

RNAscope[®] LS Multiplex Fluorescent Assay Combined with Immunofluorescence Technical Note

Introduction

This Technical Note provides guidelines for performing automated *in situ* hybridization (ISH) using an RNAscope[®] LS Multiplex Fluorescent Reagent Kit (Cat. No. 322800) combined with immunofluorescence (IF) on the Leica BOND RX System. This procedure is based on the standard RNAscope[®] LS Multiplex Fluorescent Assay and requires the Leica BOND Detection Kit for immunofluorescent detection). Before starting the procedure, create a protocol for the RNAscope[®] assay combined with IF on the RX controller with the help of your ACD FAS. For every chemical, read the Material Safety Data Sheet (MSDS) and follow handling instructions. Wear appropriate protective eyewear, clothing, and gloves. For the latest service and support information, go to www.acdbio.com/support.

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

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

Materials Required

RNAscope® LS Multiplex Fluorescent Reagent Kit

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

	RNAscope [®] LS Multiplex Reagent Kit (Cat. No. 322440)					
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			



Materials from Leica BOND RX

The RNAscope[®] LS Multiplex Fluorescent Assay requires specific materials and equipment available *only* from Leica Biosystems.

\checkmark	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	\$21.1971	Room temp (20–25°C)

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

TSA[®] Plus Fluorophores or Opal[™] Dyes

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

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

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

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

Recommended fluorophore combinations

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

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

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

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

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

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

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

Workflow

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

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

Create a 3-plex ISH protocol

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

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



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

nsert reagent Insert wash	Duplicate	Delete duplicate	Import
Step N° Reagent	Supplier	Inc. (min)	
67 ACD Multiplex TSA-F1	ACD	1:00	ACD LS Multiplex Detection Kit
68 ACD Multiplex TSA-F1	ACD	30:00	<u>(</u>
77 ACD Multiplex HRP blocker	ACD	1:00	Step details
78 ACD Multiplex HRP blocker	ACD	15:00	Reagent: Bond Wash (ACD)
87 ACD Multiplex HRP-C2	ACD	1:00	
88 ACD Multiplex HRP-C2	ACD	15:00	Incubation time (min):
97 ACD Multiplex TSA-F2	ACD	1:00	Wash:
98 ACD Multiplex TSA-F2	ACD	30:00	
107 ACD Multiplex HRP blocker	ACD	1:00	
108 ACD Multiplex HRP blocker	ACD	15:00	
117 ACD Multiplex HRP-C3	ACD	1:00	
118 ACD Multiplex HRP-C3	ACD	15:00	
127 ACD Multiplex TSA-F3	ACD	1:00	
128 ACD Multiplex TSA-F3	ACD	30:00	
137 ACD Multiplex HRP blocker	ACD	1:00	
138 ACD Multiplex HRP blocker	ACD	15:00	
147 Bond Wash	ACD	10:00	
		Ö	
		\odot	

- 5. To perform a sequential dual stain (ds stain), make sure that **First** is selected under Double-staining status. **Note:** The **Single** button is optional unless you would like to run a single stain assay with this protocol.
- 6. Select Save.
- 7. Click Next to proceed. Ignore any pop ups that may appear on the screen.
- 8. To create a protocol for each additional probe, follow steps 1–7.

Create a duplex ISH protocol

- 1. In the Protocol setup screen, select **Staining** under the Protocol group menu.
- 2. Highlight the protocol for the standard RNAscope[®] LS Multiplex Fluorescent Assay set up by your ACD FAS (for example, **ACD Multiplex Protocol P1**). Select **Copy**.
- 3. Change the protocol name for your first probe to ACD Duplex Protocol P1 bw in the Name text box, Du_P1bw in the Abbreviated name text box, and ACD Duplex Protocol P1 with Bond Wash in the Description text box.
- 4. Highlight the DAPI step (step 147). From the Reagent drop down menu, change DAPI to Bond Wash.
- 5. To perform a sequential dual stain (ds stain), make sure that **First** is selected under Double-staining status. **Note:** The **Single** button is optional unless you would like to run a single stain assay with this protocol.
- 6. Click on **Show wash steps** to view all of the wash steps.
- 7. Select **Delete duplicate** or **Delete wash** to delete steps 117-146.
- 8. Select Save.
- 9. Click **Next** to proceed. Ignore any pop ups that may appear on the screen.
- 10. To create a protocol for each additional probe, follow steps 1-7.

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	 ACD Duplex Proto 	col P1 bw		Protocol type: (ISH detection	
	Du_P1bw)			
	ACD Duplex Proto	col P1 with bond wash	1		
BOND RX					
Insert reage	Insert wash		Delete duplicate		rt
Step N*	Reagent	Supplier	Inc. (min)	Preferred detection system:	
1 *ACI	D 2.5 P1	Advanced Cell Diag	n0:00 🔺	ACD LS Multiplex Detection Kit	_
2 *ACE	D 2.5 P1	Advanced Cell Diag	m0:00	6	
3 *ACE	D 2.5 P1	Advanced Cell Diag	m120:00	Step details	
15 ACD	Multiplex Amp 1	ACD	1:00	Reagent: (*ACD 2.5 P1	
16 ACD	Multiplex Amp 1	ACD	30:00	(000	_
25 *LSI	Rinse	Advanced Cell Diag	m5:00		_
26 *LSI	Rinse	Advanced Cell Diag	m5:00	Wash:	
31 ACD	Multiplex Amp 2	ACD	1:00		
32 ACD	Multiplex Amp 2	ACD	30:00		
41 *LSI	Rinse	Advanced Cell Diag	m5:00		
42 *LSI	Rinse	Advanced Cell Diag	m5:00		
47 ACD	Multiplex Amp 3	ACD	1:00		
48 ACD	Multiplex Amp 3	ACD	15:00		
57 ACD	Multiplex HRP-C1	ACD	1:00		
58 ACD	Multiplex HRP-C1	ACD	15:00		
67 ACD	Multiplex ISA-F1	ACD	1:00		
68 ACD	Multiplex (SA-F1	ACD	30:00		
77 ACD	Multiplex HRP blocker	ACD	1:00		
79 8(3)	usuminiev HRPhlocker	11.10	15mm		
Show v					
<u> </u>					
				Referred	
Double-staini	ing status				

bbreviated name: Du_P1bw Description: ACD Duplex Proto) col P1 with bond was	n))	
Insert reagent Insert wash	Duplicate	Delete duplicate		Import
Step N* Reagent	Supplier	Inc. (min)	Preferred detection system:	
41 *LS Rinse	Advanced Cell Diag	jn5:00	ACD LS Multiplex Detection Kit	
42 *LS Rinse	Advanced Cell Diag	gn 5:00	(
47 ACD Multiplex Amp 3	ACD	1:00	Step details	
48 ACD Multiplex Amp 3	ACD	15:00	Reagent: (*ACD 2.5 P1	
57 ACD Multiplex HRP-C1	ACD	1:00		
58 ACD Multiplex HRP-C1	ACD	15:00		
67 ACD Multiplex TSA-F1	ACD	1:00	Wash:	
68 ACD Multiplex ISA-F1	ACD	30:00		
77 ACD Multiplex HRP blocker	ACD	1:00		
78 ACD Multiplex HRP blocker	ACD	15:00		
87 ACD Multiplex HRP-C2	ACD	15.00		
88 ACD Multiplex HRP-C2	ACD	1000		
97 ACD Multiplex TSA-F2	ACD	1:00		
98 ACD MURIPIEX 15A-F2	ACD	1.00		
107 ACD Multiplex HRP blocker	ACD	15:00		
105 ACD Multiplex HSP Diocker	ACD	10.00		
117 Bong wash	ACD	10.00		
Show wash steps				
			~	
Double-staining status			Preferre	

Note: The preceding two figures display all reagent steps.



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

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

Register the reagents

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

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

2. Select Add to enter reagent information.



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



🗶 Add reagent	X
Name	(TSA-F4
Abbreviated name:	(TSA-F4
Туре:	Ancillary
Supplier:	
Available bulks:	Compatible bulks:
	*BWash *DI
Preferred: 🔘	Hazardous: 🔘
Save	Cancel

Note: You may leave the Supplier text box empty.

6. Select Save.

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

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



🖉 New protocol prope	erties					
	Refine IF Protoc RefineIF Bond Polymer R	ol Define IF protocol		Protocol type:	(IHC staining	
BOND RX) (Insert wash	Duplicate	Delete reagent)	-	Import
Step N*	Reagent	Supplier	Inc. (min)	Preferred detectio	in system:	
1 *Peroxic	le Block	Leica Microsystems	5:00	Bond Polymer	Refine Detection	
5 *MARKE	ER	Leica Microsystems	15:00	Constanting		
9 *Post Pr	imary	Leica Microsystems	8:00	Step details		
13 "Polyme	ar Dan n c	Leica Microsystems	8:00	Reagent: (*F	Peroxide Block	▼)
17 "Mixed I	JAB Refine	Leica Microsystems	0:00	Incubation time		(5:00
18 "Mixed	JAB Refine	Leica Microsystems	TU:00			
Show was	h steps					
Double-staining e	status O First	Second			Save	Preferred: Cancel

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

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

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



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

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



pbreviated name: (RefinelF			
Description: Bond Polyme	r Refine IF protocol		
BOND RX			
Insert reagent Insert wa	ash Duplicate	Delete reagent	Import
Step N* Reagent	Supplier	Inc. (min)	Preferred detection system:
1 *MARKER	Leica Microsystems	15:00	Bond Polymer Refine Detection
2 *Bond Wash Solution	Leica Microsystems	0:00	(
3 *Bond Wash Solution	Leica Microsystems	0:00	Step details
4 *Bond Wash Solution	Leica Microsystems	0:00	Reagent: *MARKER
5 *Post Primary	Leica Microsystems	8:00	
6 *Bond Wash Solution	Leica Microsystems	2:00	
7 *Bond Wash Solution	Leica Microsystems	2:00	Wash:
8 *Bond Wash Solution	Leica Microsystems	2:00	
9 *Polymer	Leica Microsystems	8:00	
10 *Bond Wash Solution	Leica Microsystems	2:00	
11 *Bond Wash Solution	Leica Microsystems	2:00	
12 *Bond Wash Solution	Leica Microsystems	0:00	
13 TSA-F4		1:00	
14 TSA-F4		10:00	
15 *Bond Wash Solution	Leica Microsystems	0:00	
16 *Bond Wash Solution	Leica Microsystems	0:00	
17 *Bond Wash Solution	Leica Microsystems	1:00	
18 *Bond Wash Solution	Leica Microsystems	1:00	
14 *Bond Wash Solution	Leica Microsystems	9	
Show wash steps			
			Preferred:

7. Select Save.

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

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

Build a study1. Select the Slide setup icon at the top of the screen.



📕 BOND RX - 9	system status screen (processing module	1)			>
File Window	Item ID Configuration Maintenance Help				
L	E 🖻 🗳 🤞		U	eica BOND R	
RX 1	System status			System	Protocol
	Run 254: Unlocked	Run 255: Unlocked	Run 256: Unlocked	"Dewax" "DI "BWash "Alc	•ER1 •ER2

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

BOND RX -	Slide screen	🔺
L		Leica BOND RX
RX 1	Slide setup Add study Edit study Delete study Study D: 3 Study name	Copy study Add slide Add panel
	Add study Study ID: Test Study name: Study commants: Researcher: Study N*: Bd1 Resear Dispense volume: 100 µL Preparation protocol: TBake and Dewax OK Cancel	
	Positive tissue controls: 0 Negative t Total studies: 23	ssue controls: 0 Total states: 180 Slide setup summary Print labels

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

Add an ISH and IHC protocol to each slide 1. Select Add slide.



📕 BOND RX - SI	lide screen							_ 🗆 🗙
File Window I	tem ID Configuration Ma	intenance H	telp					
L		1	$ \ge $			Leica BOND	RX	
RX 1	Slide setup Add study Study ID Test Run 1	Study Inanu Study Inanu Researche	t study t study Tost Run 1 Study name	Delete study	Copy stu	Add slide Add	d panel	
	Positiv	e tissue cont Total stu	rols: 0 lies: 1	Negative	tissue controls: (Total slides: (Slide setup summary	Print labe	

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

ደ Add slide		1
		(01YU
		(156
	(ізн-інс	
Comments:	(tissue probe	
Tissue type: Test tissue Negative tissue Positive tissue	Dispense volume: 100 µL 150 µL	
Staining mode: Single Resear Single Sequential DS Parallel DS:	ch 🔹	
Marker:		
Protocols		
Preparation. *Bake	and Dewax	•
Add slide	C	Close

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

🗳 Add slide			
	Slide ID: 01YU		
	Study N*: 156		
	dy name:		
	mments:		
	Study ID (ISH-IHC		
	mments: (tissue probe		
Tissue type: Test tissue Negative tissue Positive tissue	Dispense volume: 100 μL 150 μL		
Staining mode: Sequential D 3	Research		
First V	Second		
Process:	O IHC O ISH		
Marker:	Mock Probe (ACD)		
Protocols			
Staining:	ACD Multiplex Protocol P1 bw		
Preparation:	*Bake and Dewax 🔹		
HIER:	*ACD HIER 15 min with ER2 (95)		
Enzyme:	*ACD 15 min Protease		
Denaturation:	(*···· v)		
Hybridization:	ISH Hybridization 1 min		

- 6. Under **Protocols**:
 - Select a protocol from the Staining drop down menu for each probe. Make sure that each probe is associated with a different protocol (for example, ACD Multiplex Protocol P1 bw for multiplex ISH or ACD Duplex Protocol P1 bw for duplex ISH).
 - b. , Select the protocol ***Bake and Dewax** from the Preparation drop down menu for standard FFPE tissues (otherwise, leave blank).
 - c. Select ***ACD HIER 15 min with ER2 (95)** as the HIER protocol or the appropriate HIER protocol for your tissue.
 - d. Select *ACD 15 min Protease for Enzyme or the appropriate HIER protocol for your tissue.
 - e. Select ACD 1 min Hybridization for Hybridization.



Side ID: 01YU Study name: Study comments: Study comments: Study ID: SH-IHC Comments: ISH-IHC Comments: ISH-IHC Comments: ISSue probe Dispense volume: 100 µL 100 µL 150 µL 150 µL Staining mode: Sequential D\$ Research First Second Forcess: • HC Marker: *CD8 (4B11) Frotocols Staining Refine IF Protocol HER: • •	L Slide properties	
Study N* 156 Study name: Study comments: Study ID: SH-IHC Comments: Issue probe Tissue type: Tissue type: Tissue type: Negative issue Positive issue Positive issue Staining mode: Sequential DS: Research First Second Process: HC Staining: Refine IF Protocol HER: Enzyme: The staining issue Staining: Refine IF Protocol HER: The staining issue Staining: Refine IF Protocol HER: The staining issue Staining: Refine IF Protocol Staining: Refine IF Protocol Staining: Refine IF Protocol		Slide ID: 01YU
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Study comments: Study D: ISH-IHC Comments: ISBUE probe Tissue type: Tissue type: Tissue type: Positive tissue Positive tissue Staining mode: Sequential D: Research First Second Process: HC HC Marker *CD8 (4B11) Protocols Staining Refine IF Protocol HER Enzyme ************************************		
Study Dr. (SH-IHC Comments: (tissue probe		
Commerts: (issue probe Tissue type: ① Test tissue ● Negative tissue ● Positive tissue Staining mode: Sequential D: Research First Second Process: ● HC Marker: *CD8 (4B11) Protocole Staining Refine IF Protocol HER: * *		(ІЗН-ІНС
Tissue type: Test tissue Negative tissue Positive tissue Staining mode: Sequential D: Research First Second Process: HC Marker: CDB (4B11) Protocols Staining Refine IF Protocol HER Enzyme 	Comments:	tissue probe
Staining mode: Sequential D Research First Second Process: HC HC Marker: CD8 (4B11) Frotocole Staining: Refine IF Protocol HER Enzyme:	Tissue type: Test tissue Negative tissue Positive tissue	Dispense volume: 100 μL 150 μL
Process: HC ISH Marker: CD8 (4B11) Protocols Staining: Refine IF Protocol HIER:	Staining mode: Sequential DS Researc	ch 🔹
Marker: *CD8 (4B11) Protocols Staining: Refine IF Protocol HER: *		
Protocols Staining: Refine IF Protocol HIER:	Marker: (*CD8 ((4811)
Staining: Refine IF Protocol	Protocols	
HER:	Staining: Refine	IF Protocol
Enzyme	HER: (*	•
	Enzyme:	T

- 7. Add the IHC protocol by selecting the **Second** tab.
- Select IHC under Process and the antibody of interest (for example, *CD8) from the Marker menu.
 Note: For antibodies to be available from the menu, you must first register any antibodies not already available through Leica.
- Under Protocols, select Refine IF Protocol from the Staining menu. Leave HIER and Enzyme blank.
 Note: Including additional HIER or Enzyme steps following ISH staining may decrease the intensity of ISH markers.
- 10. Repeat steps 1–9 for each slide.

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



Complete the study

- 1. After adding all the slides to the study, select **Close** to return to the Slide setup screen.
- 2. Select **Print labels** to print barcodes to attach to the slides.



Part 4: Imaging

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

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



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