

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

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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 RNA and protein. Workflow 2 describes how to perform Co-Detection using TSA-based fluorescent detection of RNA and protein.

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**.

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)				
V	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		

LS Fluorescent RNA-Protein Co-Detection



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® buffer and Co-Detection Antibody Diluent, and are stored as indicated in the following table:

	Ancillary Materials for Co-Detection				
\checkmark	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[®] LS Multiplex Fluorescent Assay requires specific materials and equipment available *only* from Leica Biosystems.

V	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	\$21.2001	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[®] Plus Fluorophores or Opal[™] Dyes

The RNAscope[®] LS Multiplex Fluorescent Reagent Kit (Cat. No. 322800) requires purchase of Opal[™] dyes or TSA[®] Plus fluorophores from Akoya Biosciences. Dilute the fluorophores in TSA buffer provided in the kit. The Opal[™] Polaris 780 Reagent Pack contains two reagents: Opal[™] TSA-DIG and Opal[™] Polaris 780. We recommend diluting Opal[™] TSA-DIG in TSA buffer, and diluting Opal[™] 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 [™] 520	FP1487001KT: Opal™520 Reagent Pack†	1:750-1:3000
Opal [™] 570	FP1488001KT: Opal™570 Reagent Pack†	1:750-1:3000
Opal [™] 620	FP1495001KT: Opal™620 Reagent Pack†	1:750-1:3000



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	Option1 (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 flHC 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	Opal Polaris 780	Opai 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	Opul rolaris 700	Opul Foldris 700

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	TSA-DIG	Opal [™] Polaris 780	Opal [™] Polaris 780
– C3	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.**

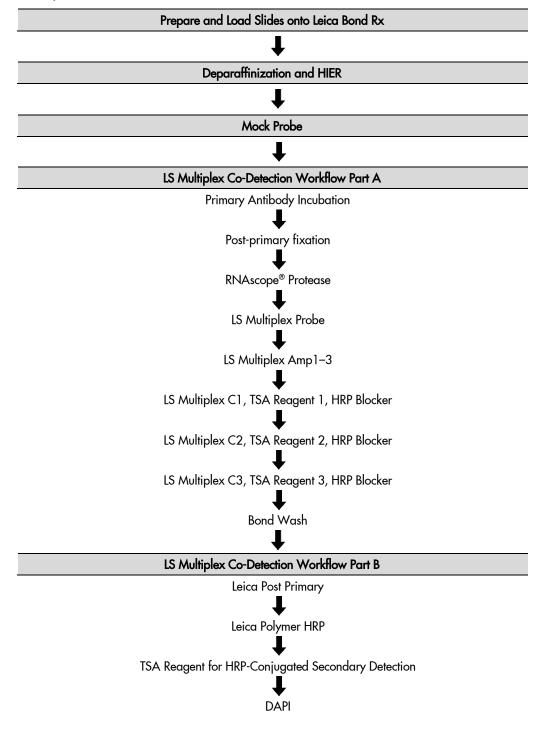


Workflow1: Fluorescent Multiplex Co-Detection Using TSA-based RNA and Protein Detection

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



LS Fluorescent RNA-Protein Co-Detection



<u>↓</u>
Mount the slides
Ļ
Evaluate the samples

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.

🌔 BOND - (bondpo	weruser) - Windows Internet Explorer						
Slide setup	Protocol setup	Slide history	Search	Help	Log out		<u>Leica</u>
Mrs. Potato Head	Reagent setup			U	ų		BIOSYSTEMS
Slinky Dog	*ACD Amp 1	ime		Abb. name *ACDAmp1	Type Ancillary	Supplier Advanced Cell Diagnostics	Pref.
	*ACD Amp 2			*ACDAmp2	Ancillary	Advanced Cell Diagnostics	~
	*ACD Amp 3			*ACDAmp3	Ancillary	Advanced Cell Diagnostics	~
	*ACD Amp 4			*ACDAmp4	Ancillary	Advanced Cell Diagnostics	~
	*ACD Amp 5 Brown			*ACDAmp5Br	Ancillary	Advanced Cell Diagnostics	~
	*ACD Amp 5 Red			*ACDAmp5Red	Ancillary	Advanced Cell Diagnostics	~
	*ACD Amp 6 Brown			*ACDAmp6Br	Ancillary	Advanced Cell Diagnostics	~
	*ACD Amp 6 Red			*ACDAmp6Red	Ancillary	Advanced Cell Diagnostics	~
	*ACD Blue			*ACDBlue	Ancillary	Advanced Cell Diagnostics	~
	*ACD Duplex AMP 1			*ACD_Du_AMP1	Ancillary	Advanced Cell Diagnostics	~
	*ACD Duplex AMP 10			*ACD_Du_AMP10	Ancillary	Advanced Cell Diagnostics	~
	*ACD Duplex AMP 2			*ACD_Du_AMP2	Ancillary	Advanced Cell Diagnostics	*
	Package type:	Rea	ent type:		Supplier:	Preferred status:	
	All reagents	▼ All			- All	Preferred	

- 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**).



🖉 BOND - (bondpowe	eruser) - Windows In	iternet Explorer						<u>_0×</u>
Slide setup	Protocol setup	Reagent setup	Slide history	Search	Help L	og out		Leica
	~	9			Add reagent		×	DIUSTOICHS
Mrs. Potato Head	Reagent	t setu	Name:	Co-Detection	on Antibody 1			
	Setup	Inv	Abbreviated name:	CoD Ab1				
	Add	Open	Туре:	Ancillary		▼		
		open	Supplier:					Pref.
Slinky Dog	Probe 14		Available bulks:			Compatible bulks:		Piel.
					>>	*BWash *DI		
	Probe 15				<<			*
	Probe 2							4
	Probe 3		 Preferred 	Hazardous				*
	Probe 4	_					_	*
	Probe 5			1	Save Cano	el		4
	Probe 6							~
	Probe 7			Prb7	Pro	be RNA		~
	Probe 8			Prb8	Pro	be RNA		4
	Probe 9			Prb9	Pro	be RNA		4
	Antibody 7			Ab7	Pri	mary antibody		4
	10% NBF			NBF	An	cillary		¥
	Package type:		Reagen	it type:		Supplier:	Preferred status:	
			▼ All		•		Preferred	Υ.

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.

Name: Abbreviated name: Description:		IA-Protein Co-Detection Part A					
Staining method: BOND RX	✓ Single	First 🖌 Second			Import protocol	Protocol type:	 Preferre ISH detectio
Preferred detection s	ystem: ACD	S Multiplex Detection Kit	•				
Step N° Wash	ı Rea	jent Su	pplier Ambie	nt Temperature	Inc. (min)	Dispense type	
1	*ACD 2.5 P1	Advanced C	ell Diagnostics 🛛 🗸		0:00	150 µL	
2	*ACD 2.5 P1	Advanced C	ell Diagnostics 🗸 🗸		0:00	150 µL	
3	*ACD 2.5 P1	Advanced C	ell Diagnostics	42	120:00	150 µL	
15	ACD Multiplex A	mp1 ACD		42	1:00	150 µL	
16	ACD Multiplex A	mp1 ACD		42	30:00	150 µL	
25	*LS Rinse	Advanced C	ell Diagnostics 🗸		5:00	150 µL	
26	*LS Rinse	Advanced C	ell Diagnostics 🗸		5:00	150 µL	
31	ACD Multiplex A	mp 2 ACD		42	1:00	150 µL	
Show wash step	AOD M. IE-1 /	9 ACD		<i>∦</i> .⊐	20-00 Insert wash	n Insert reagent	Delete ste

4. For Staining method, select First.

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				Preferre	
BOND RX					Import protocol	Protocol type:	ISH detection
Preferred detection syst	tem: 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	Í
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	
Show wash steps	AOD 14-85-1 A 2	ACD		40	იიიი Insert wash	Insert reagent	Delete ste

- 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**.

Name:	ACD Mu	Itiplex Co-Detection	Part A				
Abbreviated name:	Mx CoD	A					
Description:	ACD Mul	Itiplex RNA-Protein (Co-Detection Part A				
Staining method:	 Single 	le 🗸 First	Second		V Preferre		
BOND RX					Import p	rotocol	Protocol type: ISH detection
Preferred detection s	system:	ACD LS Multiplex	Detection Kit				
Step N° Was		Reagent	Supplier	Ambient Temper	rature Inc. (min)	Dispen	ise type
164 🗸	*Bond	Wash Solution	Leica Microsystems	~	0:00	150 µL	
165 🗸	*Bond \	Wash Solution	Leica Microsystems	~	1:00	150 µL	
166 🗸	*Bond	Wash Solution	Leica Microsystems	~	1:00	150 µL	
167 🖌	*Bond	Wash Solution	Leica Microsystems	1	10:00	150 uL	
168	Bond V	Vash		~	10:00	150 µL	
169 🗸	*Deioni	ized Water		√	0:00	150 µL	
170 🗸	*Deioni	ized Water		~	0:00	150 µL	
171 🗸	*Deioni	ized Water		~	0:00	150 µL	
 Show wash ste 	ps				Ins	ert 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**).



Name:	ACD Multiplex Co-Detecti	on Part A - O1 CD3					
Abbreviated name:	MxCoD A2						
Description:		in Co-Detection Part A Open1	CD3				
Staining method:	 Single First 	Second				✓ Preferre	
BOND RX					Import protocol	Protocol type	: ISH detect
Preferred detection sy	ystem: ACD LS Multip	lex Detection Kit	•				
Step N° Wash	h 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 Diagn	nostics	40	0:00	150 µL	
18	*ACD Enzyme	Advanced Cell Diagn	nostics	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 step	000 Multi-Lui A4	ACD		40	4-00 Insert wash	Insert reagent	Delete st
Show wash step	DS				insert wash	i insert reagent	Delete s

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.

Name:	ACD Multiplex Co-Detection P	art A - O1 CD3					
Abbreviated name:	MxCoD A2						
ACD Multiplex RNA-Protein Co-Detection Part A		o-Detection Part A Open1 CD3					
Staining method:	✓ Single ✓ First	Second					 Preferred
BOND RX					mport protocol	Protocol type	: ISH detection
Preferred detection sys	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	
E.N.	AOD M. Kalan A	10D		10	1-00	4701	¥
Show wash steps	3				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.

Name: Abbreviated name: Description:	ACD Multiplex Co-Detection Mx CoD B ACD Multiplex Co-Detection					
Staining method: BOND RX	Single First	 Second 		Import p	rotocol	Preferred Protocol type: IHC staining
Preferred detection sy	stem: Bond Polymer Re	efine Detection	•			
Step N° Wash	Reagent *Peroxide Block	Supplier Leica Microsystems	Ambient Temperatur	e Inc. (min) 5:00	Dispens	e type
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 step	S			Inser	twash I	nsert reagent Delete step



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

Name:	ACD Multiplex Co-Detection	Part B TSA				
Abbreviated name:	Mx CoD B					
Description:	ACD Multiplex Co-Detection	Part B TSA				
Staining method:	Single First	 Second 				Preferred
BOND RX				Import p	rotocol	Protocol type: IHC staining
Preferred detection sy	stem: Bond Polymer R	efine Detection				
Step N° Wash	, v	Supplier	Ambient Temperature	Inc. (min)	Dispens	e 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	4	0:00	150 µL	
18	*Mixed DAB Refine	Leica Microsystems	~	10:00	150 µL	
22	*Hematoxylin	Leica Microsystems	*	5:00	150 µL	
Show wash step	5			Inse	rt wash I	nsert 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

LS Fluorescent RNA-Protein Co-Detection



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.

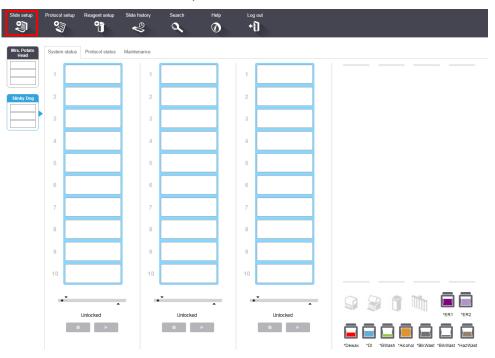
6. Select Save.

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 µL as shown.

BOND - (bondpow	reruser) - Windows Internet Explorer			
Slide setup			Help Log out	Leica
Mrs. Potato Head	Slide setup			Add study Edit study Delete study Copy study
	Study ID		Add study	X Sides 0
	CoD Run 154	Study ID:	Test	Add slide Add panel
Slinky Dog		Study name: Study comments:		
		Researcher:	[
		Study N°:	Manage researchers	
				_
		Dispense volume:	 100 μL ✓ 150 μL 	
		Preparation protocol:	*Bake and Dewax	
			OK Cancel	
	L			
	Positive tissue controls: 0			Total studies: 0
	Negative tissue controls: 0			Total slides: 0
				Study report Slide setup summary Print labels

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

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Mrs. Potato Head				Add study Edit study Delete study Copy study
	Study ID		Add study	X Slides 0
	CoD Run 154	Study ID:	Test	Add slide Add panel
Slinky Dog		Study name: Study comments:		
		Researcher:		
			Manage researchers	
		Study N°:		_
		Dispense volume:	100 μL	
		Preparation protocol:	 IS0 μL *Bake and Dewax 	
		Preparation protocol.	Dake and Dewax	
		[
		L	OK Cancel	
	L			
	Positive tissue controls: 0			Total studies: 0
	Negative tissue controls: 0			Total slides: 0
				Study report Slide setup summary Print labels

- 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

BOND - (bondpov						
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Sinky Dog	test	test Researcher Slide ID: Study N*: 8 Study com Date create 3/2/2020 1:0	nents: d:	Tissue type: Test tissue Negative tiss Staining mode: Single Single Single Single Single Marker: Protocols Preparation:	<u> </u>	Add galand
	Positive Negative			Add sid	0 Close	
						Study report Slide setup summary Print label

- 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.

🜔 BOND - (bondpo						_ D ×
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			Add	slide	<u>^</u>	
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			Process: Marker: Protocols	IHC VISH Mock Probe (ACD)		
			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*	•	
	Positive Negative		Add slide	Close		
					Study report Slide s	etup summary Print labels

10. In the Protocols tab, do the following:

- a. 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.
 - b. Verify the ***Bake and Dewax** protocol is selected 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.

LS Fluorescent RNA-Protein Co-Detection



f.

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

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		Slide ID:	Negative tissue	🥏 150 μL		
Slinky Dog		Study N°: 8	Positive tissue			
		Study comments:	Staining mode:			
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				Mock Probe (ACD)	•	
				MOCK FIDDE (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*	-	
	Positive		Add slide	Close		
	Negative			_		

- 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.

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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.

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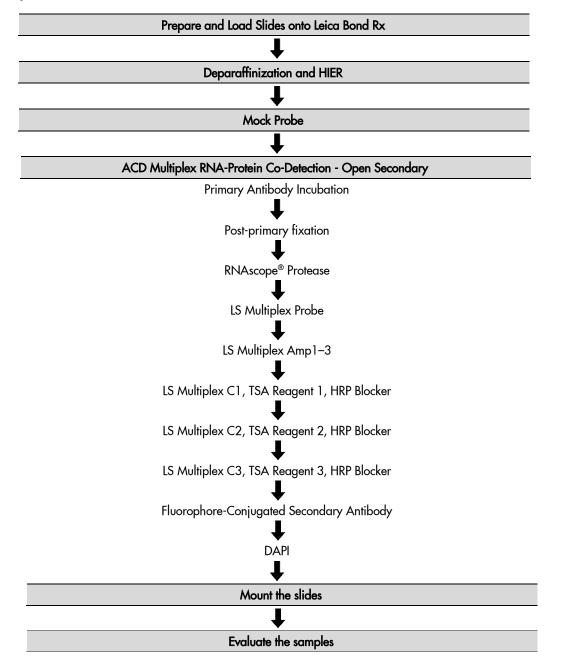
- 15. Repeat steps 5–14 for each slide.
- 16. After adding all the slides to the study, select **Close** to return to the Slide setup screen.
- 17. Select **Print labels** to print barcodes to attach to the slides.



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 Lecia 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.

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*ACD Amp 4 *ACD Amp 4 Ancilary Advanced Cell Diagnostics • *ACD Amp 5 Brown *ACD Amp5Br Ancilary Advanced Cell Diagnostics • *ACD Amp 5 Red *ACD Amp5Red Ancilary Advanced Cell Diagnostics • *ACD Amp 5 Red *ACD Amp6Br Ancilary Advanced Cell Diagnostics • *ACD Amp 6 Brown *ACD Amp6Br Ancilary Advanced Cell Diagnostics • *ACD Amp 6 Red *ACD Amp6Red Ancilary Advanced Cell Diagnostics • *ACD Blue *ACD Blue Ancilary Advanced Cell Diagnostics • *ACD Duplex AMP 1 *ACD_Du_AMP1 Andraced Cell Diagnostics • *ACD Duplex AMP 10 *ACD_Du_AMP10 Anvanced Cell Diagnostics •		*ACD Amp	2			*ACDAmp2	Ancillary	Advanced Cell Diagnostics	~
*ACD Amp 5 Brown *ACDAmp5Br Ancillary Advanced Cell Diagnostics • *ACD Amp 5 Red *ACDAmp5Red Ancillary Advanced Cell Diagnostics • *ACD Amp 5 Red *ACDAmp6Br Ancillary Advanced Cell Diagnostics • *ACD Amp 6 Brown *ACDAmp6Br Ancillary Advanced Cell Diagnostics • *ACD Amp 6 Red *ACDAmp6Red Ancillary Advanced Cell Diagnostics • *ACD Blue *ACDBlue Ancillary Advanced Cell Diagnostics • *ACD Duplex AMP 1 *ACD_Du_AMP1 Andvanced Cell Diagnostics • *ACD Duplex AMP 10 *ACD_Du_AMP1 Andvanced Cell Diagnostics •		*ACD Amp	3			*ACDAmp3	Ancillary	Advanced Cell Diagnostics	~
*ACD Amp 5 Red *ACD Amp5Red Ancillary Advanced Cell Diagnostics • *ACD Amp 6 Brown *ACD Amp6Ref Ancillary Advanced Cell Diagnostics • *ACD Amp 6 Red *ACD Amp6Ref Ancillary Advanced Cell Diagnostics • *ACD Amp 6 Red *ACD Amp6Ref Ancillary Advanced Cell Diagnostics • *ACD Blue *ACD Blue Ancillary Advanced Cell Diagnostics • *ACD Duplex AMP 1 *ACD_Du_AMP1 Ancillary Advanced Cell Diagnostics • *ACD Duplex AMP 10 *ACD_Du_AMP10 Ancillary Advanced Cell Diagnostics •		*ACD Amp	4			*ACDAmp4	Ancillary	Advanced Cell Diagnostics	~
*ACD Amp 6 Brown *ACDAmp6Br Ancillary Advanced Cell Diagnostics • *ACD Amp 6 Red *ACDAmp6Red Ancillary Advanced Cell Diagnostics • *ACD Amp 6 Red *ACDBlue Ancillary Advanced Cell Diagnostics • *ACD Blue *ACDBlue Ancillary Advanced Cell Diagnostics • *ACD Duplex AMP 1 *ACD_Du_AMP1 Ancillary Advanced Cell Diagnostics • *ACD Duplex AMP 10 *ACD_Du_AMP10 Anvanced Cell Diagnostics •		*ACD Amp	5 Brown			*ACDAmp5Br	Ancillary	Advanced Cell Diagnostics	~
*ACD Amp 6 Red *ACDAmp6Red Ancillary Advanced Cell Diagnostics • *ACD Blue *ACDBlue Ancillary Advanced Cell Diagnostics • *ACD Duplex AMP 1 *ACD_Du_AMP1 Ancillary Advanced Cell Diagnostics • *ACD Duplex AMP 10 *ACD_Du_AMP1 Ancillary Advanced Cell Diagnostics •		*ACD Amp	5 Red			*ACDAmp5Red	Ancillary	Advanced Cell Diagnostics	~
*ACD Blue *ACDBlue Ancillary Advanced Cell Diagnostics *ACD Duplex AMP 1 *ACD_Du_AMP1 Ancillary Advanced Cell Diagnostics *ACD Duplex AMP 10 *ACD_Du_AMP10 Ancillary Advanced Cell Diagnostics		*ACD Amp	6 Brown			*ACDAmp6Br	Ancillary	Advanced Cell Diagnostics	~
*ACD Duplex AMP 1 *ACD_Du_AMP1 Ancillary Advanced Cell Diagnostics *ACD Duplex AMP 10 *ACD_Du_AMP10 Ancillary		*ACD Amp	6 Red			*ACDAmp6Red	Ancillary	Advanced Cell Diagnostics	~
*ACD Duplex AMP 10 *ACD_Du_AMP10 Ancillary Advanced Cell Diagnostics		*ACD Blue				*ACDBlue	Ancillary	Advanced Cell Diagnostics	*
		*ACD Dupl	lex AMP 1			*ACD_Du_AMP1	Ancillary	Advanced Cell Diagnostics	~
*ACD Duplex AMP 2 *ACD_Du_AMP2 Ancillary Advanced Cell Diagnostics		*ACD Dupl	lex AMP 10			*ACD_Du_AMP10	Ancillary	Advanced Cell Diagnostics	~
		*ACD Dupl	lex AMP 2			*ACD_Du_AMP2	Ancillary	Advanced Cell Diagnostics	~
Package type: Reagent type: Supplier: Preferred status:		Package type:		Re	agent type:		Supplier:	Preferred status:	. *
All v All v Preferred		All reagents		- /	All		▼ All	▼ Preferred	-

- 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**).

🜔 BOND - (bondp	oweruser) - Windows	Internet Explorer							<u>_0×</u>
Slide setup	Protocol setup	Reagent setup	Slide history	Search	Help	Log out	×		Leica BIOSYSTEMS
	~				Add re	agent	^	_	
Mrs. Potato Head	Reager	nt setu	Name:	Co-De	tection Antibody 1				
	Setup	Inv	Abbreviated name	: CoD A	b1				
		Onon	Туре:	Ancilla	ary	•			
	Add	Open	Supplier:						Pref.
Slinky Dog	Probe 14		Available bulks:			Compatible bulks:		_	
	Probe 15				>	*BWash *DI			
	Probe 2				<	< Comparison of the second sec			
	Probe 3		Preferred	Hazardous					~
	Probe 4		 Preferred 	Hazardous					~
	Probe 5								~
	Probe 6				Save	Cancel			4
	Probe 7				Prb7	Probe RNA			*
2	Probe 8				Prb8	Probe RNA			~
	Probe 9				Prb9	Probe RNA			~
	Antibody 7			,	Ab7	Primary antibody			4
0 10	10% NBF			1	NBF	Ancillary			4
	Package type:		Reage	ent type:		Supplier:	Preferm	ed status:	
8						All	▼ Prefe		-



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 fluorophoreconjugated 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.

Name: Abbreviated name: Description:	ACD Multiplex Co-Detection C Mx CoD 2 ACD Multiplex RNA-Protein C	co-Detection Open Secondary				
Staining method:	 Single First 	Second			Import protocol	Preferred Protocol type: ISH detection
Preferred detection sy	stem: ACD LS Multiplex	Detection Kit				
Step N° Wash		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
Show wash step	8 OD 14.36-1 8 9	400		<i>د</i> ړ	วก-กก Insert wash	Insert reagent Delete step



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

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		Prefer			
BOND RX					Import protocol	Protocol type: ISH detect	
Preferred detection s	ystem: ACD LS Multiplex	Detection Kit					
Step N° Wasi	n 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	
Show wash step	ACD M.H.L. A	80D		40	იიიი Insert wash	Insert reagent Delete st	

- 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	lution 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	6 *Bond Wash Solution		Wash 0 MIN		
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	*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	

LS Fluorescent RNA-Protein Co-Detection



u				
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



Name:	ACD Multiplex	Co-Detection C	Open 2nd						
Abbreviated name:	Mx CoD 2								
Description:	ACD Multiplex	RNA-Protein C	o-Detection Open Secondar	у					
Staining method:	 Single 	First	Second		✓ Preferred				
BOND RX						Import pr	otocol	Protocol type	: ISH detection
Preferred detection s	ystem: AC	D LS Multiplex I	Detection Kit	•					
Step N° Was	h F	teagent	Supplier	Ambient	Temperature	Inc. (min)	Dispen	se type	
166 🖌	*Bond Wash	Solution	Leica Microsystems	~		1:00	150 µL		
167 🗸	*Bond Wash	Solution	Leica Microsystems	~		10:00	150 µL		
168	Co-Detectio	n Secondary 1		~		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 I	Nater				0.00	150 ul		,
 Show wash ste 	ps					Inse	rt wash	Insert reagent	Delete step

- 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.

Name: Abbreviated name: Description:	ACD Multiplex Co-Detection C MxCoD2-1 ACD Multiplex RNA-Protein Co						
Staining method: BOND RX	Single First	Second			Import protocol	Protocol type	Preferred e: ISH detection
Preferred detection sys							
Step N° Wash	Reagent Co-Detection Antibody 1	Supplier	Ambient	Temperature	Inc. (min) 15:00	Dispense type 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	***** *	Advanced Cell Disgnostics		10	۲۰۸۸ Insert wash	Insert reagent	▼ Delete step
		Save	Cancel				



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

Name:	ACD Mu	ultiplex Co-Detection	Open 2nd - P1 CD3					
Abbreviated name:	MxCoD2	2-1						
Description:	ACD Mu	ultiplex RNA-Protein	Co-Detection Open Secondary					
Staining method:	< Sing	gle First	Second					 Preferre
BOND RX						Import protocol	Protocol type:	ISH detection
Preferred detection s	system:	ACD LS Multiplex	Detection Kit					
Step N° Was	sh	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type	
4	Co-De	tection Antibody 1		~		15:00	150 μL	
10	10% N	IBF		~		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 ste	eps	A	Advanced Call Disgnastics		10	1.00 Insert wash	Insert reagent	Delete ste

- 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**)

Name:	ACD Mu	Itiplex Co-Detection	Open 2nd - P1 CD3					
Abbreviated name:	MxCoD2	2-1						
Description:	ACD Mu	Itiplex RNA-Protein	Co-Detection Open Seco	ndary				
Staining method:	 Sing 	le 🗹 First 🛛	Second					 Preferre
BOND RX					1	mport protocol	Protocol type:	ISH detectio
Preferred detection s	ystem:	ACD LS Multiplex	Detection Kit	•				
Step N° Wast	1	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type	
4	Co-De	tection CD3		~		15:00	150 µL	
10	10% N	BF		~		30:00	150 µL	
17	*ACD I	Enzyme	Advanced Cell Dia	agnostics	40	0:00	150 µL	
18	*ACD I	Enzyme	Advanced Cell Dia	agnostics	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 step		6.141	A0D		4 0	4-00 Insert wash	Insert reagent	Delete ste



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

BOND RX Import protocol type: ISH de BOND RX ACD LS Multiplex Detection Kit Step N° Wash Reagent Supplier 4 Co-Detection CD3 ✓ 10 10% NBF ✓ 17 *ACD Enzyme Advanced Cell Diagnostics 40 0.00 150 µL					ACD Multiplex Co-Detection (Name:
Staining method: ✓ Single ✓ First Second BOND RX Import protocol Protocol type: ISH de Preferred detection system: ACD LS Multiplex Detection Kit Step N* Wash Reagent 4 Co-Detection CD3 10 10% NBF 17 *ACD Enzyme					MxCoD2-1	Abbreviated name:
BOND RX Import protocol Protocol type: ISH de Preferred detection system: ACD LS Multiplex Detection Kit Step N* Wash Reagent Supplier Ambient Temperature Inc. (min) Dispense type 4 Co-Detection CD3 </th <th></th> <th></th> <th></th> <th>Detection Open Secondary</th> <th>ACD Multiplex RNA-Protein C</th> <th>Description:</th>				Detection Open Secondary	ACD Multiplex RNA-Protein C	Description:
Mathematical Supplier 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	Prefer			Second	 Single First 	Staining method:
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	port protocol Protocol type: ISH detect	Import protocol				BOND RX
4 Co-Detection CD3 ISO μL 10 10% NBF 30.00 150 μL 17 'ACD Enzyme Advanced Cell Diagnostics 40 0.00 150 μL	<u>_</u>			etection Kit	stem: ACD LS Multiplex	Preferred detection sy
10 10% NBF 30.00 150 μL 17 *ACD Enzyme Advanced Cell Diagnostics 40 0.00 150 μL	Inc. (min) Dispense type	re Inc. (min)	nt Temperature	Supplier Ambie	Reagent	Step N° Wash
17 *ACD Enzyme Advanced Cell Diagnostics 40 0.00 150 μL	15:00 150 µL	15:00		~	Co-Detection CD3	4
	30:00 150 µL	30:00		4	10% NBF	10
18 *ACD Enzyme Advanced Cell Diagnostics 40 30:00 150 uL	0:00 150 µL	40 0:00	40	Advanced Cell Diagnostics	*ACD Enzyme	17
	30:00 150 µL	40 30:00	40	Advanced Cell Diagnostics	*ACD Enzyme	18
22 *Open 0 Haz User 🖌 10:00 150 μL	10:00 150 µL	10:00		User 🗸	*Open 0 Haz	22
36 *Open 1 User 🖌 0:00 150 μL	0:00 150 µL	0:00		User 🗸	*Open 1	36
37 *Open 1 User 🗸 0:00 150 μL	0:00 150 µL	0:00		User 🗸	*Open 1	37
38 *Open 1 User 42 120:00 150 μL	120:00 150 µL	42 120:00	42	User	*Open 1	38
Show wash steps Insert wash Insert reagent Delet	4-00 4-00-01 Insert wash Insert reagent Delete s		13	400		

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**)

Name:	ACD Mul	Itiplex Co-Detection C	Open 2nd					
Abbreviated name:	Mx CoD	2						
Description:	ACD Mul	Itiplex RNA-Protein C	o-Detection Open Secondary					
Staining method:	 Singl 	le First	Second					 Preferred
BOND RX					Impor	t protocol	Protocol type:	ISH detection
Preferred detection s	system:	ACD LS Multiplex	Detection Kit	2				
Step N° Was	sh	Reagent	Supplier	Ambient Ten	nperature Inc. (mir	1) Disper	se type	
166 🖌	*Bond \	Wash Solution	Leica Microsystems	~	1:00	150 µL		<u>^</u>
167 🖌	*Bond \	Wash Solution	Leica Microsystems	~	10:00	150 µL		_
168	Co-Det	ection 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 🖌		izerl Water		.0	0.00	150 ul nsert wash	Insert reagent	▼ Delete step

h. Select Save.



1.

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.

Sele	ct the Sl i	de setup	icon at	the top	of the	screer	1 .					
Slide setup	Protocol setup	Reagent setup	Slide history	Search Q	Help	Log out						
Mrs. Potato Head	System status	Protocol status	Maintenance									
	1		1			1						
Slinky Dog	2		2			2						
	3		3			3						
	4		4			4						
	5		5			5						
	6		6			6						
	7		7			7						
	8		8			8						
	9		9			9						
	10		10			10						
	•			•		·						
		Unlocked		Unlocked			Unlocked				'ER1 '1	R2
		Þ						*Dewax	*DI *BWas	h *Alcohol *BikWa	at "BikWast "Ha	azWast

. .

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

				Leica
Mite Peddo Host	Slide setup Stady (D) CoD Run 154	Study ID: Study name: Study comments: Researcher: Study N°: Dispense volume: Preparation protocol:	Add study Test Manage researchers Manage researchers 100 µL W 150 µL W 150 µL *Bake and Dewax	Add shoty E dit shoty Deletes story Copy shoty Sider 0 Add shoty Add shoty Add shoty
	Positive tissue controls: 0 Negative tissue controls: 0		OK Cancel	Total studies: 0 Total sludies: 0 Study report Stude summary Port Labels



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

Slide setup	veruser) - Windows Internet Explorer Protocol setup Reagont setup	Slide history Search	Help Log out	LICIX Lica
Mitti Polisiki Head	Slide setup Stady (D Cold Run 154	Study ID: Study name. Study comments: Researchar: Study N": Dispense volume: Preparation protocot	Add study Test Test Manage researchers Manage researchers 100 µL 30 µL 3	Add study Edit study Device study Copy study Stoles 0 Add study Add panel
	Positive tissue controls: 0 Negative tissue controls: 0			Total studies: 0 Total stides: 0 Study report Stide setup summary Print Labels

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

ke setup	Protocol setup				Leic
			Add	lido	×
e Rotato	Slide			niue	Delete study Copy stu
s. Potato Head	Onde	Study ID:	Tissue Probe Antibody		
		test Researcher:	Tissue type:	Dispense volume:	
	test		 Test tissue 	100 μL	Add slide Add panel
		Slide ID:	Negative tissue		
inky Dog		Study N°: 8	Positive tissue		
		Study comments:	Staining mode:	Routine	
		Date created: 3/2/2020 1:01:39 PM	Single	Routine	
		3/2/2020 1:01:39 PM	Sequential DS		
			Parallel DS	ј інс 🔵 ізн	
			Marker:		•
			Protocols		
			Preparation:		
	Positive		Add slide	Close	
	Negative				
					Study report Slide setup summary Print lab

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



🖉 BOND - (bondpower	ruser) - Windows	Internet Explorer	×
			history Search Help Log out
2		02	
			Add slide
Mrs. Potato Head	Slide		Tissue Probe Antibody Copy study
Head		Study ID: test	
		Researcher:	Tissue type: Dispense volume: 0
	test		Image: Weight of the state of th
		Slide ID:	Negative tissue 🕢 150 µL
Slinky Dog		Study N°: 8	Positive tissue
		Study comments:	Staining mode:
		Date created:	Single Routine
		3/2/2020 1:01:39 PM	Single
			Process: IHC 🕢 ISH
			Marker. Mock Probe (ACD)
			Protocols
			Staining: ACD Multiplex Co-Detection Open 2nd - P1 CD3
			Preparation: "Bake and Dewax
			HIER: ACD HIER 30 min with ER2 (95)
			Enzyme:
			Probe Application: *DEFAULT* Penaturation: *
			Hybridization: ACD 1 Min Hybridization Probe Removal: "DEFAULT"
			Figer Removal.
	Positive		Add slide Close
	Negative		
			Study report Slide setup summary Print labels

- 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

🔏 BOND - (bondpo	weruser) - Windows	i Internet Explorer			_ [] ×
Slide setup	Protocol setup	Reagent setup Slide history	Search Help	p Lagiout ★¶	Leica
			Add	slide	×
Mrs. Potato Head	Slide	Study ID:	Tissue Probe Antibody		Delete study Copy study
neau	1.00	test	Tissue type:	Dispense volume:	0
		Researcher:	Test tissue	100 µL	
	test	Slide ID:	Negative tissue	✓ 150 µL	Add slide Add panel
Slinky Dog		Study N°:	Positive tissue		
		8 Study comments:	Staining mode:		
		Date created:	Single	Routine	
		3/2/2020 1:01:39 PM	Single		
			Process:	🔵 IHC 🕑 ISH	
			Marker:	Mock Probe (ACD)	
			Protocols		
			Staining:	ACD Multiplex Co-Detection Open 2nd - P1 CD3	
			Preparation:	*Bake and Dewax	
			HIER:	ACD HIER 30 min with ER2 (95)	
			Enzyme: Probe Application:	* •	
			Denaturation:	*DEFAULT* •	
			Hybridization:	ACD 1 Min Hybridization	
			Probe Removal:	*DEFAULT*	
	Positive		Add slide	Close	
	Negative				
				Study report	Slide setup summary Print labels
				Study report	Save setup summary

- 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[®] EX, Mantra[™], Vectra[®], or Polaris[®] Systems. The following table lists the corresponding filter setting for each dye.

Opal [™] system	TSA [®] Plus System	Filter setting
Opal [™] 520	TSA [®] Plus fluorescein	FITC
Opal [™] 570	TSA [®] Plus Cyanine 3	СуЗ
Opal [™] 620		Texas Red
Opal [™] 690	TSA [®] Plus Cyanine 5	Су5/Су5.5
Opal [™] 780		Су7

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.

Slide setup	Protocol setup	Reagent setup	Slide history	Search	Help	Log out +					Leica
Mrs. Potato Head	System status	Protocol status	Maintenance								
	1		1			1					
Slinky Dog	2		2			2					
	3		3			3					
	4		4			4					
	5		5			5					
	6		6			6					
	7		7			7					
	8		8			8					
	9		9			9					
	10		10			10					
		nlocked	÷	Unlocked		+* Unlock				j m	'ER1 'ER2
	-						Þ	Dewax	TDI "BWash	*Alcohol *Bik/Nar	t *BikWast *HazWast

- 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.

Slide setup	Protocol setup Reagent s	etup Slide history	Search	Help	Log out			Leia
Mrs. Potato Head	Protocol setu	þ				Copy	pen Delete	Repo
	Pro	tocol name 🔺	Pro	stocol type	Description	Modified by	Mod. date	Pref.
	*AccuCyte CTC HIER	ER2 8 mins	Heat	pretreatment	AccuCyte CTC HIER ER2 8 mins	Leica	2/21/2020	~
	*ACD HIER 15 min wit	h ER2 (95)	Heat	pretreatment	ACD RNAscope heat pretreatment	Leica	2/21/2020	~
Slinky Dog	*HIER 10 min with ER	1	Heat	pretreatment	10 min Heat Retrieval using ER1	Leica	2/21/2020	~
7:13 AM	"HIER 10 min with ER	2	Heat	pretreatment	10 min Heat Retrieval using ER2	Leica	2/21/2020	~
	'HIER 20 min with ER	I	Heat	pretreatment	20 min Heat Retrieval using ER1	Leica	2/21/2020	~
	"HIER 20 min with ER	2	Heat	pretreatment	20 min Heat Retrieval using ER2	Leica	2/21/2020	~
	*HIER 25 min with ER	1 (97)	Heat	pretreatment	25 min Heat Retrieval using ER1	Leica	2/21/2020	~
	*HIER 30 min with ER	1	Heat	pretreatment	30 min Heat Retrieval using ER1	Leica	2/21/2020	~
	*HIER 30 min with ER	2	Heat	pretreatment	30 min Heat Retrieval using ER2	Leica	2/21/2020	~
	*HIER 40 min with ER	1	Heat	pretreatment	40 min Heat Retrieval using ER1	Leica	2/21/2020	~
	*HIER 40 min with ER	2	Heat	pretreatment	40 min Heat Retrieval using ER2	Leica	2/21/2020	~
	'HIER 5 min with ER1		Heat	pretreatment	5 min Heat Retrieval using ER1	Leica	2/21/2020	~
	*RNAscope 2.5 LSx Ta	irget Retrieval (88)	Heat	pretreatment	RNAscope 2.5 LSx heat retrieval 88C	Leica	2/21/2020	~
	*RNAscope 2.5 LSx Ta	rget Retrieval (95)	Heat	pretreatment	RNAscope 2.5 LSx heat pretreatment 95C	Leica	2/21/2020	~
	"ViewRNA HIER 10 m	n, ER1 (95)	Heat	pretreatment	10 min heat pretreatment, ER1 @ 95C for	Leica	2/21/2020	~
	SécurDNA HIED 10 mi	= ED2 (00)	Hant	orotrogtmont	10 min haat anatraatmant ED1 @ 000 for	Loiso	2/24/2020	.,
	Protocol group: Prestaining	Protocol type		Staining	status: Protocol origin:		referred status:	

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



Name: Abbreviated name:	ACD HIER 30 min with ER2 ACDHet30	(95)					
Description:	ACD RNAscope heat pretrea	atment					
							Preferre
BOND RX					Import protocol	Protocol typ	e: Heat pretreatme
Step N° Wash	Reagent	Supplier	Ambient 1	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	5						

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

Name: Abbreviated name:	ACD HIER 30 min with ER2 ACDHet30	(55)					
Description:	ACD RNAscope heat pretrea	atment					
							Preferre
BOND RX					Import protocol	Protocol ty	pe: Heat pretreatme
							
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							

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.

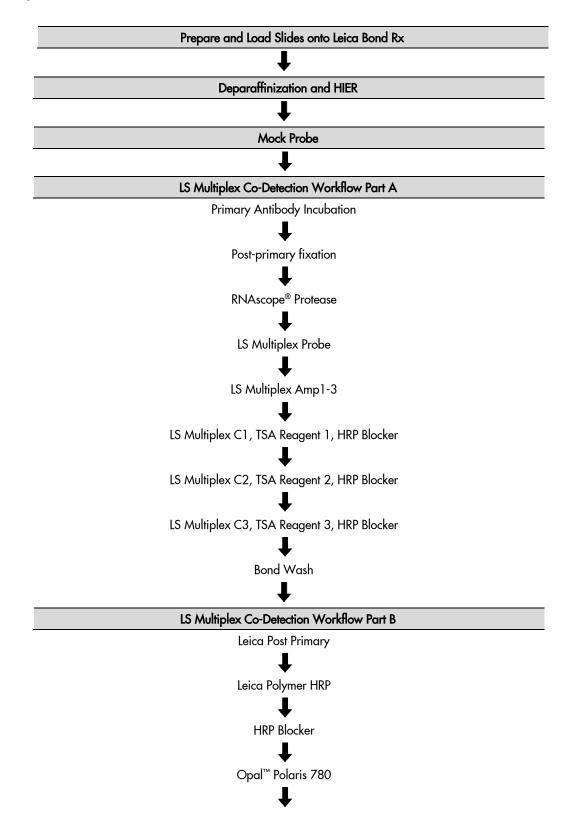
Opal[™] Polaris 780 is a two-component system requiring TSA-DIG followed by Polaris 780. The 780 fluorophore is extremely sensitive to cleavage by HRP activity and must be applied last. To enable 780 detection of any RNA channel, you can split Opal[™] Polaris 780 detection split so that TSA-DIG incubation 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.

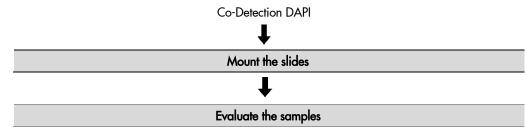


IMPORTANT! You can run a maximum of two slide trays/SSAs simultaneously to co-detect three RNA targets with one protein target.

Workflow steps







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

LS Fluorescent RNA-Protein Co-Detection



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



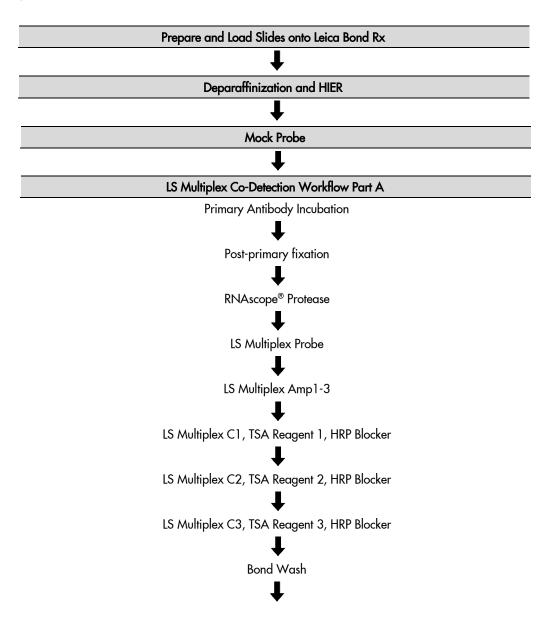
Step No.	Reagent	Step type	Incubation time	Temperature
37	*De-ionized Water	Wash	0 MIN	Ambient
38	*De-ionized Water	Wash	0 MIN	Ambient

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

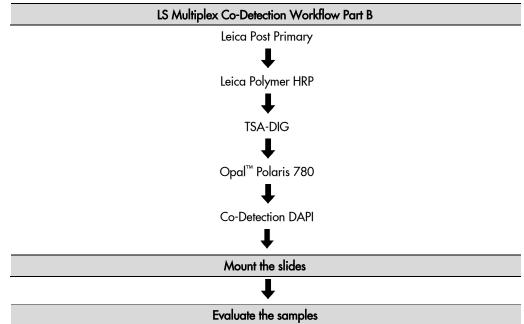
This section provides instructions for creating Sequential Dual Stain protocols for Multiplex RNA-Protein Co-Detection on the Leica BOND RX System using TSA-based RNA detection and Opal[™] Polaris 780 for protein detection.

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.

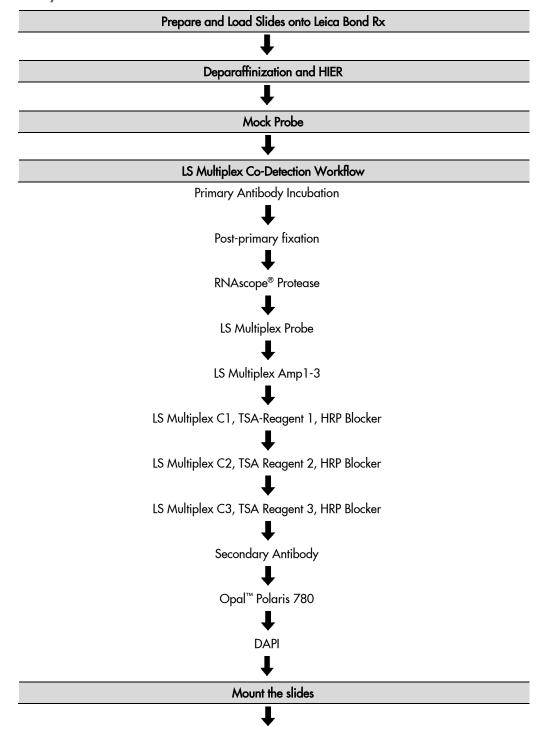
Opal[™] Polaris 780 is a two-component system requiring TSA-DIG followed by Polaris 780. The 780 fluorophore is extremely sensitive to cleavage by HRP activity and must be applied last. To enable 780 detection of any RNA channel, you can split Opal[™] Polaris 780 detection so that TSA-DIG incubation 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 postprimary 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℃
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



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