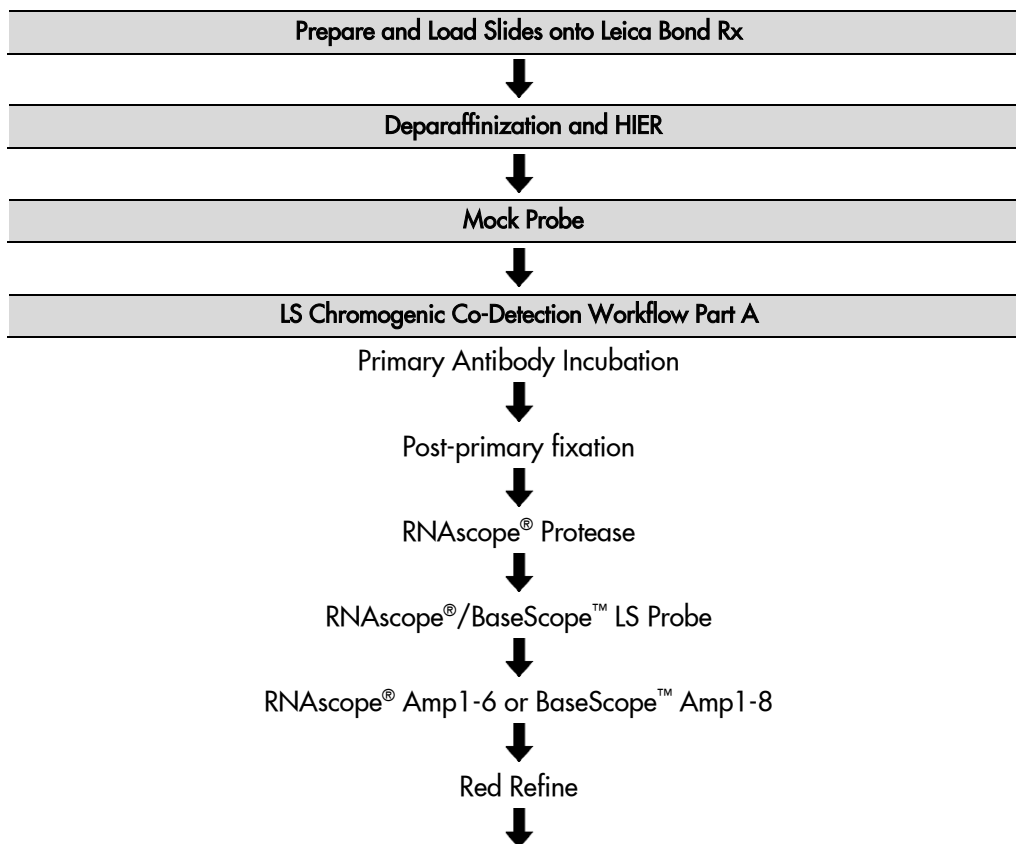


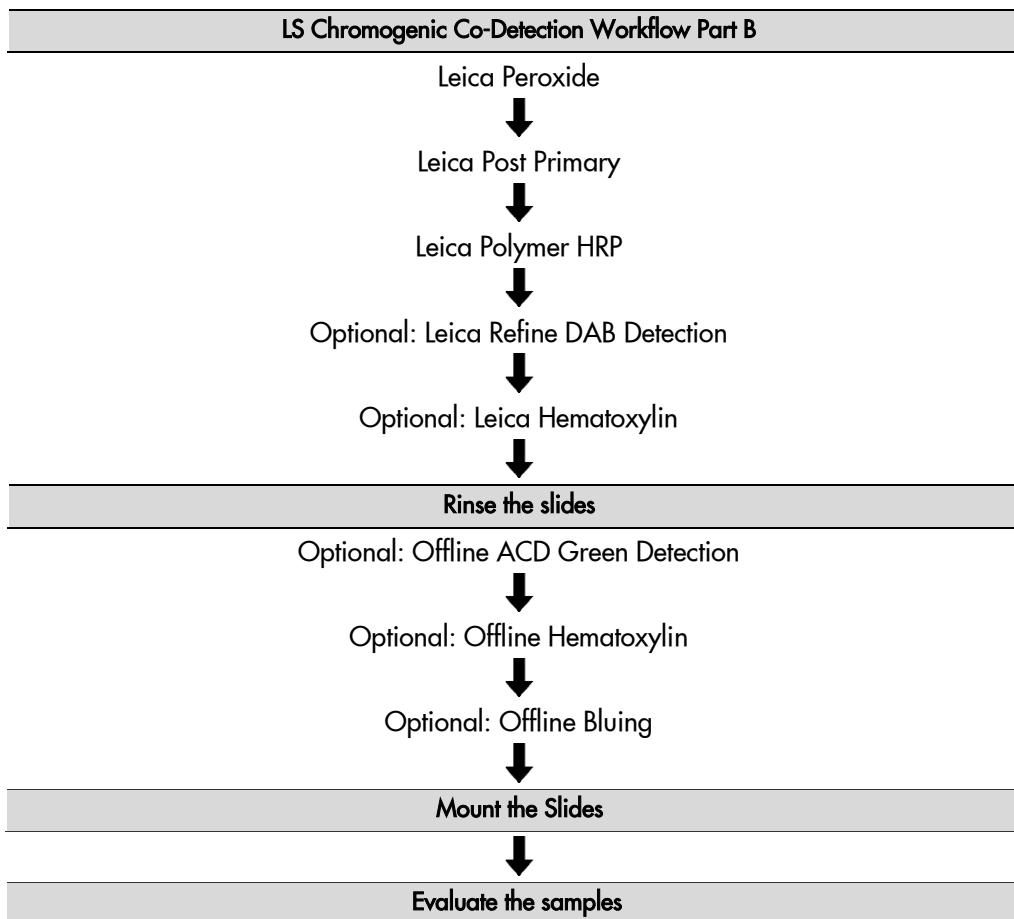
RNAscope® 2.5 LS or BaseScope™ RED Assay combined with Immunohistochemistry: Integrated Co-Detection Workflow (ICW) on Leica Bond RX

Introduction

This Technical Note provides guidelines for performing automated chromogenic co-detection of RNA and protein on the Leica BOND RX System. The Integrated Co-Detection Workflow (ICW) combines ACD's chromogenic Red ISH detection assays using either the RNAscope® 2.5 LS Reagent Kit- RED (Cat. No. 322100) or the BaseScope™ LS Reagent Kit (Cat. No. 323600) with fully automated DAB or semi-automated Green immunohistochemistry (IHC). In addition to ACD's Red ISH assays, you will need the Leica BOND Polymer Refine Red Kit for ISH detection and the Leica BOND Polymer Refine Kit for immunohistochemistry. Before starting the procedure, create protocols for RNA-Protein Co-Detection Part A and RNA-Protein Co-Detection Part B on the BOND RX controller with the help of your ACD FAS. 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.

Workflow





Chromogen Combinations for ICW

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

ACD ISH Assay	ISH Chromogen	IHC Chromogen	Reagents for ISH Detection	IHC Detection System/Reagents
RNAscope®	Red	Green	RNAscope® 2.5 LS Reagent Kit – RED; Leica BOND Refine Red Detection Kit	Co-Detection Antibody Diluent Leica BOND Refine Detection Kit; RNAscope® 2.5 LS Green Accessory Pack
RNAscope®	Red	DAB	RNAscope® 2.5 LS Reagent Kit – RED; Leica BOND Refine Red Detection Kit	Co-Detection Antibody Diluent Leica BOND Refine Detection Kit
BaseScope™	Red	Green	BaseScope™ LS Reagent Kit; Leica BOND Refine Red Detection Kit	Co-Detection Antibody Diluent Leica BOND Refine Detection Kit; RNAscope® 2.5 LS Green Accessory Pack
BaseScope™	Red	DAB	BaseScope™ LS Reagent Kit; Leica BOND Refine Red Detection Kit	Co-Detection Antibody Diluent Leica BOND Refine Detection Kit; RNAscope® 2.5 LS Green Accessory Pack

Materials Required

ACD LS Chromogenic ISH Detection Kits

RNAscope® 2.5 LS Reagent Kit- RED

The RNAscope® 2.5 LS Reagent Kit - RED (Cat. No. 322150) provides reagents to stain ~60 standard slides on Leica Biosystems' BOND RX System. The RNAscope® 2.5 LS Probes are available separately. The reagents are Ready-To-Use (RTU) and are stored as indicated in the following table:

RNAscope® 2.5 LS Reagent Kit - RED (Cat. No. 322150)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	RNAscope® 2.5 LS Hydrogen Peroxide	21 mL x 1 bottle	2–8°C
	RNAscope® 2.5 LS Protease III	21 mL x 1 bottle	2–8°C
	RNAscope® 2.5 LS AMP 1	21 mL x 1 bottle	2–8°C
	RNAscope® 2.5 LS AMP 2	21 mL x 1 bottle	2–8°C
	RNAscope® 2.5 LS AMP 3	21 mL x 1 bottle	2–8°C
	RNAscope® 2.5 LS AMP 3	21 mL x 1 bottle	2–8°C
	RNAscope® 2.5 LS AMP 4 – RED	21 mL x 1 bottle	2–8°C
	RNAscope® 2.5 LS AMP 5 – RED	21 mL x 1 bottle	2–8°C
	RNAscope® 2.5 LS AMP 6 – RED	21 mL x 1 bottle	2–8°C
	RNAscope® 2.5 LS Rinse	29 mL x 2 bottles	2–8°C

BaseScope™ LS Reagent Kit

The BaseScope™ LS Reagent Kit (Cat. No. 323600) provides reagents to stain ~60 slides on Leica Biosystems' BOND RX System. BaseScope™ LS Probes are available separately. The reagents are Ready-To-Use (RTU) and are stored as indicated in the following table:

BaseScope™ LS Reagent Kit (Cat. No. 323600)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	RNAscope® 2.5 LS Hydrogen Peroxide	21 mL x 1 bottle	2–8°C
	RNAscope® 2.5 LS Protease III	21 mL x 1 bottle	2–8°C
	BaseScope™ LS AMP 1	21 mL x 1 bottle	2–8°C
	BaseScope™ LS AMP 2	21 mL x 1 bottle	2–8°C
	BaseScope™ LS AMP 3	21 mL x 1 bottle	2–8°C
	BaseScope™ LS AMP 4	21 mL x 1 bottle	2–8°C
	BaseScope™ LS AMP 5	21 mL x 1 bottle	2–8°C
	BaseScope™ LS AMP 6	21 mL x 1 bottle	2–8°C
	BaseScope™ LS AMP 7	21 mL x 1 bottle	2–8°C
	BaseScope™ LS AMP 8	21 mL x 1 bottle	2–8°C
	RNAscope® 2.5 LS Rinse	29 mL x 4 bottles	2–8°C

RNAscope® 2.5 LS Green Accessory Pack

Optional: If green IHC staining is desired, we recommend the RNAscope® 2.5 LS Green Accessory Pack (Cat.

No. 322550). This accessory pack provides reagents to stain ~60 standard slides offline, following co-detection, on Leica Biosystems' BOND RX System. The reagents are Ready-To-Use (RTU) and are stored as indicated in the following table:

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

Additional Reagents

Additional Reagents for Co-Detection			
Reagent	Source / Ordering Info	Quantity	Storage
Co-Detection Antibody Diluent	ACD/Cat No. 323160	120 mL x 1 bottle	2–8°C
RNAscope® LS Protease IV (BaseScope™ only)	ACD/Cat No. 322140 (5 mL)	21 mL	2–8°C
Primary Antibody Concentrate	User	As needed	Per manufacturer's recommendation
10% Neutral Buffered Formalin	User	5–10 mL	Per manufacturer's recommendation

Required Materials from Leica BOND RX

The Integrated Co-Detection Workflow (ICW) requires specific materials and equipment available *only* from Leica Biosystems.

<input checked="" type="checkbox"/>	Component	Cat. No.	Storage
	BOND 30 mL Open Containers	OP309700	Room temp (20–25°C)
	BOND 7mL Open Containers	OP79193	Room temp (20–25°C)
	BOND Universal Covertiles 100 pack	S21.2001	Room temp (20–25°C)
	BOND Polymer Refine Detection (DAB) and Hematoxylin *	DS9800	2–8°C
	BOND Epitope Retrieval Solution 1-1L (RTU)	AR9961	2–8°C
	BOND Epitope Retrieval Solution 2-1L (RTU)	AR9640	2–8°C
	BOND Dewax Solution – 1L (RTU)	AR9222	2–8°C
	BOND Wash Solution 10X Concentrate – 1L	AR9590	2–8°C
	BOND Aspirating Probe Cleaning System	CS9100	2–8°C
	BOND Mixing Stations	S21.1971	Room temp (20–25°C)
	BOND Polymer Refine Red Detection and Hematoxylin*	CS9390	2–8°C

*Do not substitute with any other chromogen kit.

Run the Assay

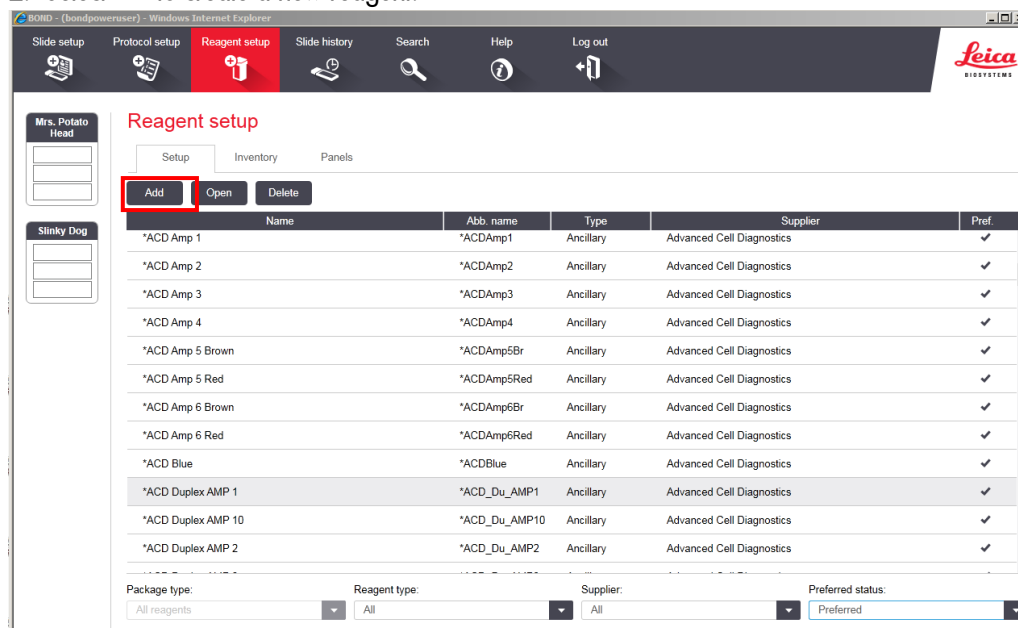
Part 1: Add and Register Reagents for Co-Detection

The Integrated Co-Detection Workflow requires the addition of new reagents in the software, including Co-Detection Antibody and 10% NBF. To ensure optimal ISH detection, perform IHC with a concentrated primary antibody diluted in Co-Detection Antibody Diluent. Using Ready-to-Use (RTU) primary antibody in

this workflow may result in suboptimal RNA detection. To use in this Co-Detection protocol, the antibody must be placed in a Leica Open Container. Register the antibody as an ancillary reagent on the Leica BOND RX instrument. To ensure optimal IHC detection, post-primary fixation must be performed. Place the NBF reagent in a 7mL Open Container registered as an ancillary reagent. Keep the container closed when not on the Leica BOND RX instrument.

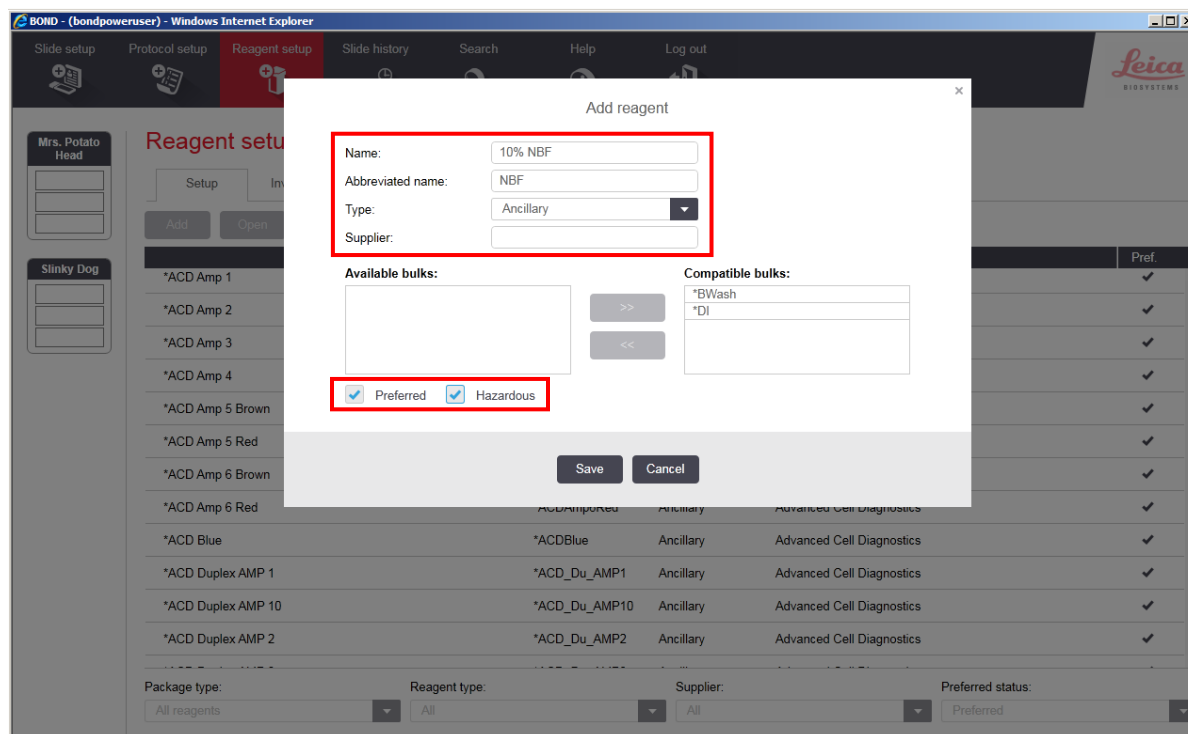
Add new co-detection reagents

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



3. Enter the name **10% NBF** in the Name text box.
4. Enter **NBF** in the Abbreviated name text box.

5. Select **Ancillary** in the Type drop-down menu.



Note: You may leave the Supplier text box empty.

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

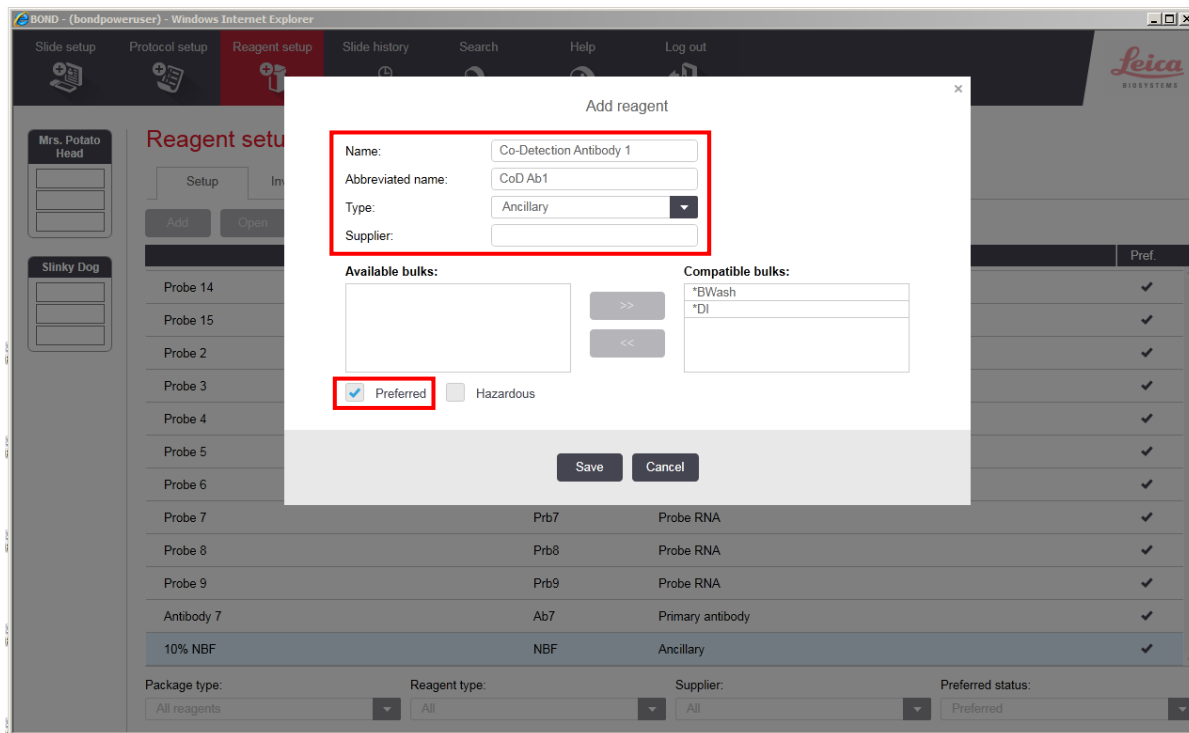
IMPORTANT! For waste disposal, follow local guidelines.

7. Select Add to create an additional new reagent.
8. To create a generic Co-Detection antibody reagent, enter **Co-Detection Antibody 1** in the Name text box.
9. Enter **CoD Ab1** in the Abbreviated name text box.
10. Select **Ancillary** in the Type drop-down menu.

Note: You may leave the Supplier text box empty.

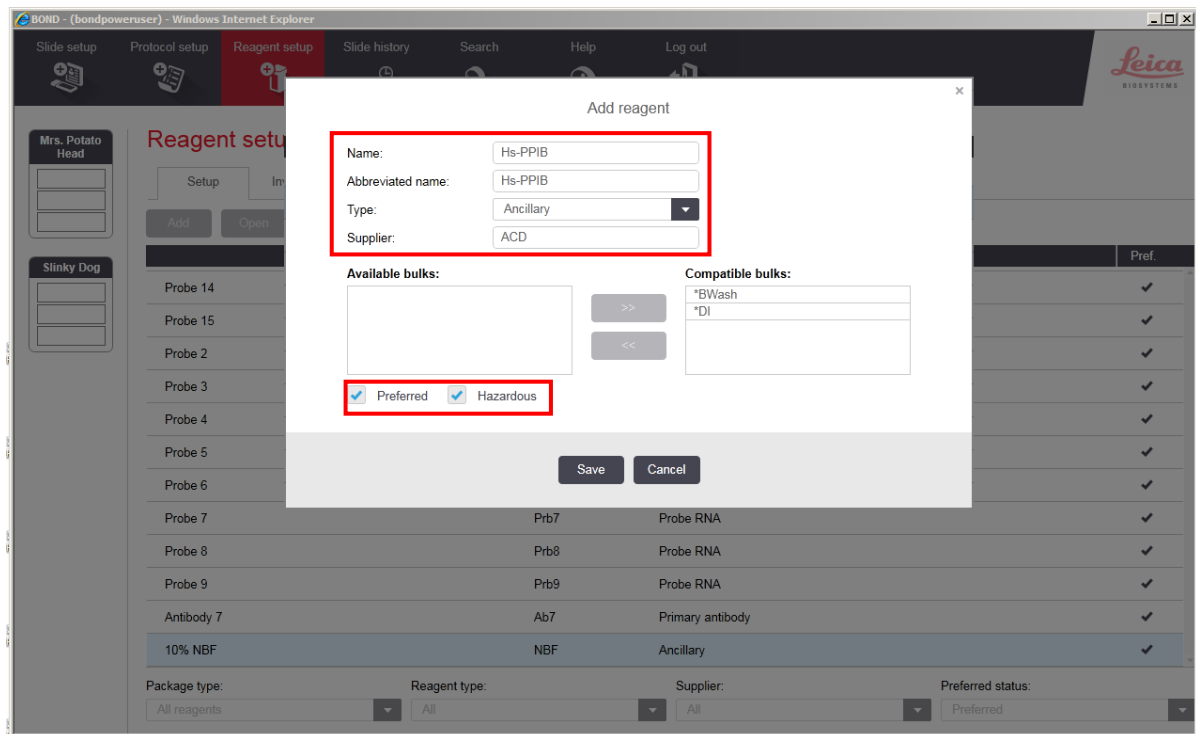
11. Select **Preferred**, then **Save**.
12. Register additional Co-Detection antibody reagents as needed.

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**).



13. To register a probe for use with the co-detection workflow, select **Add** to create an additional new reagent.
14. Enter a probe name under **Name** and enter abbreviated name.
15. Select **Ancillary** as Reagent Type.

IMPORTANT! For probes, you must select **Ancillary** as the reagent type. Make sure that the name of the probe is unique and does not match the name of any existing reagent registered as a **Probe**.



16. Select **Preferred** and **Hazardous**, then **Save**.
17. Register additional probes as needed.

Part 2: Create Co-Detection Software Protocols

This section provides instructions for creating two staining protocols for ICW. Use the protocols together in a sequential dual stain procedure on the Leica BOND RX System. Part A applies primary antibody followed by post-primary fixation, RNAscope® Pretreatment, and ISH staining. Part B applies and detects secondary antibody. If you choose Green IHC, perform the chromogen and counterstaining steps offline. If you choose Brown IHC, these steps are fully automated.

Create Part A: primary antibody and ISH detection

Note: The figures and table from the following procedure display the steps for the Leica RNAscope® protocol. You can use the same procedure to modify the Leica BaseScope™ protocol as well.

1. In the Protocol setup screen, select **Staining** under the Protocol group menu.
2. Highlight the ***ACD 2.5 Red Rev B** protocol. Select **Copy**.
3. Change the protocol name for your first probe to **ACD 2.5 RNA-Protein Co-Detection Part A** in the Name text box, **RP-CoD A** in the Abbreviated name text box, and **ACD 2.5 Red RNAscope-Protein Co-Detection Part A** in the Description text box.
4. For Staining method, select **First**.

New protocol properties

Name:

Abbreviated name:

Description:

Staining method: Single First Second Preferred

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

Preferred detection system:

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
1		*ACD 2.5 P1	Advanced Cell Diagnostics	<input checked="" type="checkbox"/>		0:00	150 µL
2		*ACD 2.5 P1	Advanced Cell Diagnostics	<input checked="" type="checkbox"/>		0:00	150 µL
3		*ACD 2.5 P1	Advanced Cell Diagnostics	<input type="checkbox"/>	42	120:00	150 µL
15		*ACD Amp 1	Advanced Cell Diagnostics	<input type="checkbox"/>	42	1:00	150 µL
16		*ACD Amp 1	Advanced Cell Diagnostics	<input type="checkbox"/>	42	30:00	150 µL
25		*LS Rinse	Advanced Cell Diagnostics	<input checked="" type="checkbox"/>		5:00	150 µL
26		*LS Rinse	Advanced Cell Diagnostics	<input checked="" type="checkbox"/>		5:00	150 µL
31		*ACD Amp 2	Advanced Cell Diagnostics	<input type="checkbox"/>	42	1:00	150 µL
32		*ACD Amp 2	Advanced Cell Diagnostics	<input type="checkbox"/>	42	15:00	150 µL

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

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

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

Note: BaseScope™ uses an alternative protease reagent for ICW. For steps 17–18, select the appropriate protease: ***ACD Enzyme** for RNAscope® or ***Enzyme 1** for BaseScope™.

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

Step No.	Reagent	Step Type	Incubation Time	Temperature
16	*Bond Wash Solution	Wash	0 MIN	Ambient
17	*ACD Enzyme /*Enzyme 1	Reagent	0 MIN	40°C
18	*ACD Enzyme /*Enzyme 1	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

Note: Add steps 1–35 at the start of the protocol. After adding these steps, previous protocol steps should begin at step 36.

7. Delete two ***Bond Wash Solution** 1 MIN steps directly before the *LS Rinse steps that precede ***Mixed Red Refine**. For RNAscope®, these are steps 120–121. For BaseScope™, these are steps 152–153.

Edit protocol properties

Name:

Abbreviated name:

Description:

Staining method: Single First Second Preferred

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

Preferred detection system:

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
119	✓	*Bond Wash Solution	Leica Microsystems	✓		1:00	150 µL
120	✓	*Bond Wash Solution	Leica Microsystems	✓		1:00	150 µL
121	✓	*Bond Wash Solution	Leica Microsystems	✓		1:00	150 µL
122		*LS Rinse	Advanced Cell Diagnostics	✓		5:00	150 µL
123		*LS Rinse	Advanced Cell Diagnostics	✓		5:00	150 µL
124		*Mixed Red Refine	Leica Microsystems	✓		1:00	150 µL
125		*Mixed Red Refine	Leica Microsystems	✓		10:00	150 µL
126	✓	*Deionized Water		✓		0:00	150 µL

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

8. The Wash step after ***Mixed Red Refine** is the final step of the Part A protocol. For RNAscope[®], delete all steps following step 124 ***Deionized Water**. For BaseScope[™], delete all steps following step 156 ***Deionized Water**.

Edit protocol properties

Name:

Abbreviated name:

Description:

Staining method: Single First Second Preferred

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

Preferred detection system:

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
117	✓	*Bond Wash Solution	Leica Microsystems	✓		1:00	150 µL
118	✓	*Bond Wash Solution	Leica Microsystems	✓		1:00	150 µL
119	✓	*Bond Wash Solution	Leica Microsystems	✓		1:00	150 µL
120		*LS Rinse	Advanced Cell Diagnostics	✓		5:00	150 µL
121		*LS Rinse	Advanced Cell Diagnostics	✓		5:00	150 µL
122		*Mixed Red Refine	Leica Microsystems	✓		1:00	150 µL
123		*Mixed Red Refine	Leica Microsystems	✓		10:00	150 µL
124	✓	*Deionized Water		✓		0:00	150 µL

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

9. Change the Wash step after ***Mixed Red Refine** from ***Deionized Water** to ***Bond Wash Solution**.

ACD 2.5 RNA-Protein Co-Detection Part A

RP-CoD A

ACD 2.5 Red RNAscope-Protein Co-Detection Part A

Single First Second Preferred

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

Preferred detection system: Bond Polymer Refine Red Detection

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
117	<input checked="" type="checkbox"/>	*Bond Wash Solution	Leica Microsystems	<input checked="" type="checkbox"/>		1:00	150 µL
118	<input checked="" type="checkbox"/>	*Bond Wash Solution	Leica Microsystems	<input checked="" type="checkbox"/>		1:00	150 µL
119	<input checked="" type="checkbox"/>	*Bond Wash Solution	Leica Microsystems	<input checked="" type="checkbox"/>		1:00	150 µL
120	<input type="checkbox"/>	*LS Rinse	Advanced Cell Diagnostics	<input checked="" type="checkbox"/>		5:00	150 µL
121	<input type="checkbox"/>	*LS Rinse	Advanced Cell Diagnostics	<input checked="" type="checkbox"/>		5:00	150 µL
122	<input type="checkbox"/>	*Mixed Red Refine	Leica Microsystems	<input checked="" type="checkbox"/>		1:00	150 µL
123	<input type="checkbox"/>	*Mixed Red Refine	Leica Microsystems	<input checked="" type="checkbox"/>		10:00	150 µL
124	<input checked="" type="checkbox"/>	*Deionized Water		<input checked="" type="checkbox"/>		0:00	150 µL

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

Save Cancel

10. Select **Save**.

ACD 2.5 RNA-Protein Co-Detection Part A

RP-CoD A

ACD 2.5 Red RNAscope-Protein Co-Detection Part A

Single First Second Preferred

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

Preferred detection system: Bond Polymer Refine Red Detection

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
117	<input checked="" type="checkbox"/>	*Bond Wash Solution	Leica Microsystems	<input checked="" type="checkbox"/>		1:00	150 µL
118	<input checked="" type="checkbox"/>	*Bond Wash Solution	Leica Microsystems	<input checked="" type="checkbox"/>		1:00	150 µL
119	<input checked="" type="checkbox"/>	*Bond Wash Solution	Leica Microsystems	<input checked="" type="checkbox"/>		1:00	150 µL
120	<input type="checkbox"/>	*LS Rinse	Advanced Cell Diagnostics	<input checked="" type="checkbox"/>		5:00	150 µL
121	<input type="checkbox"/>	*LS Rinse	Advanced Cell Diagnostics	<input checked="" type="checkbox"/>		5:00	150 µL
122	<input type="checkbox"/>	*Mixed Red Refine	Leica Microsystems	<input checked="" type="checkbox"/>		1:00	150 µL
123	<input type="checkbox"/>	*Mixed Red Refine	Leica Microsystems	<input checked="" type="checkbox"/>		10:00	150 µL
124	<input checked="" type="checkbox"/>	*Bond Wash Solution	Leica Microsystems	<input checked="" type="checkbox"/>		0:00	150 µL

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

Save Cancel

11. Click **Yes** to proceed.

Note: Additional Part A protocols must be created for each new probe/primary antibody combination.

12. To create a protocol for each additional probe and primary antibody, follow these steps:

- Highlight the **ACD 2.5 RNA-Protein Co-Detection Part A** protocol. Select **Copy**.
- Change the protocol name by adding your antibody and probe name in the Name text box (for

example, ACD 2.5 RNA-Protein Co-Detection Part A – CD3 Hs-PPIB). Change the Abbreviated name text and Description text box accordingly.

- c. For Staining method, select **First**.

New protocol properties

Name: ACD 2.5 RNA-Protein Co-Detection P1A - CD3 HsPPIB

Abbreviated name: RP-CoDA2

Description: ACD 2.5 Red RNA-Protein Co-Detection P1A - CD3 HsPPIB

Staining method: Single First Second Preferred

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

Preferred detection system: Bond Polymer Refine Red Detection

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

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

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

- d. If using 5.2 software, select the appropriate tab for your instrument (BOND RXm or BOND RX).

- e. Select ***Co-Detection Antibody 1**. Change the Reagent to your registered ancillary antibody (for example, **Co-Detection CD3**).

New protocol properties

Name: ACD 2.5 RNA-Protein Co-Detection P1A - CD3 HsPPIB

Abbreviated name: RP-CoDA2

Description: ACD 2.5 Red RNA-Protein Co-Detection P1A - CD3 HsPPIB

Staining method: Single First Second Preferred

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

Preferred detection system: Bond Polymer Refine Red Detection

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		*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
en		*ACD 2.5 P1	Advanced Cell Diagnostics		42	1:00	150 µL

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

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

- f. Select ***ACD 2.5 P1**. Change the **Reagent** to your registered ancillary probe (for example, **Hs-PPIB**).

Edit protocol properties

Name:
 Abbreviated name:
 Description:
 Staining method: Single First Second Preferred

Import protocol Protocol type: ISH detection

Preferred detection system:

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
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		Hs-PPIB	ACD	✓		0:00	150 µL
37		Hs-PPIB	ACD	✓		0:00	150 µL
38		Hs-PPIB	ACD		42	120:00	150 µL

Show wash steps Insert wash | Insert reagent | Delete step

IMPORTANT! Make sure to change all three probe steps.

g. Select **Save**.

Create Part B: semi-automated green or fully automated brown IHC detection

Note: You can use the Part B protocol for both RNAscope® and BaseScope™ Co-Detection

1. In the Protocol setup screen, select **IHC Staining** under the Protocol type menu.
2. To create an IHC protocol, highlight ***IHC Protocol F** in the protocol set up page and select **Copy**.

BOND - (bondpoweruser) - Windows Internet Explorer

Side setup Protocol setup Reagent setup Slide history Search Help Log out

Leica
BIOSYSTEMS

Protocol setup

Protocol name	Protocol type	Description	Modified by	Mod. date	Pref.
*AccuCyte CTC IF Protocol	IHC staining	AccuCyte CTC IF protocol	Leica	2/21/2020	✓
*AccuCyte CTC IHC Protocol	IHC staining	AccuCyte CTC IHC protocol	Leica	2/21/2020	✓
*IF Protocol	IHC staining	IF protocol	Leica	2/21/2020	✓
*IHC Open Dispense Template	IHC staining	IHC template with Open Ancillary and Chromogen...	Leica	2/21/2020	✓
*IHC Protocol F	IHC staining	Bond Polymer Refine IHC protocol	Leica	2/21/2020	✓
*IHC Protocol F RX 37M	IHC staining	IHC Protocol F with marker step at 37C	Leica	2/21/2020	✓
*IHC Protocol F RX 40M	IHC staining	IHC Protocol F with marker step at 40C	Leica	2/21/2020	✓
*IHC Protocol J	IHC staining	Bond Polymer Refine Red IHC protocol	Leica	2/21/2020	✓
*IHC Protocol J RX 37M	IHC staining	IHC Protocol J with marker step at 37C	Leica	2/21/2020	✓
*IHC Protocol J RX 40M	IHC staining	IHC Protocol J with marker step at 40C	Leica	2/21/2020	✓
*IHC Protocol K	IHC staining	ChromoPlex 1 Dual IHC protocol	Leica	2/21/2020	✓
*IHC Protocol K - 50 Test	IHC staining	ChromoPlex 1 Dual IHC protocol	Leica	2/21/2020	✓
*IHC Protocol N	IHC staining	IHC Protocol using 1/60 mixed chromogen	Leica	3/10/2020	✓
*IHC Protocol S	IHC staining	IHC HRP FLEX Protocol with Blue Chromogen	Leica	3/10/2020	✓
*IHC Protocol T	IHC staining	IHC HRP FLEX Protocol with Green Chromogen	Leica	3/10/2020	✓

Protocol group: Protocol type: Staining status: Protocol origin: Preferred status:

3. Change the protocol name to **ACD 2.5 RNA-Protein Co-Detection Part B** in the Name text box, **RP-CoD B** in the Abbreviated name text box, and **ACD 2.5 Red RNAscope-Protein Co-Detection Part B** in the Description text box.

4. For Staining Method, select **Second**.

New protocol properties

Name: ACD 2.5 RNA-Protein Co-Detection Part B
 Abbreviated name: RP-CoD B
 Description: Bond Polymer Refine IHC protocol
 Staining method: Single First Second Preferred

BOND RX Import protocol Protocol type: IHC staining

Preferred detection system: Bond Polymer Refine Detection

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
1		*Peroxide Block	Leica Microsystems	✓		5.00	150 µL
5		*MARKER	Leica Microsystems	✓		15.00	150 µL
9		*Post Primary	Leica Microsystems	✓		8.00	150 µL
13		*Polymer	Leica Microsystems	✓		8.00	150 µL
17		*Mixed DAB Refine	Leica Microsystems	✓		0.00	150 µL
18		*Mixed DAB Refine	Leica Microsystems	✓		10.00	150 µL
22		*Hematoxylin	Leica Microsystems	✓		5.00	150 µL

Show wash steps Insert wash | Insert reagent | Delete step

Save Cancel

5. If using 5.2 software, select the appropriate tab for your instrument (BOND RXm or BOND RX).

6. Select **Show wash steps**.

New protocol properties

Name: ACD 2.5 RNA-Protein Co-Detection Part B
 Abbreviated name: RP-CoD B
 Description: Bond Polymer Refine IHC protocol
 Staining method: Single First Second Preferred

BOND RX Import protocol Protocol type: IHC staining

Preferred detection system: Bond Polymer Refine Detection

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
1		*Peroxide Block	Leica Microsystems	✓		5.00	150 µL
2	✓	*Bond Wash Solution	Leica Microsystems	✓		0.00	150 µL
3	✓	*Bond Wash Solution	Leica Microsystems	✓		0.00	Open
4	✓	*Bond Wash Solution	Leica Microsystems	✓		0.00	150 µL
5		*MARKER	Leica Microsystems	✓		15.00	150 µL
6	✓	*Bond Wash Solution	Leica Microsystems	✓		0.00	150 µL
7	✓	*Bond Wash Solution	Leica Microsystems	✓		0.00	150 µL
8	✓	*Bond Wash Solution	Leica Microsystems	✓		0.00	150 µL

Show wash steps Insert wash | Insert reagent | Delete step

Save Cancel

7. Set up semi-automated green or fully automated brown IHC detection by choosing one of the following procedures:

a. For semi-automated green IHC detection:

- i. Modify the protocol according to the following table. Verify that the final protocol is 11 steps.

Note: Green chromogen and counterstaining are performed offline.

- ii. (Optional) Change the incubation time for ***Post Primary** and ***Polymer** to **16 MIN**.

Step No.	Reagent	Step Type	Incubation Time	Temperature
1	*Peroxide Block	Reagent	5 MIN	Ambient
2	*Bond Wash Solution	Wash	0 MIN	Ambient
3	*Bond Wash Solution	Wash	0 MIN	Ambient
4	*Bond Wash Solution	Wash	0 MIN	Ambient
5	*Post Primary	Reagent	8 MIN /16 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 /16 MIN	Ambient
10	*Bond Wash Solution	Wash	2 MIN	Ambient
11	*Bond Wash Solution	Wash	2 MIN	Ambient

iii. Select **Save**.

b. For fully automated brown IHC detection:

i. Modify the protocol according to the following table. Verify that the protocol is 21 steps.

Step No.	Reagent	Step Type	Incubation Time	Temperature
1	*Peroxide Block	Reagent	5 MIN	Ambient
2	*Bond Wash Solution	Wash	0 MIN	Ambient
3	*Bond Wash Solution	Wash	0 MIN	Ambient
4	*Bond Wash Solution	Wash	0 MIN	Ambient
5	*Post Primary	Reagent	8 MIN / 16 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 / 16 MIN	Ambient
10	*Bond Wash Solution	Wash	2 MIN	Ambient
11	*Bond Wash Solution	Wash	2 MIN	Ambient
12	*Deionized Water	Wash	0 MIN	Ambient
13	*Mixed Refine DAB	Wash	0 MIN	Ambient
14	*Mixed Refine DAB	Wash	10 MIN	Ambient
15	*Deionized Water	Wash	0 MIN	Ambient
16	*Deionized Water	Wash	0 MIN	Ambient
17	*Deionized Water	Wash	0 MIN	Ambient
18	*Hematoxylin	Reagent	5 MIN	Ambient
19	*Deionized Water	Wash	0 MIN	Ambient
20	*Bond Wash Solution	Wash	0 MIN	Ambient
21	*Deionized Water	Wash	0 MIN	Ambient

ii. Select **Save**.

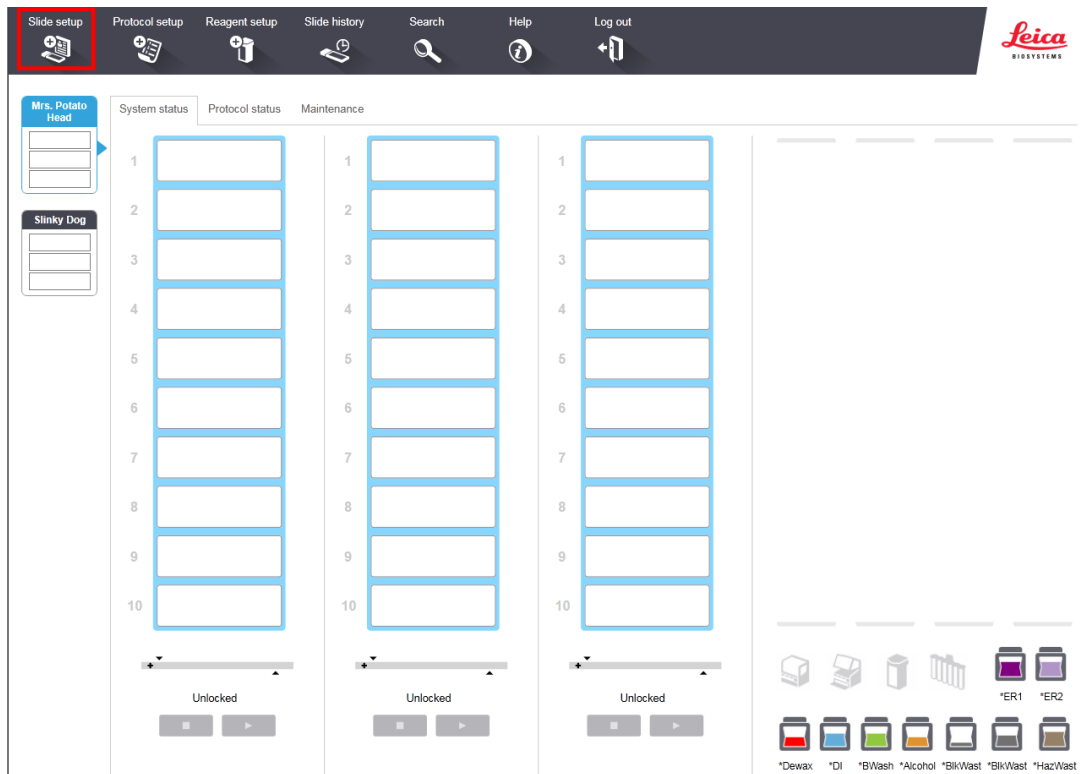
Create an HIER protocol for use with Co-Detection protocols

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.

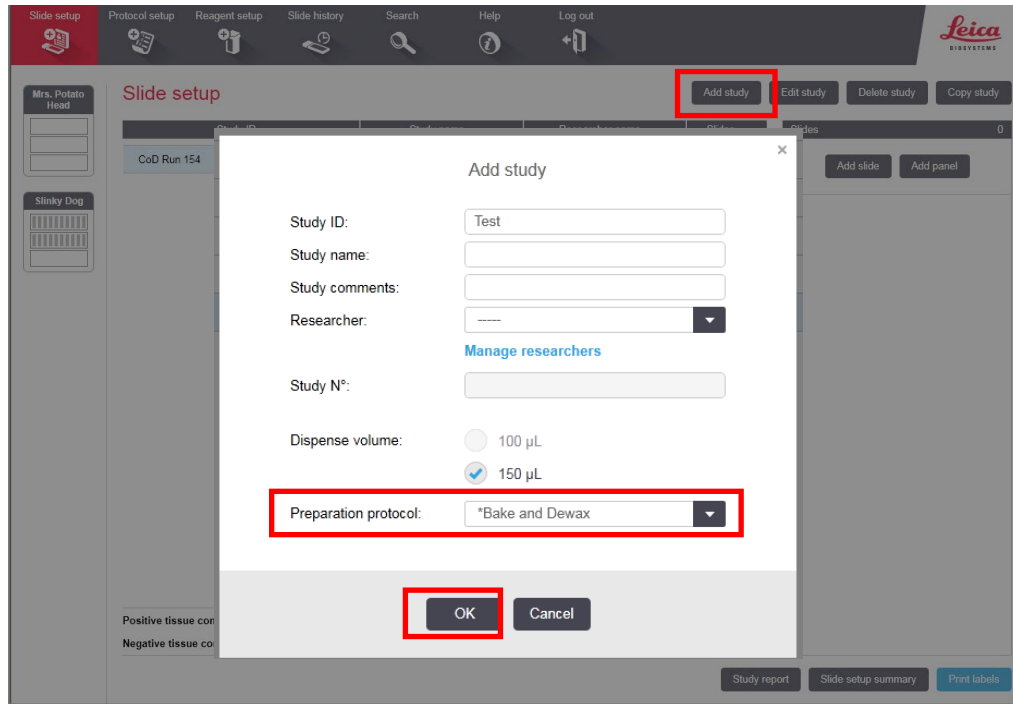
Part 3: Set up a Study for Co-Detection

Build a study

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



2. Select **Add study** and enter a name in the Study ID field (keep the Dispense volume at **150 µL** as shown).
3. For FFPE tissues, select ***Bake and Dewax** as the Preparation protocol (leave blank for other tissue types).
4. Select **OK**.



Add a slide for ICW in FFPE samples

IMPORTANT! We recommend using an extended heat-induced epitope retrieval (HIER) incubation for optimal RNA and protein co-detection. Before continuing, create the **ACD HIER 30 min with ER2 (95)** protocol in **Appendix A**.

Note: To set up slides for fresh frozen samples, refer to **Appendix B**. To set up slides for fixed frozen samples, refer to **Appendix C**.

1. Select **Add slide**.
2. Enter the probe name and primary antibody under the Comments field.

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

The screenshot shows the 'Add slide' form for 'Hs-PPIB / CD3'. On the left, there is a sidebar with study information: Study ID: test, Researcher: ---, Slide ID: ---, Study N°: 8, Study comments: ---, Date created: 3/2/2020 1:01:39 PM. The main form has sections for 'Tissue type' (Test tissue selected), 'Dispense volume' (150 µL selected), 'Staining mode' (Single and Routine dropdowns, with 'Sequential DS' selected and highlighted in a red box), 'Marker' (IHC and ISH radio buttons), and 'Preparation' (*Bake and Dewax dropdown).

4. Under the **First** tab, select **ISH**

5. Select **Mock Probe** as Marker.

6. Under Protocols:

- For each probe, select a protocol from the Staining drop down menu. Make sure that each probe/primary antibody combination is associated with a different Part A protocol (for example, **ACD 2.5 RNA-Protein Co-Detection Part A – Hs-PPIB CD3**).
- For HIER protocol, select **ACD HIER 30 min with ER2 (95)** as the HIER protocol.
- Select ***---** for Enzyme.

Note: The Part A protocol already includes a protease step. Additional Enzyme pretreatment can negatively impact IHC detection.

- Select ***DEFAULT*** for Probe Application and Probe Removal.
- Select ***---** for Denaturation and **ACD 1 Min Hybridization** for Hybridization.

The screenshot shows the 'Add slide' form with the 'Protocols' section expanded. A red box highlights the following dropdown menus: Staining (ACD 2.5 RNA-Protein 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), and Probe Removal (*DEFAULT*). The 'Staining mode' is now 'Sequential DS' and 'Process' is 'ISH'.

7. Under the **Second** tab, select IHC.
8. Select ***Negative** as Marker.

9. Under Protocols:
 - f. Select **ACD 2.5 RNA-Protein Co-Detection Part B** from the Staining drop down menu.
 - g. For HIER, select ***---**.
 - h. For Enzyme, select ***---**.
10. Select **Add Slide**
11. Repeat steps 1–10 for each slide.

Complete the study

1. After adding slides to the study, select **Close** to return to the Slide setup screen.
 2. Select **Print labels** to print barcodes and attach to the slides.
 3. Place slides into the Leica BOND Rx Slide Staining Assemblies (SSAs) and carefully place Leica Covertiles on each slide.
 4. Place the SSA in the Leica BOND RX, and press the button to load the tray onto the machine.
- Note:** If performing the RNAscope® assay, you can run up to three SSAs simultaneously using ICW. For the BaseScope™ assay, run up to two SSAs simultaneously.
5. Once the slides have been scanned, select the **PLAY** (triangular) button on the screen located under the start tray to start the run. Alternatively, right-click on the scanned label images and select **Delayed Start** to start the run at a future time.

Note: If using **Delayed Start**, set the staining procedure to begin within six hours of loading reagents.

Part 4: Detect green IHC staining off the instrument for RED

Note: If you are performing Brown IHC detection, it is already included in the automated protocol. Proceed to *Dry and Mount the Samples*.

Prepare reagents and equipment

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

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

Detect Green staining off the instrument

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

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

2. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Agitate slides by moving the slide rack up and down in the staining dish.
3. Repeat Step 2 with fresh 1X Wash Buffer.
4. Briefly spin down the contents of the Green B tube to be sure content is at the bottom of the tube before opening the cap.
5. Prepare 200 μ L of GREEN working solution per slide using a 1:50 ratio of Green B to Green A. Mix well.

IMPORTANT! Use the GREEN solution within **5 MIN**. Do not expose to direct sunlight or UV light.

6. Take each slide one at a time from the Tissue-Tek[®] Slide Rack and tap and/or flick to remove the excess liquid.
7. Pipette ~200 μ L GREEN solution onto each tissue section. Ensure sections are covered.
8. Incubate the slides for **15 –30 MIN** at **RT** until the desired level of signal intensity is achieved.
9. To remove the GREEN working solution from the slides, tilt each slide one at a time over a waste container and tap the corner on the edge of the container. Immediately insert the slide into a Tissue-Tek[®] Slide Rack submerged in a Tissue-Tek[®] Staining Dish filled with distilled water.
10. Quickly rinse the slides with fresh distilled water for less than 30 seconds.

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

Counterstain the slides

1. Move the Tissue-Tek[®] Slide Rack into the staining dish containing 50% Hematoxylin I staining solution for **30 SEC** at **RT**. Tissue should turn purple.

IMPORTANT! Proceed quickly to the next step. GREEN substrate can fade if in Hematoxylin for longer than 30 seconds.

2. Immediately transfer the slide rack into a staining dish filled with tap water. Do not let the slides remain in the water for more than 30 seconds.
3. Repeat Step 2 once or twice.

Dry and mount the samples

1. Remove the slide rack from the staining dish and dry slides in a **60°C** dry oven for **30 MIN**.

IMPORTANT! GREEN substrate is alcohol sensitive. Do not dehydrate the slides in alcohol.

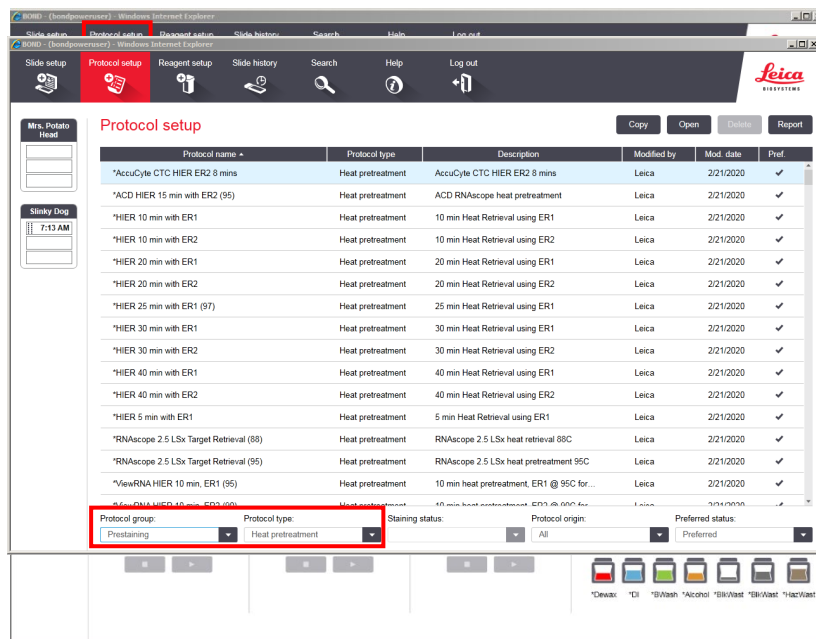
2. Cool the slides for **5 MIN** at **RT**.
3. Briefly dip one slide into fresh pure xylene and immediately place 1–2 drops of VectaMount[®] Mounting Medium on the slide before the xylene dries.
4. Carefully place a 24 mm x 50 mm coverslip over the tissue section. Avoid trapping air bubbles.
5. Repeat steps 2 and 3 for each slide.
6. Air dry the slides for **5 MIN**.

Appendix A. Creating a new HIER Protocol

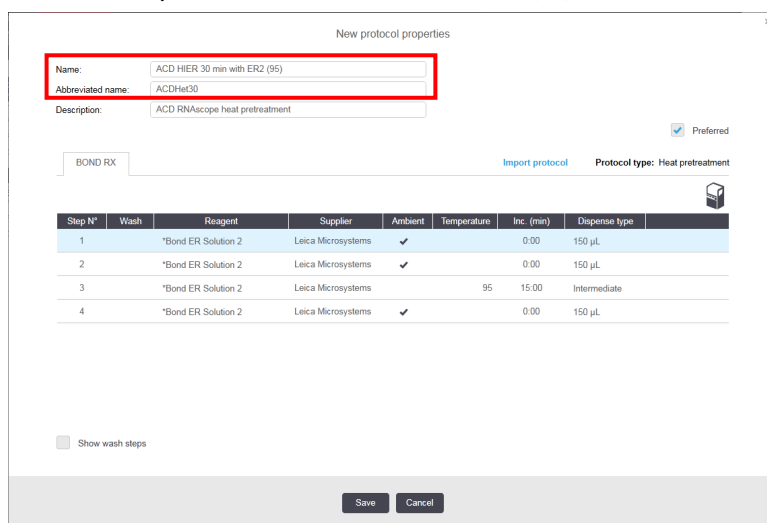
For optimal RNA and protein detection, we recommend using an extended heat-induced epitope retrieval (HIER) step. Follow the steps to edit the epitope retrieval procedure in the software.

Create a prestaining protocol

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



2. Under the Protocol group menu, select **Prestaining**. Under Protocol type menu, select **Heat Pretreatment**.
3. Highlight the ***ACD HIER 15min with ER2 (95)** protocol. Select **Copy**.
4. Rename the protocol as **ACD HIER 30min with ER2 (95)**. Rename the abbreviated name as **ACDHet30**.



5. Highlight the third ***BOND ER Solution 2** step. Change the incubation time to **30 MIN**.
6. Select **Save**.

Appendix B. LS Chromogenic ICW using Fresh Frozen Samples

Part 1. Prepare Fresh Frozen Samples

Prepare reagents and equipment

1. Remove tissue and trim to fit into cryomolds.
2. Freeze on dry ice or liquid Nitrogen within **5 MIN** of harvest.
3. Embed frozen tissue in cryo-embedding medium and freeze blocks.
4. Store the frozen block in an air-tight container at **-80°C**.

Note: Embedded tissue may be stored for at least three months

5. Equilibrate block to **-20°C** in a cryostat **~1 HR**.
6. Cut 10–20 µm sections and mount **ONLY** onto SuperFrost® Plus slides.
7. Dry slides at **-20°C** for **1 HR**.
8. Store in air-tight slide boxes at **-80°C** until use.

New protocol properties

Name: ACD HIER 30 min with ER2 (95)
 Abbreviated name: ACDHie30
 Description: ACD RNAscope heat pretreatment Preferred

BOND RX [Import protocol](#) Protocol type: Heat pretreatment

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
1		*Bond ER Solution 2	Leica Microsystems	✓		0:00	150 µL
2		*Bond ER Solution 2	Leica Microsystems	✓		0:00	150 µL
3		*Bond ER Solution 2	Leica Microsystems		95	30:00	Intermediate
4		*Bond ER Solution 2	Leica Microsystems	✓		0:00	150 µL

Show wash steps

Save Cancel

Note: Use sectioned tissue within three months.

Prepare the slides

1. Add fresh 10% NBF or 4% PFA to a Tissue Tek® Staining Dish.
2. Remove slides from **-80 °C** and place in a Tissue Tek® Slide Rack.
3. Immediately immerse slides in staining dish containing 10% NBF or 4% PFA. Fix for 90 MIN at ROOM TEMPERATURE (RT).
4. Prepare four Tissue Tek® Staining Dishes with about 200mL of fresh 50% EtOH, 70% EtOH, and 100% EtOH (2x).
5. After 90 min, remove slides from the fixative and immediately place in 50% EtOH for 5 MIN at RT.
6. Place the slides in 70% EtOH for 5 MIN at RT.
7. Place the slides in 100% EtOH for 5 MIN at RT.
8. Repeat with fresh 100% EtOH for 5 MIN at RT.
9. Remove slides from 100% EtOH and allow to air dry for **5 MIN** at **RT** on absorbent paper.

Note: Slides may be stored in 100% EtOH at **-20°C** for up to **1 WEEK**.

Part 2. Run the Assay

Setup a Protocol

1. Refer to Part 1 on page 4 to add and register new reagents.
2. Refer to **Part 2** on page 8 to create co-detection software protocols. If needed, modify the Protease step in the **Part A** protocol according to the following table:

Assay	Protease Reagent	Step No.	Registration Name	Step Type	Incubation Time	Temperature
LS 2.5 RNAscope® Red	Protease IV	17	*Enzyme 1	Reagent	0 MIN	Ambient
	Protease IV	18	*Enzyme 1	Reagent	30 MIN	Ambient
BaseScope™ LS†	Protease IV	17	*Enzyme 1	Reagent	0 MIN	40°C
	Protease IV	18	*Enzyme 1	Reagent	30 MIN	40°C

† The table lists the standard Protease conditions for BaseScope™ LS. We recommend using standard conditions unless your tissue type requires additional time and temperature optimization. If stronger Protease treatment is needed, add an additional 30 minute Enzyme reagent step directly following Step 18.

Setup a study

1. Go to the **Slide setup** screen. Select **Add Study** and add study information.
2. For Preparation, select *---.

IMPORTANT! The ***Frozen Slide Delay** preparation selection is not recommended for fresh frozen samples, as it may result in non-uniform staining.

3. Select Add slide.
4. Enter the probe name and primary antibody under the Comments field.
5. Select Sequential DS from the Staining mode drop down menu.
6. Under the First tab, select ISH. Select Mock Probe as Marker.
7. Under Protocols:
 - a. Select a protocol from the Staining drop down menu for each probe. Make sure that each probe/primary antibody combination is associated with a different Part A protocol (for example, **ACD 2.5 RNA-Protein Co-Detection Part A – Hs-PPIB CD3**).
 - b. For HIER protocol, select *---.
 - c. For Enzyme, Select *---.
 - d. The Part A protocol already includes a protease step. Additional Enzyme pretreatment can negatively impact IHC detection. Select ***DEFAULT*** for Probe Application and Probe Removal.
 - e. Select *--- for Denaturation and **ACD 1 min Hybridization** for Hybridization .
8. Under the **Second** tab, select IHC. Select ***Negative** as Marker.
9. Under Protocols:
 - f. Select **ACD 2.5 RNA-Protein Co-Detection Part B** from the Staining drop down menu.

Note: Part B will remain constant for each Probe/Primary Antibody combination

- g. For HIER and Enzyme, select *---.
10. Select **Add Slide**.

Repeat steps 1–8 for each additional slide. Complete the study

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

3. Place slides into the Leica BOND RX Slide Staining Assemblies (SSAs) and carefully place Leica Covertiles on each slide.
4. Place the SSA in the Leica BOND RX, and press the button to load the tray onto the machine.

Note: If performing the RNAscope® assay, you can run up to three SSAs simultaneously using ICW. For the BaseScope™ assay, run up to two SSAs simultaneously.

5. Once the slides have been scanned, select the **PLAY** (triangular) button on the screen located under the start tray to start the run.

Appendix C. LS Chromogenic ICW using Fixed Frozen Samples

Part 1. Prepare Fixed Frozen Samples

Post fixation

1. Remove slides from -80 °C and place in a Tissue Tek® Slide Rack.
2. Bake slides at 60 °C for 30–60 MIN
3. Immerse slides in staining dish containing 10% NBF or 4% PFA. Fix for **15 MIN** at **4°C**.

Note: Formalin that has been stored for more than six months, exposed to air for more than one week, or used repeatedly can result in suboptimal post-fixation.

4. Prepare four Tissue Tek® Staining Dishes with about 200mL of fresh 50% EtOH, 70% EtOH, and 100% EtOH (2x).
5. After 15min post-fixation, remove slides from the fixative and immediately place in 50% EtOH for 5 MIN at RT.
6. Place the slides in 70% EtOH for 5 MIN at RT.
7. Place the slides in 100% EtOH for 5 MIN at RT.
8. Repeat with fresh 100% EtOH for 5 MIN at RT.
9. Remove slides from 100% EtOH and allow to air dry for **5 MIN** at **RT** on absorbent paper.

Note: You can store slides in 100% EtOH at -20°C for up to one week.

Part 2. Run the Assay

IMPORTANT! This procedure uses the heat-induced epitope retrieval (HIER) protocol **ACD HIER 5 min with ER2 (95)**. Before continuing, make sure you have this protocol on your instrument. For an example of how to create a new HIER protocol, see **Appendix A**.

Setup a protocol

1. Refer to Part 1 on page 4 to add and register new reagents.
2. Refer to Part 2 on page 8 to create co-detection software protocols.

Note: For fixed frozen samples, changes to protease conditions are unnecessary.

Setup a study

1. Go to the **Slide setup** screen. Select **Add Study** and add study information.
2. For Preparation, select *--.

IMPORTANT! The ***Frozen Slide Delay** preparation selection is not recommended for fixed frozen samples, as it can result in non-uniform staining.

3. Select **Add slide**.
4. Enter the probe name and primary antibody under the Comments field.

5. Select **Sequential DS** from the Staining mode drop down menu.
6. Under the **First** tab, select ISH. Select **Mock Probe** as Marker.
7. Under Protocols:
 - a. Select a protocol from the Staining drop down menu for each probe. Make sure that each probe/primary antibody combination is associated with a different Part A protocol (for example, **ACD 2.5 RNA-Protein Co-Detection Part A – Hs-PPIB CD3**).
 - b. For HIER protocol, select **ACD HIER 5 min with ER2 (95)**.
 - c. For Enzyme, Select * - - -.
 - d. The Part A protocol already includes a protease step. Additional Enzyme pretreatment can negatively impact IHC detection. Select ***DEFAULT*** for Probe Application and Probe Removal.
 - e. Select ***---** for Denaturation and **ACD 1 min Hybridization** for Hybridization.
8. Under the **Second** tab, select IHC. Select ***Negative** for Marker.
9. Under Protocols:
 - a. Select **ACD 2.5 RNA-Protein Co-Detection Part B** from the Staining drop down menu.

Note: Part B will remain constant for each Probe/Primary Antibody combination

 - b. For HIER and Enzyme, select ***---**.
10. Select **Add Slide**.
11. Repeat steps 1–8 for each additional slide.

Complete the study

1. After adding all the slides to the study, select **Close** to return to the Slide setup screen.
 2. Select **Print labels** to print barcodes to attach to the slides.
 3. Place slides into the Leica BOND RX Slide Staining Assemblies (SSAs) and carefully place Leica Covertiles on each slide.
 4. Place the SSA in the Leica BOND RX, and press the button to load the tray onto the machine.
- Note:** If performing the RNAscope® assay, you can run up to three SSAs simultaneously using ICW. For the BaseScope™ assay, run up to two SSAs simultaneously.
5. Once the slides have been scanned, select the **PLAY** (triangular) button on the screen located under the start tray to start the run

Appendix D. ICW Troubleshooting Guide

You may need to use a higher primary antibody concentration for the co-detection workflow than you would normally use for IHC alone. To optimize protein detection, we recommend optimizing the antibody concentration for the co-detection workflow.

The post-primary fixation and pretreatment conditions in this Tech Note provide optimal RNA and protein detection across most tissue samples. If further optimization is required for a specific sample or target of interest, adjust the following parameters:

Reagent	Incubation Temperature	Recommended Incubation Time	Optimization Range
HIER	95°C	30 MIN	15–30 MIN @ 88–95°C
Primary Antibody	Ambient	15 MIN	15–60 MIN
10% NBF	Ambient	30 MIN	15–60 MIN
Protease	40°C*	30 MIN	15–30 MIN†
Post Primary	Ambient	8 MIN	8–16 MIN
Polymer	Ambient	8 MIN	8–16 MIN

* For fresh frozen samples, we recommend incubating at ambient temperature. Higher temperatures can compromise RNA quality.

† Some samples may require stronger protease treatment. For these samples, add an additional Enzyme reagent step directly following step 18.

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