*BOND ER Solution 2** step. Change the incubation time to 30 minutes.

New protocol properties

Name: ACD HIER 30 min with ER2 (95)  
 Abbreviated name: ACDHet30  
 Description: ACD RNAscope heat pretreatment

Preferred

BOND RX Import protocol Protocol type: Heat pretreatment

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
1		*Bond ER Solution 2	Leica Microsystems	✓		0.00	150 µL
2		*Bond ER Solution 2	Leica Microsystems	✓		0.00	150 µL
3		*Bond ER Solution 2	Leica Microsystems		95	30.00	Intermediate
4		*Bond ER Solution 2	Leica Microsystems	✓		0.00	150 µL

Show wash steps

Save Cancel

6. Select **Save**.

## Appendix B. Multiplex Co-Detection Using Opal™ Polaris 780 for RNA Detection and TSA fluorophore for Protein Detection

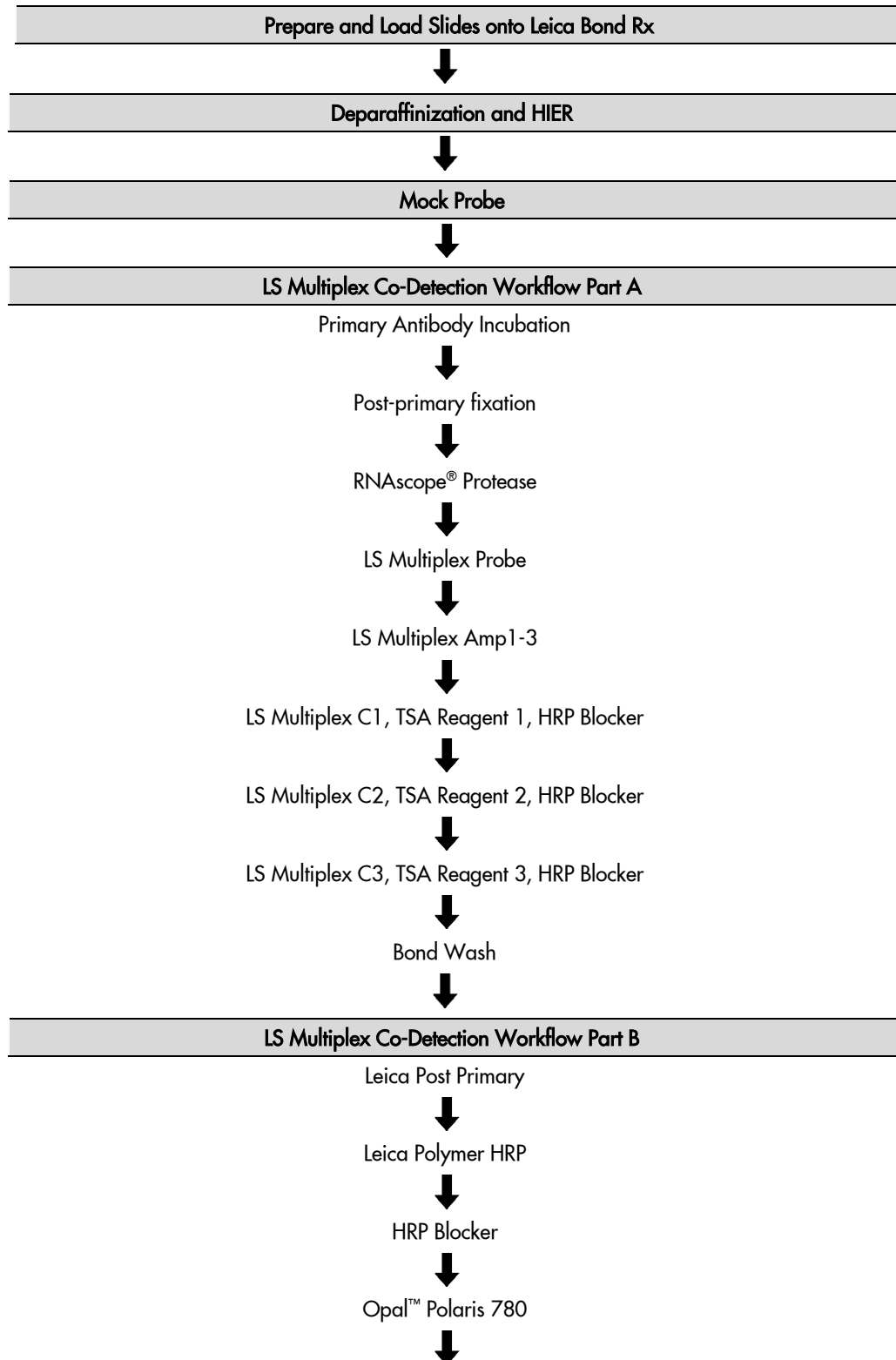
The Multiplex Co-Detection workflow is compatible with Opal™ Polaris 780 RNA detection. This section provides instructions for creating the Sequential Dual Stain protocols for Multiplex RNA-Protein Co-Detection on the Leica BOND RX System using TSA-based detection for both RNA and protein including Polaris 780 for RNA detection.

Opal™ Polaris 780 is a two-component system requiring TSA-DIG followed by Polaris 780. The 780 fluorophore is extremely sensitive to cleavage by HRP activity and must be applied last. To enable 780 detection of any RNA channel, you can split Opal™ Polaris 780 detection split so that TSA-DIG incubation is followed by detection of the remaining RNA targets and protein detection. In this adjusted Co-Detection workflow, apply Polaris 780 as the final step before counter staining and mounting.

**Note:** Depending on the desired RNAscope® channel for 780 detection, you can apply TSA-DIG as TSA Reagent 1, TSA Reagent 2, or TSA Reagent 3.

**IMPORTANT!** You can run a maximum of two slide trays/SSAs simultaneously to co-detect three RNA targets with one protein target.

## Workflow steps



Co-Detection DAPI



Mount the slides



Evaluate the samples

## Add new Co-Detection Reagents

Create the following reagents to perform Multiplex RNA-Protein Co-Detection on the Leica BOND RX System using TSA-based detection for both RNA and protein including Opal™ Polaris 780 for RNA detection.

Reagent	Reagent Registration Name	Reagent Type	Reagent Status
Primary Antibody	Co-Detection Antibody 1 (2, etc)*	Ancillary	Preferred
10% NBF	10% NBF	Ancillary	Preferred, Hazardous†
RNAscope® Probe	Probe 1 (2, etc)*	Ancillary	Preferred, Hazardous†
TSA-DIG	TSA-DIG	Ancillary	Preferred
Opal™ Polaris 780	Polaris 780	Ancillary	Preferred
Opal™ TSA Fluorophore	TSA-F4	Ancillary	Preferred
DAPI	Co-Detection DAPI	Ancillary	Preferred, Hazardous†

\* To use RNAscope® probes and antibodies with the Co-Detection workflow, you must register them as **Ancillary** reagent types with unique names not shared by any probes or antibodies registered as different reagent types.

† Please refer to the reagent SDS and local guidelines for the proper collection and disposal of all generated waste.

## Part A of the Sequential Dual Stain software protocol with Opal™ Polaris ISH detection

The following table displays Part A of the Sequential Dual Stain software protocol for performing Multiplex Co-Detection using TSA-based detection with Polaris 780 for RNA detection and TSA-based protein detection.

The post probe heated bond washes, shown below at steps 39–41, are specialized wash steps that are heated by the instrument and cannot be deleted. You can delete other wash steps.

Step No.	Reagent	Step Type	Incubation Time	Temperature
1	*Bond Wash Solution	Wash	0 MIN	Ambient
2	*Bond Wash Solution	Wash	0 MIN	Ambient
3	*Bond Wash Solution	Wash	0 MIN	Ambient
4	Co-Detection Antibody 1	Reagent	15 MIN	Ambient
5	*Bond Wash Solution	Wash	0 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	*Bond Wash Solution	Wash	0 MIN	Ambient
10	10% NBF	Reagent	30 MIN	Ambient
11	*Bond Wash Solution	Wash	0 MIN	Ambient
12	*Bond Wash Solution	Wash	0 MIN	Ambient
13	*Bond Wash Solution	Wash	0 MIN	Ambient
14	*Bond Wash Solution	Wash	2 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature
15	*Bond Wash Solution	Wash	2 MIN	Ambient
16	*Bond Wash Solution	Wash	0 MIN	Ambient
17	*ACD Enzyme	Reagent	0 MIN	40 °C
18	*ACD Enzyme	Reagent	30 MIN	40 °C
19	*Bond Wash Solution	Wash	0 MIN	Ambient
20	*Bond Wash Solution	Wash	0 MIN	Ambient
21	*Bond Wash Solution	Wash	0 MIN	Ambient
22	*Open 0 Haz	Reagent	10 MIN	Ambient
23	*Bond Wash Solution	Wash	0 MIN	Ambient
24	*Bond Wash Solution	Wash	0 MIN	Ambient
25	*Bond Wash Solution	Wash	0 MIN	Ambient
26	*Bond Wash Solution	Wash	0 MIN	Ambient
27	*Bond Wash Solution	Wash	0 MIN	Ambient
28	*Bond Wash Solution	Wash	0 MIN	Ambient
29	*Bond Wash Solution	Wash	0 MIN	Ambient
30	*Bond Wash Solution	Wash	0 MIN	Ambient
31	*Bond Wash Solution	Wash	0 MIN	Ambient
32	*Bond Wash Solution	Wash	0 MIN	Ambient
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	*ACD 2.5 P1	Reagent	0 MIN	Ambient
37	*ACD 2.5 P1	Reagent	0 MIN	Ambient
38	*ACD 2.5 P1	Reagent	120 MIN	42°C
39	*Bond Wash Solution	Wash	0 MIN	42°C
40	*Bond Wash Solution	Wash	1 MIN	42°C
41	*Bond Wash Solution	Wash	5 MIN	42°C
42	*Bond Wash Solution	Wash	0 MIN	Ambient
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	0 MIN	Ambient
47	*Bond Wash Solution	Wash	1 MIN	Ambient
48	*Bond Wash Solution	Wash	1 MIN	Ambient
49	*Bond Wash Solution	Wash	0 MIN	Ambient
50	ACD Multiplex Amp 1	Reagent	1 MIN	42°C
51	ACD Multiplex Amp 1	Reagent	30 MIN	42°C
52	*Bond Wash Solution	Wash	0 MIN	Ambient
53	*Bond Wash Solution	Wash	0 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature
54	*Bond Wash Solution	Wash	0 MIN	Ambient
55	*Bond Wash Solution	Wash	3 MIN	Ambient
56	*Bond Wash Solution	Wash	3 MIN	Ambient
57	*Bond Wash Solution	Wash	0 MIN	Ambient
58	*Bond Wash Solution	Wash	0 MIN	Ambient
59	*Bond Wash Solution	Wash	0 MIN	Ambient
60	*LS Rinse	Reagent	5 MIN	Ambient
61	*LS Rinse	Reagent	5 MIN	Ambient
62	*Bond Wash Solution	Wash	0 MIN	Ambient
63	*Bond Wash Solution	Wash	0 MIN	Ambient
64	*Bond Wash Solution	Open Wash	0 MIN	Ambient
65	*Bond Wash Solution	Wash	0 MIN	Ambient
66	ACD Multiplex Amp 2	Reagent	1 MIN	42°C
67	ACD Multiplex Amp 2	Reagent	30 MIN	42°C
68	*Bond Wash Solution	Wash	0 MIN	Ambient
69	*Bond Wash Solution	Wash	0 MIN	Ambient
70	*Bond Wash Solution	Wash	0 MIN	Ambient
71	*Bond Wash Solution	Wash	3 MIN	Ambient
72	*Bond Wash Solution	Wash	3 MIN	Ambient
73	*Bond Wash Solution	Wash	0 MIN	Ambient
74	*Bond Wash Solution	Wash	0 MIN	Ambient
75	*Bond Wash Solution	Wash	0 MIN	Ambient
76	*LS Rinse	Reagent	5 MIN	Ambient
77	*LS Rinse	Reagent	5 MIN	Ambient
78	*Bond Wash Solution	Wash	0 MIN	Ambient
79	*Bond Wash Solution	Wash	1 MIN	Ambient
80	*Bond Wash Solution	Wash	1 MIN	Ambient
81	*Bond Wash Solution	Wash	1 MIN	Ambient
82	ACD Multiplex Amp 3	Reagent	1 MIN	42°C
83	ACD Multiplex Amp 3	Reagent	15 MIN	42°C
84	*Bond Wash Solution	Wash	0 MIN	Ambient
85	*Bond Wash Solution	Wash	0 MIN	Ambient
86	*Bond Wash Solution	Wash	0 MIN	Ambient
87	*Bond Wash Solution	Wash	1 MIN	Ambient
88	*Bond Wash Solution	Wash	1 MIN	Ambient
89	*Bond Wash Solution	Wash	1 MIN	Ambient
90	*Bond Wash Solution	Wash	1 MIN	Ambient
91	*Bond Wash Solution	Wash	1 MIN	Ambient
92	ACD Multiplex HRP-C1	Reagent	1 MIN	42°C

Step No.	Reagent	Step Type	Incubation Time	Temperature
93	ACD Multiplex HRP-C1	Reagent	15 MIN	42°C
94	*Bond Wash Solution	Wash	0 MIN	Ambient
95	*Bond Wash Solution	Wash	0 MIN	Ambient
96	*Bond Wash Solution	Wash	0 MIN	Ambient
97	*Bond Wash Solution	Wash	1 MIN	Ambient
98	*Bond Wash Solution	Wash	1 MIN	Ambient
99	*Bond Wash Solution	Wash	1 MIN	Ambient
100	*Bond Wash Solution	Wash	1 MIN	Ambient
101	*Bond Wash Solution	Wash	1 MIN	Ambient
102	ACD Multiplex TSA-F1**	Reagent	1 MIN	Ambient
103	ACD Multiplex TSA-F1**	Reagent	30 MIN	Ambient
104	*Bond Wash Solution	Wash	0 MIN	Ambient
105	*Bond Wash Solution	Wash	0 MIN	Ambient
106	*Bond Wash Solution	Wash	0 MIN	Ambient
107	*Bond Wash Solution	Wash	1 MIN	Ambient
108	*Bond Wash Solution	Wash	1 MIN	Ambient
109	*Bond Wash Solution	Wash	1 MIN	Ambient
110	*Bond Wash Solution	Wash	1 MIN	Ambient
111	ACD Multiplex HRP blocker	Reagent	1 MIN	42°C
112	ACD Multiplex HRP blocker	Reagent	15 MIN	42°C
113	*Bond Wash Solution	Wash	0 MIN	Ambient
114	*Bond Wash Solution	Wash	0 MIN	Ambient
115	*Bond Wash Solution	Wash	0 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 Multiplex HRP-C2	Reagent	1 MIN	42°C
120	ACD Multiplex HRP-C2	Reagent	15 MIN	42°C
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	ACD Multiplex TSA-F2**	Reagent	1 MIN	Ambient
128	ACD Multiplex TSA-F2**	Reagent	30 MIN	Ambient
129	*Bond Wash Solution	Wash	0 MIN	Ambient
130	*Bond Wash Solution	Wash	0 MIN	Ambient
131	*Bond Wash Solution	Wash	0 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature
132	*Bond Wash Solution	Wash	1 MIN	Ambient
133	*Bond Wash Solution	Wash	1 MIN	Ambient
134	*Bond Wash Solution	Wash	1 MIN	Ambient
135	ACD Multiplex HRP blocker	Reagent	1 MIN	42°C
136	ACD Multiplex HRP blocker	Reagent	15 MIN	42°C
137	*Bond Wash Solution	Wash	0 MIN	Ambient
138	*Bond Wash Solution	Wash	0 MIN	Ambient
139	*Bond Wash Solution	Wash	0 MIN	Ambient
140	*Bond Wash Solution	Wash	1 MIN	Ambient
141	*Bond Wash Solution	Wash	1 MIN	Ambient
142	*Bond Wash Solution	Wash	1 MIN	Ambient
143	ACD Multiplex HRP-C3	Reagent	1 MIN	42°C
144	ACD Multiplex HRP-C3	Reagent	15 MIN	42°C
145	*Bond Wash Solution	Wash	0 MIN	Ambient
146	*Bond Wash Solution	Wash	0 MIN	Ambient
147	*Bond Wash Solution	Wash	0 MIN	Ambient
148	*Bond Wash Solution	Wash	1 MIN	Ambient
149	*Bond Wash Solution	Wash	1 MIN	Ambient
150	*Bond Wash Solution	Wash	1 MIN	Ambient
151	TSA-DIG†	Reagent	1 MIN	Ambient
152	TSA-DIG†	Reagent	30 MIN	Ambient
153	*Bond Wash Solution	Wash	0 MIN	Ambient
154	*Bond Wash Solution	Wash	0 MIN	Ambient
155	*Bond Wash Solution	Wash	0 MIN	Ambient
156	*Bond Wash Solution	Wash	1 MIN	Ambient
157	*Bond Wash Solution	Wash	1 MIN	Ambient
158	ACD Multiplex HRP blocker	Reagent	1 MIN	42°C
159	ACD Multiplex HRP blocker	Reagent	15 MIN	42°C
160	*Bond Wash Solution	Wash	0 MIN	Ambient
161	*Bond Wash Solution	Wash	0 MIN	Ambient
162	*Bond Wash Solution	Wash	0 MIN	Ambient
163	*Bond Wash Solution	Wash	1 MIN	Ambient
164	*Bond Wash Solution	Wash	1 MIN	Ambient
165	Bond Wash	Reagent	10 MIN	Ambient
166	*De-ionized Water	Wash	0 MIN	Ambient
167	*De-ionized Water	Wash	0 MIN	Ambient
168	*De-ionized Water	Wash	0 MIN	Ambient

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

† Depending on channel preference, TSA-DIG can replace ACD Multiplex TSA-F1 (steps 102–103) or ACD Multiplex TSA-F2 (steps 127–128). You can then replace the TSA-DIG steps listed in the table with ACD Multiplex TSA-F3 (steps 151–152).

## Part B of the Sequential Dual Stain software protocol with Opal™ Polaris ISH detection

The following table displays Part B of the Sequential Dual Stain software protocol for performing Multiplex Co-Detection using TSA-based Polaris 780 RNA detection and TSA-based protein detection.

Step No.	Reagent	Step type	Incubation time	Temperature
1	*Post Primary	Reagent	8 MIN	Ambient
2	*Bond Wash Solution	Wash	2 MIN	Ambient
3	*Bond Wash Solution	Wash	2 MIN	Ambient
4	*Bond Wash Solution	Wash	2 MIN	Ambient
5	*Polymer	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	TSA-F4	Reagent	1 MIN	Ambient
10	TSA-F4	Reagent	10 MIN	Ambient
15	*Bond Wash Solution	Wash	0 MIN	Ambient
16	*Bond Wash Solution	Wash	0 MIN	Ambient
17	*Bond Wash Solution	Wash	0 MIN	Ambient
18	*Bond Wash Solution	Wash	1 MIN	Ambient
19	*Bond Wash Solution	Wash	1 MIN	Ambient
20	ACD Multiplex HRP blocker	Reagent	1 MIN	42°C
21	ACD Multiplex HRP blocker	Reagent	15 MIN	42°C
22	*Bond Wash Solution	Wash	0 MIN	Ambient
23	*Bond Wash Solution	Wash	0 MIN	Ambient
24	*Bond Wash Solution	Wash	1 MIN	Ambient
25	*Bond Wash Solution	Wash	1 MIN	Ambient
26	*Bond Wash Solution	Wash	1 MIN	Ambient
27	Polaris 780	Reagent	1 MIN	Ambient
28	Polaris 780	Reagent	30 MIN	Ambient
29	*Bond Wash Solution	Wash	0 MIN	Ambient
30	*Bond Wash Solution	Wash	0 MIN	Ambient
31	*Bond Wash Solution	Wash	1 MIN	Ambient
32	*Bond Wash Solution	Wash	1 MIN	Ambient
33	*Bond Wash Solution	Wash	1 MIN	Ambient
34	Co-Detection DAPI	Reagent	10 min	Ambient
35	*De-ionized Water	Wash	0 MIN	Ambient
36	*De-ionized Water	Wash	0 MIN	Ambient



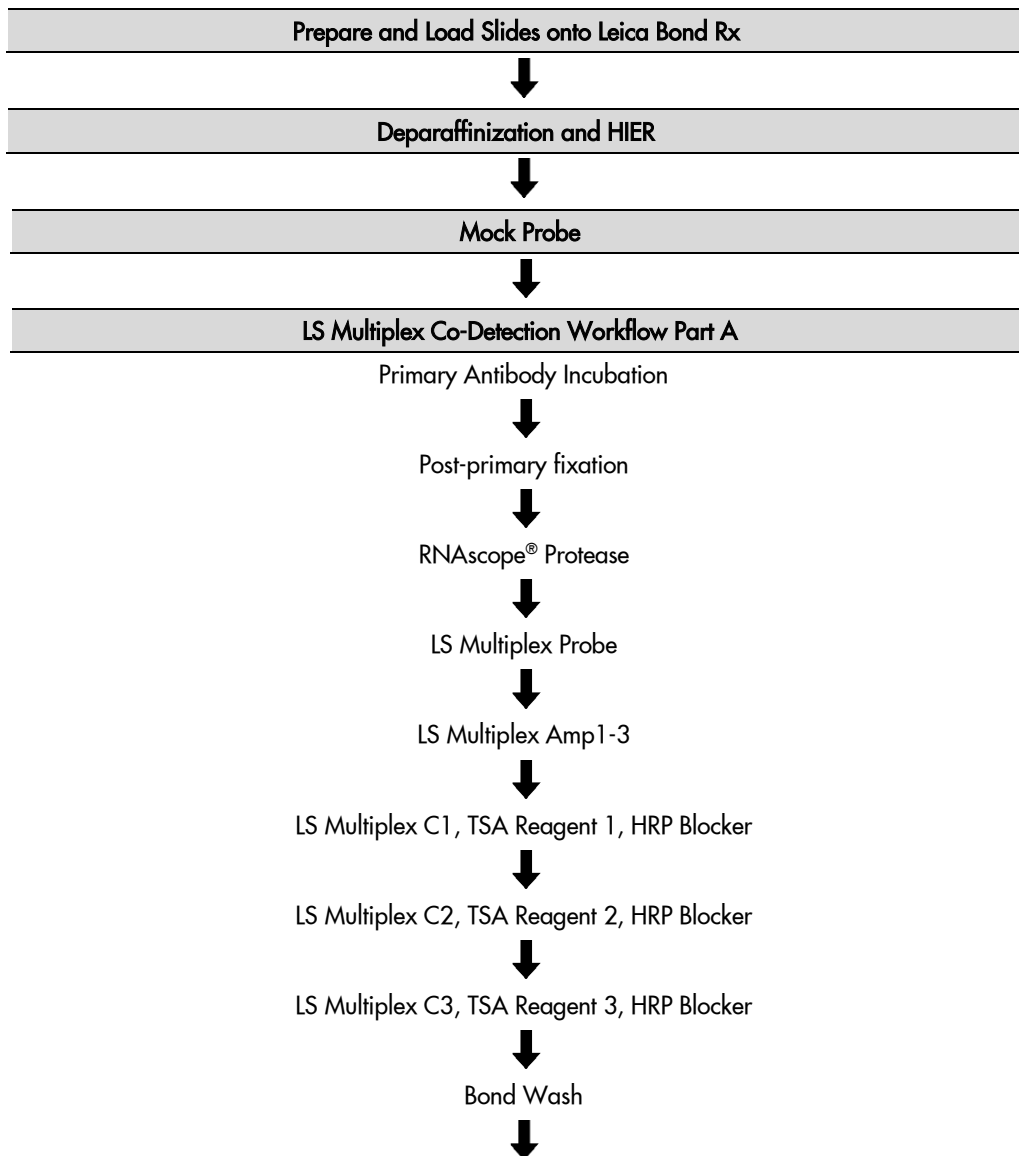
Step No.	Reagent	Step type	Incubation time	Temperature
37	*De-ionized Water	Wash	0 MIN	Ambient
38	*De-ionized Water	Wash	0 MIN	Ambient

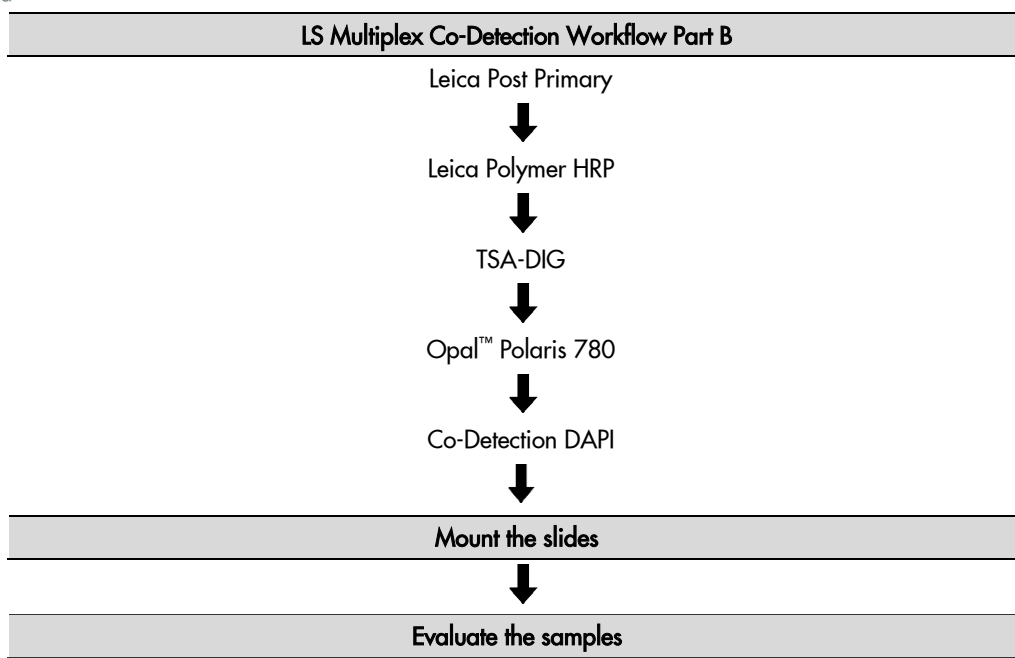
## Appendix C. Multiplex Co-Detection Using TSA Fluorophores for RNA Detection and Opal™ Polaris 780 for Protein Detection

This section provides instructions for creating Sequential Dual Stain protocols for Multiplex RNA-Protein Co-Detection on the Leica BOND RX System using TSA-based RNA detection and Opal™ Polaris 780 for protein detection.

**IMPORTANT!** You can run a maximum of two slide trays/SSAs simultaneously to co-detect three RNA targets with one protein target.

### Workflow steps





### Add new Co-Detection Reagents

Create the following reagents to perform Multiplex RNA-Protein Co-Detection on the Leica BOND RX System using TSA-based RNA detection and Opal™ Polaris 780 for protein detection.

Reagent	Reagent Registration Name	Reagent Type	Reagent Status
Primary Antibody	Co-Detection Antibody 1 (2, etc)*	Ancillary	Preferred
10% NBF	10% NBF	Ancillary	Preferred, Hazardous†
RNAscope® Probe	Probe 1 (2, etc)*	Ancillary	Preferred, Hazardous†
TSA-DIG	TSA-DIG	Ancillary	Preferred
Opal™ Polaris 780	Polaris 780	Ancillary	Preferred
DAPI	Co-Detection DAPI	Ancillary	Preferred

\* To use RNAscope® probes and antibodies with the Co-Detection workflow, you must register them as **Ancillary** reagent types with unique names not shared by any probes or antibodies registered as different reagent types.

† Please refer to the reagent SDS and local guidelines for the proper collection and disposal of all generated waste.

## Part A of the Sequential Dual Stain software protocol with Opal™ Polaris ISH detection

The following table displays Part A of the Sequential Dual Stain software protocol for performing Multiplex Co-Detection, using TSA-based detection for RNA detection and Opal™ Polaris 780 protein detection.

The post probe heated bond washes, shown below at steps 39–41, are specialized wash steps that are heated by the instrument and cannot be deleted. You can delete other wash steps.

Step No.	Reagent	Step Type	Incubation Time	Temperature
1	*Bond Wash Solution	Wash	0 MIN	Ambient
2	*Bond Wash Solution	Wash	0 MIN	Ambient
3	*Bond Wash Solution	Wash	0 MIN	Ambient
4	Co-Detection Antibody 1	Reagent	15 MIN	Ambient
5	*Bond Wash Solution	Wash	0 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	*Bond Wash Solution	Wash	0 MIN	Ambient
10	10% NBF	Reagent	30 MIN	Ambient
11	*Bond Wash Solution	Wash	0 MIN	Ambient
12	*Bond Wash Solution	Wash	0 MIN	Ambient
13	*Bond Wash Solution	Wash	0 MIN	Ambient
14	*Bond Wash Solution	Wash	2 MIN	Ambient
15	*Bond Wash Solution	Wash	2 MIN	Ambient
16	*Bond Wash Solution	Wash	0 MIN	Ambient
17	*ACD Enzyme	Reagent	0 MIN	40 °C
18	*ACD Enzyme	Reagent	30 MIN	40 °C
19	*Bond Wash Solution	Wash	0 MIN	Ambient
20	*Bond Wash Solution	Wash	0 MIN	Ambient
21	*Bond Wash Solution	Wash	0 MIN	Ambient
22	*Open 0 Haz	Reagent	10 MIN	Ambient
23	*Bond Wash Solution	Wash	0 MIN	Ambient
24	*Bond Wash Solution	Wash	0 MIN	Ambient
25	*Bond Wash Solution	Wash	0 MIN	Ambient
26	*Bond Wash Solution	Wash	0 MIN	Ambient
27	*Bond Wash Solution	Wash	0 MIN	Ambient
28	*Bond Wash Solution	Wash	0 MIN	Ambient
29	*Bond Wash Solution	Wash	0 MIN	Ambient
30	*Bond Wash Solution	Wash	0 MIN	Ambient
31	*Bond Wash Solution	Wash	0 MIN	Ambient
32	*Bond Wash Solution	Wash	0 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature
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	*ACD 2.5 P1	Reagent	0 MIN	Ambient
37	*ACD 2.5 P1	Reagent	0 MIN	Ambient
38	*ACD 2.5 P1	Reagent	120 MIN	42°C
39	*Bond Wash Solution	Wash	0 MIN	42°C
40	*Bond Wash Solution	Wash	1 MIN	42°C
41	*Bond Wash Solution	Wash	5 MIN	42°C
42	*Bond Wash Solution	Wash	0 MIN	Ambient
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	0 MIN	Ambient
47	*Bond Wash Solution	Wash	1 MIN	Ambient
48	*Bond Wash Solution	Wash	1 MIN	Ambient
49	*Bond Wash Solution	Wash	0 MIN	Ambient
50	ACD Multiplex Amp 1	Reagent	1 MIN	42°C
51	ACD Multiplex Amp 1	Reagent	30 MIN	42°C
52	*Bond Wash Solution	Wash	0 MIN	Ambient
53	*Bond Wash Solution	Wash	0 MIN	Ambient
54	*Bond Wash Solution	Wash	0 MIN	Ambient
55	*Bond Wash Solution	Wash	3 MIN	Ambient
56	*Bond Wash Solution	Wash	3 MIN	Ambient
57	*Bond Wash Solution	Wash	0 MIN	Ambient
58	*Bond Wash Solution	Wash	0 MIN	Ambient
59	*Bond Wash Solution	Wash	0 MIN	Ambient
60	*LS Rinse	Reagent	5 MIN	Ambient
61	*LS Rinse	Reagent	5 MIN	Ambient
62	*Bond Wash Solution	Wash	0 MIN	Ambient
63	*Bond Wash Solution	Wash	0 MIN	Ambient
64	*Bond Wash Solution	Open Wash	0 MIN	Ambient
65	*Bond Wash Solution	Wash	0 MIN	Ambient
66	ACD Multiplex Amp 2	Reagent	1 MIN	42°C
67	ACD Multiplex Amp 2	Reagent	30 MIN	42°C
68	*Bond Wash Solution	Wash	0 MIN	Ambient
69	*Bond Wash Solution	Wash	0 MIN	Ambient
70	*Bond Wash Solution	Wash	0 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature
71	*Bond Wash Solution	Wash	3 MIN	Ambient
72	*Bond Wash Solution	Wash	3 MIN	Ambient
73	*Bond Wash Solution	Wash	0 MIN	Ambient
74	*Bond Wash Solution	Wash	0 MIN	Ambient
75	*Bond Wash Solution	Wash	0 MIN	Ambient
76	*LS Rinse	Reagent	5 MIN	Ambient
77	*LS Rinse	Reagent	5 MIN	Ambient
78	*Bond Wash Solution	Wash	0 MIN	Ambient
79	*Bond Wash Solution	Wash	1 MIN	Ambient
80	*Bond Wash Solution	Wash	1 MIN	Ambient
81	*Bond Wash Solution	Wash	1 MIN	Ambient
82	ACD Multiplex Amp 3	Reagent	1 MIN	42°C
83	ACD Multiplex Amp 3	Reagent	15 MIN	42°C
84	*Bond Wash Solution	Wash	0 MIN	Ambient
85	*Bond Wash Solution	Wash	0 MIN	Ambient
86	*Bond Wash Solution	Wash	0 MIN	Ambient
87	*Bond Wash Solution	Wash	1 MIN	Ambient
88	*Bond Wash Solution	Wash	1 MIN	Ambient
89	*Bond Wash Solution	Wash	1 MIN	Ambient
90	*Bond Wash Solution	Wash	1 MIN	Ambient
91	*Bond Wash Solution	Wash	1 MIN	Ambient
92	ACD Multiplex HRP-C1	Reagent	1 MIN	42°C
93	ACD Multiplex HRP-C1	Reagent	15 MIN	42°C
94	*Bond Wash Solution	Wash	0 MIN	Ambient
95	*Bond Wash Solution	Wash	0 MIN	Ambient
96	*Bond Wash Solution	Wash	0 MIN	Ambient
97	*Bond Wash Solution	Wash	1 MIN	Ambient
98	*Bond Wash Solution	Wash	1 MIN	Ambient
99	*Bond Wash Solution	Wash	1 MIN	Ambient
100	*Bond Wash Solution	Wash	1 MIN	Ambient
101	*Bond Wash Solution	Wash	1 MIN	Ambient
102	ACD Multiplex TSA-F1	Reagent	1 MIN	Ambient
103	ACD Multiplex TSA-F1	Reagent	30 MIN	Ambient
104	*Bond Wash Solution	Wash	0 MIN	Ambient
105	*Bond Wash Solution	Wash	0 MIN	Ambient
106	*Bond Wash Solution	Wash	0 MIN	Ambient
107	*Bond Wash Solution	Wash	1 MIN	Ambient
108	*Bond Wash Solution	Wash	1 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature
109	*Bond Wash Solution	Wash	1 MIN	Ambient
110	*Bond Wash Solution	Wash	1 MIN	Ambient
111	ACD Multiplex HRP blocker	Reagent	1 MIN	42°C
112	ACD Multiplex HRP blocker	Reagent	15 MIN	42°C
113	*Bond Wash Solution	Wash	0 MIN	Ambient
114	*Bond Wash Solution	Wash	0 MIN	Ambient
115	*Bond Wash Solution	Wash	0 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 Multiplex HRP-C2	Reagent	1 MIN	42°C
120	ACD Multiplex HRP-C2	Reagent	15 MIN	42°C
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	ACD Multiplex TSA-F2	Reagent	1 MIN	Ambient
128	ACD Multiplex TSA-F2	Reagent	30 MIN	Ambient
129	*Bond Wash Solution	Wash	0 MIN	Ambient
130	*Bond Wash Solution	Wash	0 MIN	Ambient
131	*Bond Wash Solution	Wash	0 MIN	Ambient
132	*Bond Wash Solution	Wash	1 MIN	Ambient
133	*Bond Wash Solution	Wash	1 MIN	Ambient
134	*Bond Wash Solution	Wash	1 MIN	Ambient
135	ACD Multiplex HRP blocker	Reagent	1 MIN	42°C
136	ACD Multiplex HRP blocker	Reagent	15 MIN	42°C
137	*Bond Wash Solution	Wash	0 MIN	Ambient
138	*Bond Wash Solution	Wash	0 MIN	Ambient
139	*Bond Wash Solution	Wash	0 MIN	Ambient
140	*Bond Wash Solution	Wash	1 MIN	Ambient
141	*Bond Wash Solution	Wash	1 MIN	Ambient
142	*Bond Wash Solution	Wash	1 MIN	Ambient
143	ACD Multiplex HRP-C3	Reagent	1 MIN	42°C
144	ACD Multiplex HRP-C3	Reagent	15 MIN	42°C
145	*Bond Wash Solution	Wash	0 MIN	Ambient
146	*Bond Wash Solution	Wash	0 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature
147	*Bond Wash Solution	Wash	0 MIN	Ambient
148	*Bond Wash Solution	Wash	1 MIN	Ambient
149	*Bond Wash Solution	Wash	1 MIN	Ambient
150	*Bond Wash Solution	Wash	1 MIN	Ambient
151	ACD Multiplex TSA-F3	Reagent	1 MIN	Ambient
152	ACD Multiplex TSA-F3	Reagent	30 MIN	Ambient
153	*Bond Wash Solution	Wash	0 MIN	Ambient
154	*Bond Wash Solution	Wash	0 MIN	Ambient
155	*Bond Wash Solution	Wash	0 MIN	Ambient
156	*Bond Wash Solution	Wash	1 MIN	Ambient
157	*Bond Wash Solution	Wash	1 MIN	Ambient
158	ACD Multiplex HRP blocker	Reagent	1 MIN	42°C
159	ACD Multiplex HRP blocker	Reagent	15 MIN	42°C
160	*Bond Wash Solution	Wash	0 MIN	Ambient
161	*Bond Wash Solution	Wash	0 MIN	Ambient
162	*Bond Wash Solution	Wash	0 MIN	Ambient
163	*Bond Wash Solution	Wash	1 MIN	Ambient
164	*Bond Wash Solution	Wash	1 MIN	Ambient
165	Bond Wash	Reagent	10 MIN	Ambient
166	*De-ionized Water	Wash	0 MIN	Ambient
167	*De-ionized Water	Wash	0 MIN	Ambient
168	*De-ionized Water	Wash	0 MIN	Ambient

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

## Part B of Sequential Dual Stain software protocol including Opal™ Polaris ISH detection

The following table displays Part B of the Sequential Dual Stain software protocol for performing Multiplex Co-Detection, using TSA-based detection for RNA detection and Opal™ Polaris 780 for protein detection.

Step No.	Reagent	Step type	Incubation time	Temperature
1	*Post Primary	Reagent	8 MIN	Ambient
2	*Bond Wash Solution	Wash	2 MIN	Ambient
3	*Bond Wash Solution	Wash	2 MIN	Ambient
4	*Bond Wash Solution	Wash	2 MIN	Ambient
5	*Polymer	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	TSA-DIG	Reagent	1 MIN	Ambient
10	TSA-DIG	Reagent	10 MIN	Ambient

Step No.	Reagent	Step type	Incubation time	Temperature
11	*Bond Wash Solution	Wash	0 MIN	Ambient
12	*Bond Wash Solution	Wash	0 MIN	Ambient
13	*Bond Wash Solution	Wash	0 MIN	Ambient
14	*Bond Wash Solution	Wash	1 MIN	Ambient
15	*Bond Wash Solution	Wash	1 MIN	Ambient
16	ACD Multiplex HRP blocker	Reagent	1 MIN	42°C
17	ACD Multiplex HRP blocker	Reagent	15 MIN	42°C
18	*Bond Wash Solution	Wash	0 MIN	Ambient
19	*Bond Wash Solution	Wash	0 MIN	Ambient
20	*Bond Wash Solution	Wash	1 MIN	Ambient
21	*Bond Wash Solution	Wash	1 MIN	Ambient
22	*Bond Wash Solution	Wash	1 MIN	Ambient
23	Polaris 780	Reagent	1 MIN	Ambient
24	Polaris 780	Reagent	10 MIN	Ambient
25	*Bond Wash Solution	Wash	0 MIN	Ambient
26	*Bond Wash Solution	Wash	0 MIN	Ambient
27	*Bond Wash Solution	Wash	1 MIN	Ambient
27	*Bond Wash Solution	Wash	1 MIN	Ambient
29	*Bond Wash Solution	Wash	1 MIN	Ambient
30	Co-Detection DAPI	Reagent	10 MIN	Ambient
31	*De-ionized Water	Wash	0 MIN	Ambient
32	*De-ionized Water	Wash	0 MIN	Ambient
33	*De-ionized Water	Wash	0 MIN	Ambient
34	*De-ionized Water	Wash	0 MIN	Ambient

## *Appendix D. Multiplex Co-Detection Using Opal™ Polaris 780 for RNA Detection and Fluorophore-conjugated Secondary for Protein Detection*

The Multiplex Co-Detection workflow is compatible with use of Opal™ Polaris 780 for RNA detection. This section provides instructions for creating a Single Stain software protocol for Multiplex RNA-Protein Co-Detection on the Leica BOND RX System using TSA-based Opal™ Polaris 780 RNA detection and a fluorophore-conjugated secondary antibody for protein detection.

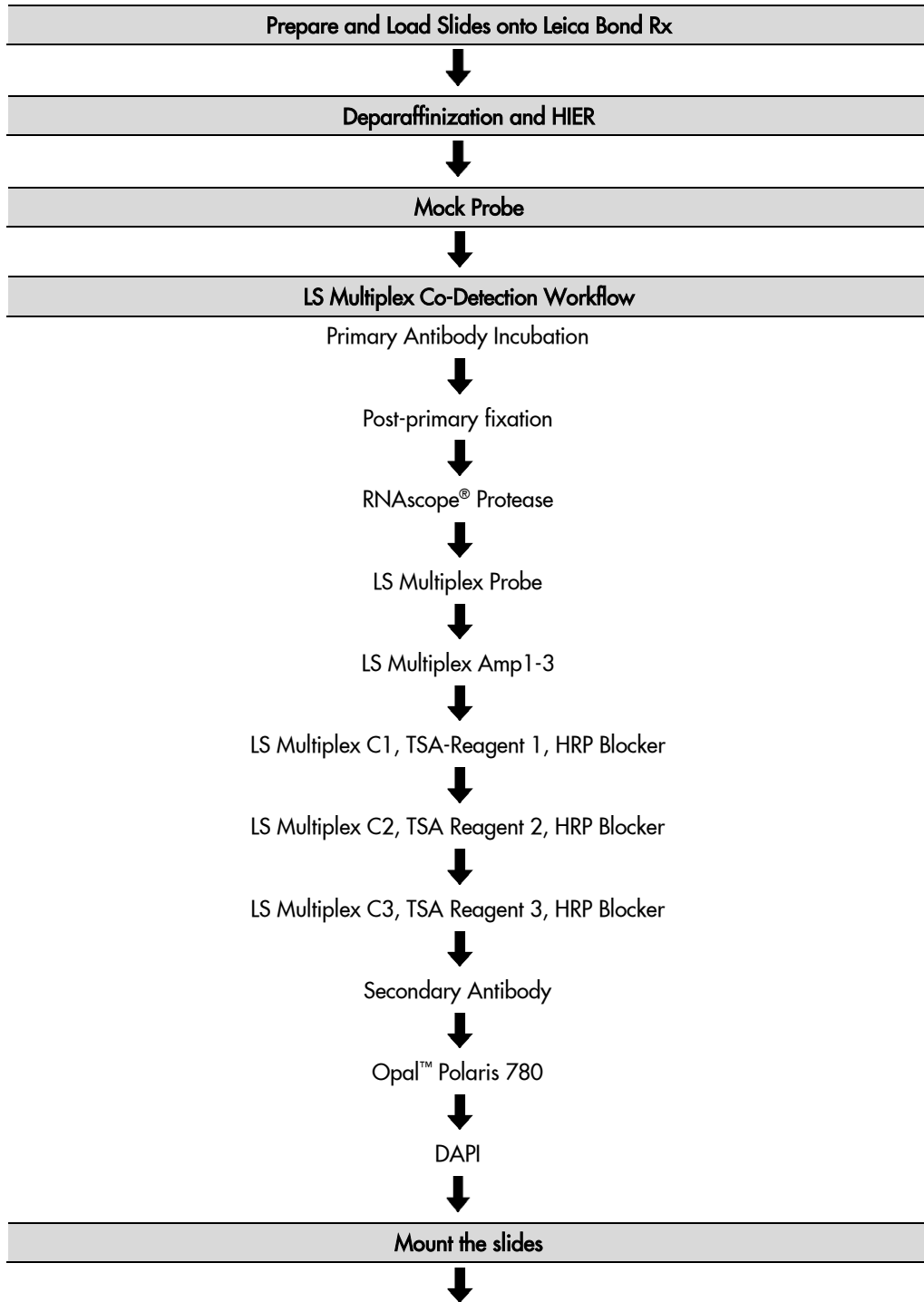
Opal™ Polaris 780 is a two-component system requiring TSA-DIG followed by Polaris 780. The 780 fluorophore is extremely sensitive to cleavage by HRP activity and must be applied last. To enable 780 detection of any RNA channel, you can split Opal™ Polaris 780 detection so that TSA-DIG incubation is followed by detection of any remaining RNA targets and protein detection. In this adjusted Co-Detection workflow, Polaris 780 is applied after secondary antibody as the final step before counter staining and mounting.

Depending on the desired RNAscope® channel for 780 detection, TSA-DIG may be applied as TSA Reagent 1, TSA Reagent 2, or TSA Reagent 3.



**IMPORTANT!** You can run a maximum of two slide trays/SSAs simultaneously to co-detect three RNA targets with one protein target.

## Overview of Assay Workflow



**Evaluate the samples**

## Add new Co-Detection Reagents

Create the following reagents to perform Multiplex RNA-Protein Co-Detection on the Leica BOND RX System using Opal™ Polaris 780 TSA-based RNA detection and a fluorophore-conjugated secondary antibody for protein detection.

Reagent	Reagent Registration Name	Reagent Type	Reagent Status
Primary Antibody	Co-Detection Antibody 1 (2, etc)*	Ancillary	Preferred
Secondary Antibody	Co-Detection Secondary	Ancillary	Preferred
10% NBF	10% NBF	Ancillary	Preferred, Hazardous†
RNAscope® Probe	Probe 1 (2, etc)*	Ancillary	Preferred, Hazardous†
TSA-DIG	TSA-DIG	Ancillary	Preferred
Opal™ Polaris 780	Polaris 780	Ancillary	Preferred
DAPI	Co-Detection DAPI	Ancillary	Preferred, Hazardous†

\* To use RNAscope® probes and antibodies with the Co-Detection workflow, you must register them as **Ancillary** reagent types with unique names not shared by any probes or antibodies registered as different reagent types.

† Please refer to the reagent SDS and local guidelines for the proper collection and disposal of all generated waste.

## Create a single stain software protocol with Opal™ Polaris ISH detection

The following table displays the single stain Part A software protocol for performing Multiplex Co-Detection, using Opal™ Polaris 780 TSA-based RNA detection and a fluorophore-conjugated secondary antibody for protein detection.

The post probe heated bond washes, shown below at steps 39–41, are specialized wash steps that are heated by the instrument and cannot be deleted. You can delete other wash steps.

Step No.	Reagent	Step Type	Incubation Time	Temperature
1	*Bond Wash Solution	Wash	0 MIN	Ambient
2	*Bond Wash Solution	Wash	0 MIN	Ambient
3	*Bond Wash Solution	Wash	0 MIN	Ambient
4	Co-Detection Antibody 1	Reagent	15 MIN	Ambient
5	*Bond Wash Solution	Wash	0 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	*Bond Wash Solution	Wash	0 MIN	Ambient
10	10% NBF	Reagent	30 MIN	Ambient
11	*Bond Wash Solution	Wash	0 MIN	Ambient
12	*Bond Wash Solution	Wash	0 MIN	Ambient
13	*Bond Wash Solution	Wash	0 MIN	Ambient
14	*Bond Wash Solution	Wash	2 MIN	Ambient
15	*Bond Wash Solution	Wash	2 MIN	Ambient
16	*Bond Wash Solution	Wash	0 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature
17	*ACD Enzyme	Reagent	0 MIN	40°C
18	*ACD Enzyme	Reagent	30 MIN	40°C
19	*Bond Wash Solution	Wash	0 MIN	Ambient
20	*Bond Wash Solution	Wash	0 MIN	Ambient
21	*Bond Wash Solution	Wash	0 MIN	Ambient
22	*Open 0 Haz	Reagent	10 MIN	Ambient
23	*Bond Wash Solution	Wash	0 MIN	Ambient
24	*Bond Wash Solution	Wash	0 MIN	Ambient
25	*Bond Wash Solution	Wash	0 MIN	Ambient
26	*Bond Wash Solution	Wash	0 MIN	Ambient
27	*Bond Wash Solution	Wash	0 MIN	Ambient
28	*Bond Wash Solution	Wash	0 MIN	Ambient
29	*Bond Wash Solution	Wash	0 MIN	Ambient
30	*Bond Wash Solution	Wash	0 MIN	Ambient
31	*Bond Wash Solution	Wash	0 MIN	Ambient
32	*Bond Wash Solution	Wash	0 MIN	Ambient
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	*ACD 2.5 P1	Reagent	0 MIN	Ambient
37	*ACD 2.5 P1	Reagent	0 MIN	Ambient
38	*ACD 2.5 P1	Reagent	120 MIN	42°C
39	*Bond Wash Solution	Wash	0 MIN	42°C
40	*Bond Wash Solution	Wash	1 MIN	42°C
41	*Bond Wash Solution	Wash	5 MIN	42°C
42	*Bond Wash Solution	Wash	0 MIN	Ambient
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	0 MIN	Ambient
47	*Bond Wash Solution	Wash	1 MIN	Ambient
48	*Bond Wash Solution	Wash	1 MIN	Ambient
49	*Bond Wash Solution	Wash	0 MIN	Ambient
50	ACD Multiplex Amp 1	Reagent	1 MIN	42°C
51	ACD Multiplex Amp 1	Reagent	30 MIN	42°C
52	*Bond Wash Solution	Wash	0 MIN	Ambient
53	*Bond Wash Solution	Wash	0 MIN	Ambient
54	*Bond Wash Solution	Wash	0 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature
55	*Bond Wash Solution	Wash	3 MIN	Ambient
56	*Bond Wash Solution	Wash	3 MIN	Ambient
57	*Bond Wash Solution	Wash	0 MIN	Ambient
58	*Bond Wash Solution	Wash	0 MIN	Ambient
59	*Bond Wash Solution	Wash	0 MIN	Ambient
60	*LS Rinse	Reagent	5 MIN	Ambient
61	*LS Rinse	Reagent	5 MIN	Ambient
62	*Bond Wash Solution	Wash	0 MIN	Ambient
63	*Bond Wash Solution	Wash	0 MIN	Ambient
64	*Bond Wash Solution	Open Wash	0 MIN	Ambient
65	*Bond Wash Solution	Wash	0 MIN	Ambient
66	ACD Multiplex Amp 2	Reagent	1 MIN	42°C
67	ACD Multiplex Amp 2	Reagent	30 MIN	42°C
68	*Bond Wash Solution	Wash	0 MIN	Ambient
69	*Bond Wash Solution	Wash	0 MIN	Ambient
70	*Bond Wash Solution	Wash	0 MIN	Ambient
71	*Bond Wash Solution	Wash	3 MIN	Ambient
72	*Bond Wash Solution	Wash	3 MIN	Ambient
73	*Bond Wash Solution	Wash	0 MIN	Ambient
74	*Bond Wash Solution	Wash	0 MIN	Ambient
75	*Bond Wash Solution	Wash	0 MIN	Ambient
76	*LS Rinse	Reagent	5 MIN	Ambient
77	*LS Rinse	Reagent	5 MIN	Ambient
78	*Bond Wash Solution	Wash	0 MIN	Ambient
79	*Bond Wash Solution	Wash	1 MIN	Ambient
80	*Bond Wash Solution	Wash	1 MIN	Ambient
81	*Bond Wash Solution	Wash	1 MIN	Ambient
82	ACD Multiplex Amp 3	Reagent	1 MIN	42°C
83	ACD Multiplex Amp 3	Reagent	15 MIN	42°C
84	*Bond Wash Solution	Wash	0 MIN	Ambient
85	*Bond Wash Solution	Wash	0 MIN	Ambient
86	*Bond Wash Solution	Wash	0 MIN	Ambient
87	*Bond Wash Solution	Wash	1 MIN	Ambient
88	*Bond Wash Solution	Wash	1 MIN	Ambient
89	*Bond Wash Solution	Wash	1 MIN	Ambient
90	*Bond Wash Solution	Wash	1 MIN	Ambient
91	*Bond Wash Solution	Wash	1 MIN	Ambient
92	ACD Multiplex HRP-C1	Reagent	1 MIN	42°C

Step No.	Reagent	Step Type	Incubation Time	Temperature
93	ACD Multiplex HRP-C1	Reagent	15 MIN	42°C
94	*Bond Wash Solution	Wash	0 MIN	Ambient
95	*Bond Wash Solution	Wash	0 MIN	Ambient
96	*Bond Wash Solution	Wash	0 MIN	Ambient
97	*Bond Wash Solution	Wash	1 MIN	Ambient
98	*Bond Wash Solution	Wash	1 MIN	Ambient
99	*Bond Wash Solution	Wash	1 MIN	Ambient
100	*Bond Wash Solution	Wash	1 MIN	Ambient
101	*Bond Wash Solution	Wash	1 MIN	Ambient
102	TSA-DIG†	Reagent	1 MIN	Ambient
103	TSA-DIG†	Reagent	30 MIN	Ambient
104	*Bond Wash Solution	Wash	0 MIN	Ambient
105	*Bond Wash Solution	Wash	0 MIN	Ambient
106	*Bond Wash Solution	Wash	0 MIN	Ambient
107	*Bond Wash Solution	Wash	1 MIN	Ambient
108	*Bond Wash Solution	Wash	1 MIN	Ambient
109	*Bond Wash Solution	Wash	1 MIN	Ambient
110	*Bond Wash Solution	Wash	1 MIN	Ambient
111	ACD Multiplex HRP blocker	Reagent	1 MIN	42°C
112	ACD Multiplex HRP blocker	Reagent	15 MIN	42°C
113	*Bond Wash Solution	Wash	0 MIN	Ambient
114	*Bond Wash Solution	Wash	0 MIN	Ambient
115	*Bond Wash Solution	Wash	0 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 Multiplex HRP-C2	Reagent	1 MIN	42°C
120	ACD Multiplex HRP-C2	Reagent	15 MIN	42°C
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	ACD Multiplex TSA-F2†	Reagent	1 MIN	Ambient
128	ACD Multiplex TSA-F2†	Reagent	30 MIN	Ambient
129	*Bond Wash Solution	Wash	0 MIN	Ambient
130	*Bond Wash Solution	Wash	0 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature
131	*Bond Wash Solution	Wash	0 MIN	Ambient
132	*Bond Wash Solution	Wash	1 MIN	Ambient
133	*Bond Wash Solution	Wash	1 MIN	Ambient
134	*Bond Wash Solution	Wash	1 MIN	Ambient
135	ACD Multiplex HRP blocker	Reagent	1 MIN	42°C
136	ACD Multiplex HRP blocker	Reagent	15 MIN	42°C
137	*Bond Wash Solution	Wash	0 MIN	Ambient
138	*Bond Wash Solution	Wash	0 MIN	Ambient
139	*Bond Wash Solution	Wash	0 MIN	Ambient
140	*Bond Wash Solution	Wash	1 MIN	Ambient
141	*Bond Wash Solution	Wash	1 MIN	Ambient
142	*Bond Wash Solution	Wash	1 MIN	Ambient
143	ACD Multiplex HRP-C3	Reagent	1 MIN	42°C
144	ACD Multiplex HRP-C3	Reagent	15 MIN	42°C
145	*Bond Wash Solution	Wash	0 MIN	Ambient
146	*Bond Wash Solution	Wash	0 MIN	Ambient
147	*Bond Wash Solution	Wash	0 MIN	Ambient
148	*Bond Wash Solution	Wash	1 MIN	Ambient
149	*Bond Wash Solution	Wash	1 MIN	Ambient
150	*Bond Wash Solution	Wash	1 MIN	Ambient
151	ACD Multiplex TSA-F3†	Reagent	1 MIN	Ambient
152	ACD Multiplex TSA-F3†	Reagent	30 MIN	Ambient
153	*Bond Wash Solution	Wash	0 MIN	Ambient
154	*Bond Wash Solution	Wash	0 MIN	Ambient
155	*Bond Wash Solution	Wash	0 MIN	Ambient
156	*Bond Wash Solution	Wash	1 MIN	Ambient
157	*Bond Wash Solution	Wash	1 MIN	Ambient
158	ACD Multiplex HRP blocker	Reagent	1 MIN	42°C
159	ACD Multiplex HRP blocker	Reagent	15 MIN	42°C
160	*Bond Wash Solution	Wash	0 MIN	Ambient
161	*Bond Wash Solution	Wash	0 MIN	Ambient
162	*Bond Wash Solution	Wash	0 MIN	Ambient
163	*Bond Wash Solution	Wash	1 MIN	Ambient
164	*Bond Wash Solution	Wash	1 MIN	Ambient
165	Co-Detection Secondary	Reagent	60 MIN	Ambient
166	*Bond Wash Solution	Wash	0 MIN	Ambient
167	*Bond Wash Solution	Wash	0 MIN	Ambient
168	*Bond Wash Solution	Wash	1 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature
169	*Bond Wash Solution	Wash	1 MIN	Ambient
170	*Bond Wash Solution	Wash	1 MIN	Ambient
171	Polaris 780	Reagent	1 MIN	Ambient
172	Polaris 780	Reagent	10 MIN	Ambient
173	*Bond Wash Solution	Wash	0 MIN	Ambient
174	*Bond Wash Solution	Wash	0 MIN	Ambient
175	*Bond Wash Solution	Wash	1 MIN	Ambient
176	*Bond Wash Solution	Wash	1 MIN	Ambient
177	*Bond Wash Solution	Wash	1 MIN	Ambient
178	DAPI	Reagent	10 min	Ambient
179	*De-ionized Water	Wash	0 MIN	Ambient
180	*De-ionized Water	Wash	0 MIN	Ambient
181	*De-ionized Water	Wash	0 MIN	Ambient
182	*De-ionized Water	Wash	0 MIN	Ambient

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

† Depending on channel preference, TSA-DIG may be applied in place of ACD Multiplex TSA-F2 (steps 127–128) or ACD Multiplex TSA-F3 (steps 151–151). In this case, ACD Multiplex TSA-F1 would be applied in place of TSA-DIG (steps 102-103).

## Appendix E. Multiplex Co-Detection Troubleshooting Guide

Prior to running the Co-Detection workflow, we recommend establishing optimal TSA fluorophore concentrations for Multiplex ISH detection using the LS Multiplex assay. Please refer to the *RNAscope® LS Multiplex Reagent Kit User Manual for BDZ 11* (USM-322800). You can then incorporate optimized TSA fluorophore concentrations for multiplex RNA detection in the Co-Detection workflow.

The Co-Detection workflow may need a higher primary antibody concentration than immunofluorescence alone. To optimize protein detection, we recommend titrating antibody and fluorophore concentrations. The post-primary fixation and pretreatment conditions in this Tech Note provides optimal RNA and protein detection across most tissue samples. Adjust the following parameters if you need further optimization.

Reagent	Incubation Temperature	Recommended Incubation Time	Optimization Range
HIER	95°C	30 MIN	15-30 MIN, 88-95°C
Primary Antibody	Ambient	15 MIN	15–60 MIN
10% NBF	Ambient	30 MIN	15–60 MIN
Protease	40°C	30 MIN	15–30 MIN
Secondary Antibody	Ambient	60 MIN	8–120 MIN

**For Research Use Only. Not For Diagnostic Use.**

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 at <http://www.acdbio.com/store/terms>. Advanced Cell Diagnostics, Inc. reserves the right to change its products and services at any time to incorporate technological developments. This document is subject to change without notice. Although this document has been prepared with every precaution to ensure accuracy, Advanced Cell Diagnostics, Inc. assumes no liability for any errors, omissions, or for any damages resulting from the use of this information.

© 2020 Advanced Cell Diagnostics. All rights reserved. RNAscope is a registered trademark of Advanced Cell Diagnostics, Inc. All other trademarks belong to their respective owners.

**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](mailto:support@acdbio.com)  
[www.acdbio.com](http://www.acdbio.com)