miRNAscope™ RED LS 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 miRNA/ ASO/ siRNA/short RNAs and protein on the Leica BOND RX System. The Integrated Co-Detection Workflow (ICW) combines ACD's miRNAscope LS Reagent Kit RED (Cat. No. 324600) 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 miRNA-Protein Co-Detection Part A and miRNA-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

Prepare and Load Slides onto Leica Bond Rx

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Deparaffinization and HIER

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Mock Probe

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LS Chromogenic Co-Detection Workflow Part A

Primary Antibody Incubation

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Crosslinking

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RNAscope™ Protease

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miRNAscope LS Probe

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miRNAscope Amp1-6

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Red Refine

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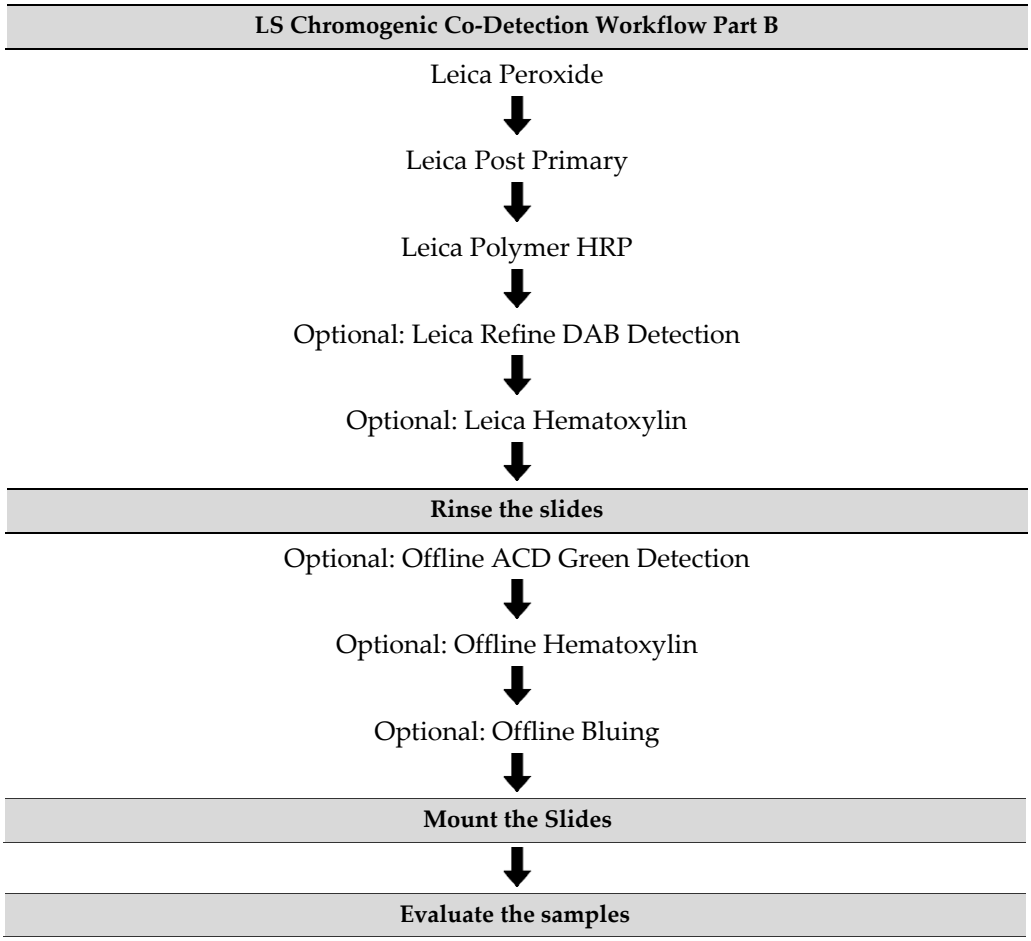


Chromogen Combinations for ICW

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
miRNAscope	Red	Green	miRNAscope 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
miRNAscope	Red	DAB	miRNAscope LS Reagent Kit – RED; Leica BOND Refine Red Detection Kit	Co-Detection Antibody Diluent Leica BOND Refine Detection Kit

Materials Required

ACD LS Chromogenic ISH Detection Kits

miRNAscope LS Reagent Kit- RED

The miRNAscope LS Reagent Kit - RED (Cat. No. 324600) provides reagents to stain ~60 standard slides on Leica Biosystems' BOND RX System. The miRNAscope 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)			
☑	**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
	miRNAscope LS AMP 1	21 mL x 1 bottle	2–8°C
	miRNAscope LS AMP 2	21 mL x 1 bottle	2–8°C
	miRNAscope LS AMP 3	21 mL x 1 bottle	2–8°C
	miRNAscope LS AMP 4 – RED	21 mL x 1 bottle	2–8°C
	miRNAscope LS AMP 5 – RED	21 mL x 1 bottle	2–8°C
	miRNAscope 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

RNAscope 2.5 LS Green Accessory Pack (Optional)

For green IHC staining, we recommend the RNAscope 2.5 LS Green Accessory Pack (Cat. No. 322550). Following co-detection, this accessory pack provides reagents to stain ~60 standard slides offline 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	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.

☑	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.

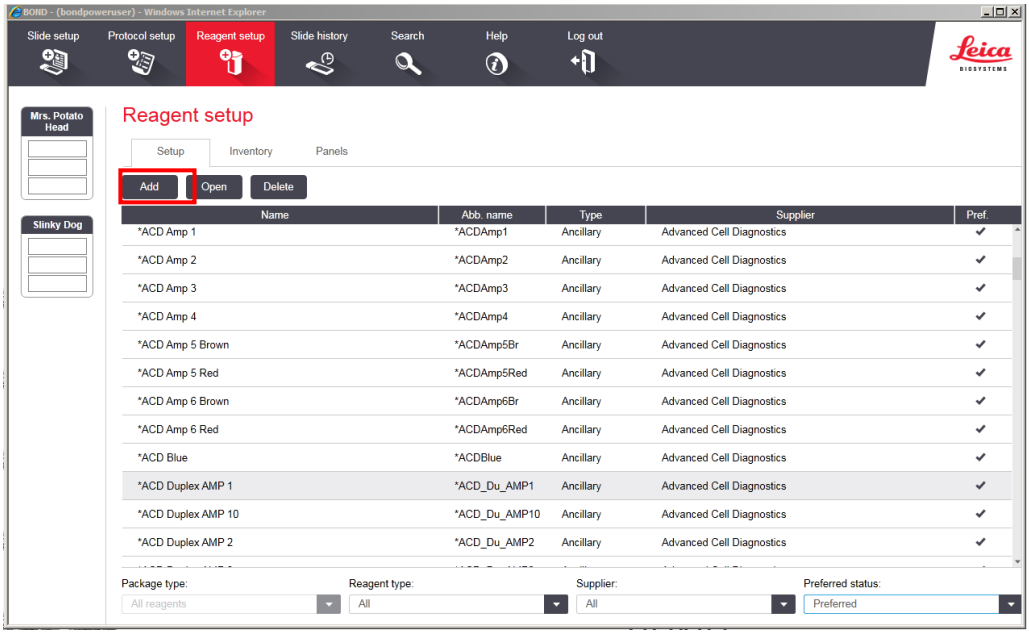
Run the Assay

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 could result in suboptimal RNA detection. You must place the antibody in a Leica Open Container and register the antibody as an ancillary reagent on the Leica BOND RX instrument. To ensure optimal IHC detection in this workflow, crosslink with 10% NBF. Place the NBF reagent in a 7mL Open Container registered as an ancillary reagent, and 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. To add 10% NBF as a new co-detection reagent, do the following steps:

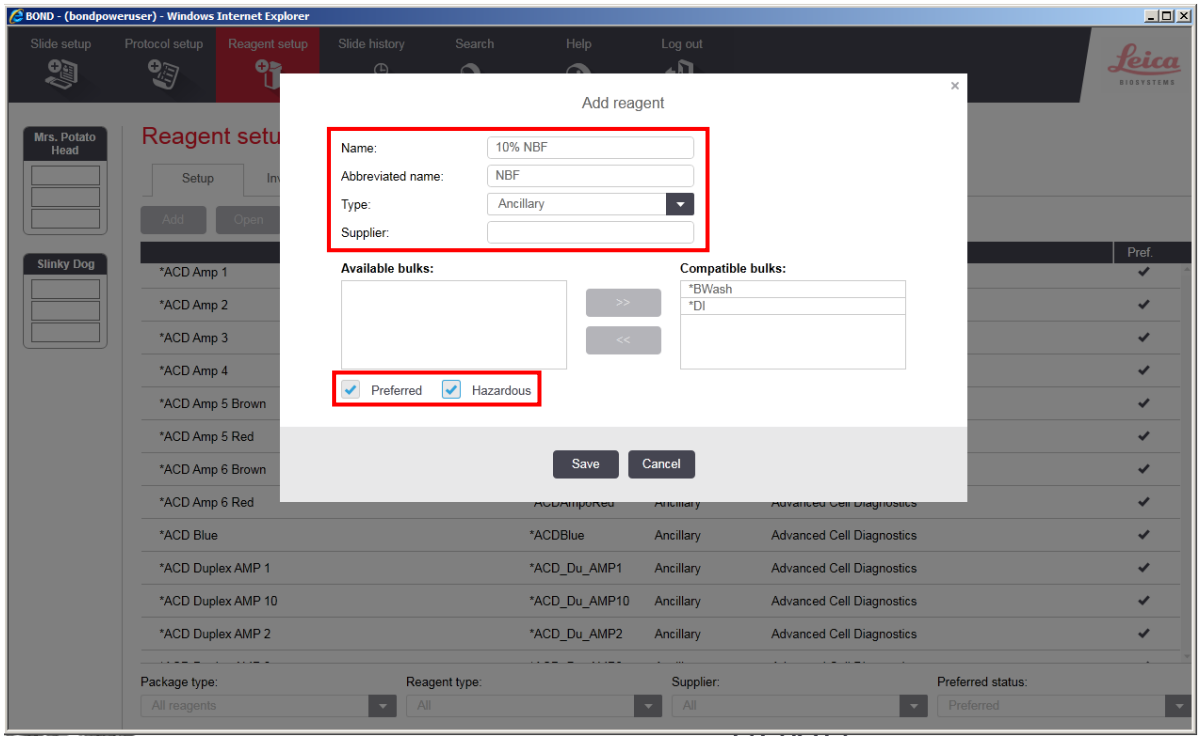


3. Select **Add**.
4. Enter the name **10% NBF** in the Name text box.
5. Enter **NBF** in the Abbreviated name text box.
6. Select **Ancillary** in the Type drop-down menu.

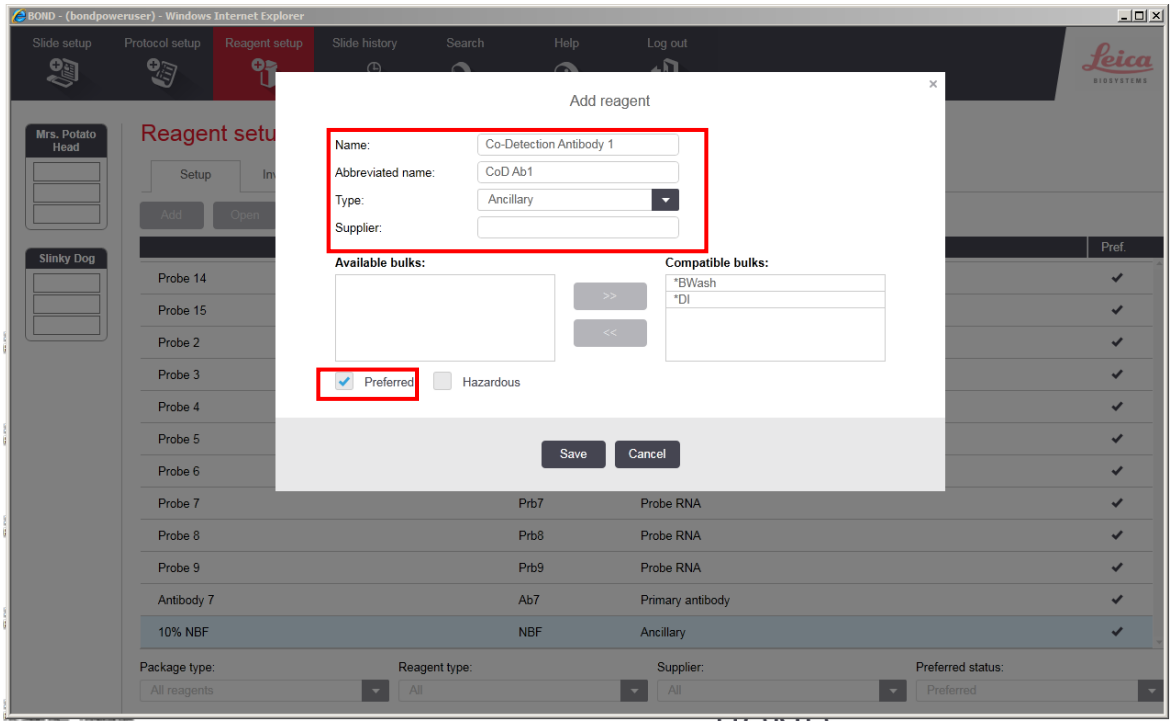
Note: You may leave the Supplier text box empty.

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

IMPORTANT! For waste disposal, follow local guidelines.



8. To create a generic Co-Detection antibody reagent, do the following steps:
9. Select **Add**.
10. Enter **Co-Detection Antibody 1** in the Name text box.
11. Enter **CoD Ab1** in the Abbreviated name text box.



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

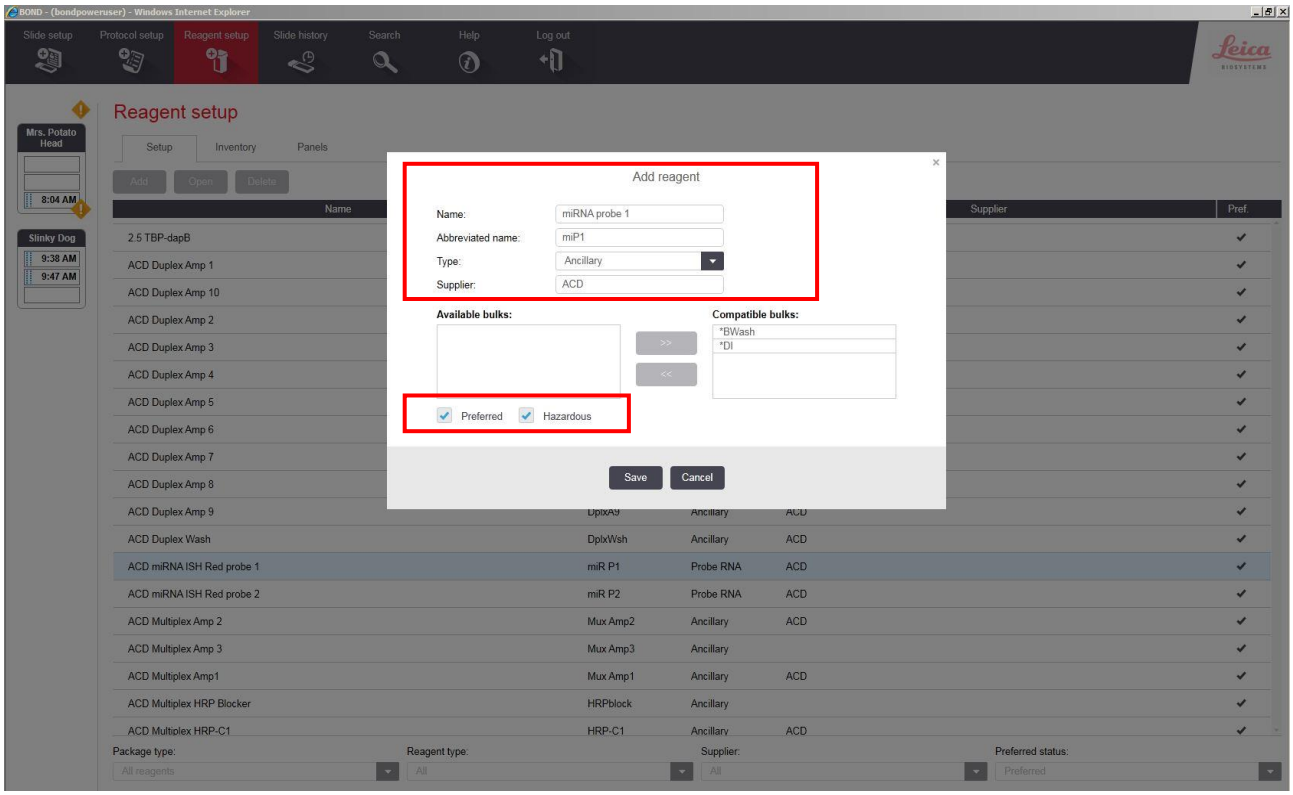
Note: You may leave the Supplier text box empty.

13. Select **Preferred**, then **Save**.
14. 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).

15. To register a probe for use with the ICW workflow, do the following steps:
16. Select **Add**.
17. Enter a probe name under **Name** and enter an abbreviated name.
18. Select **Ancillary** as Reagent Type.
19. Select **Preferred** and **Hazardous**, then **Save**.
20. Register additional probes as needed.

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.

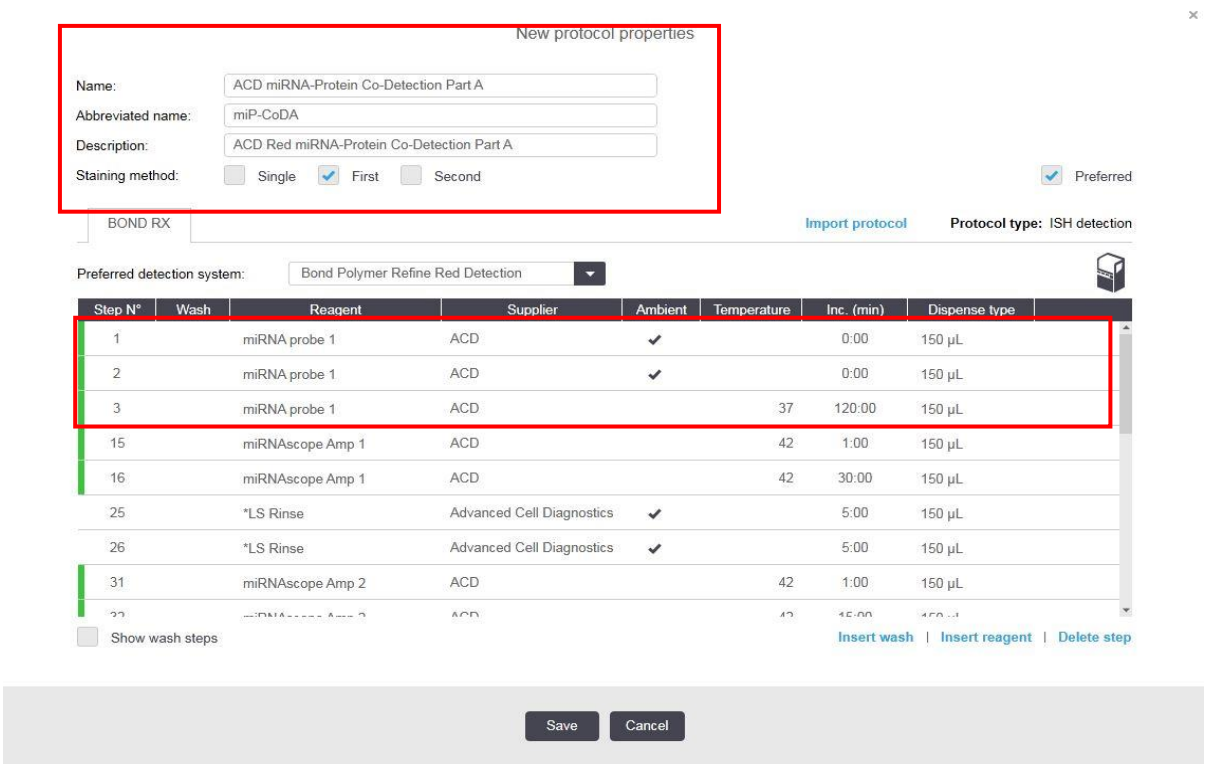


Part 2: Create Co-Detection Software Protocols

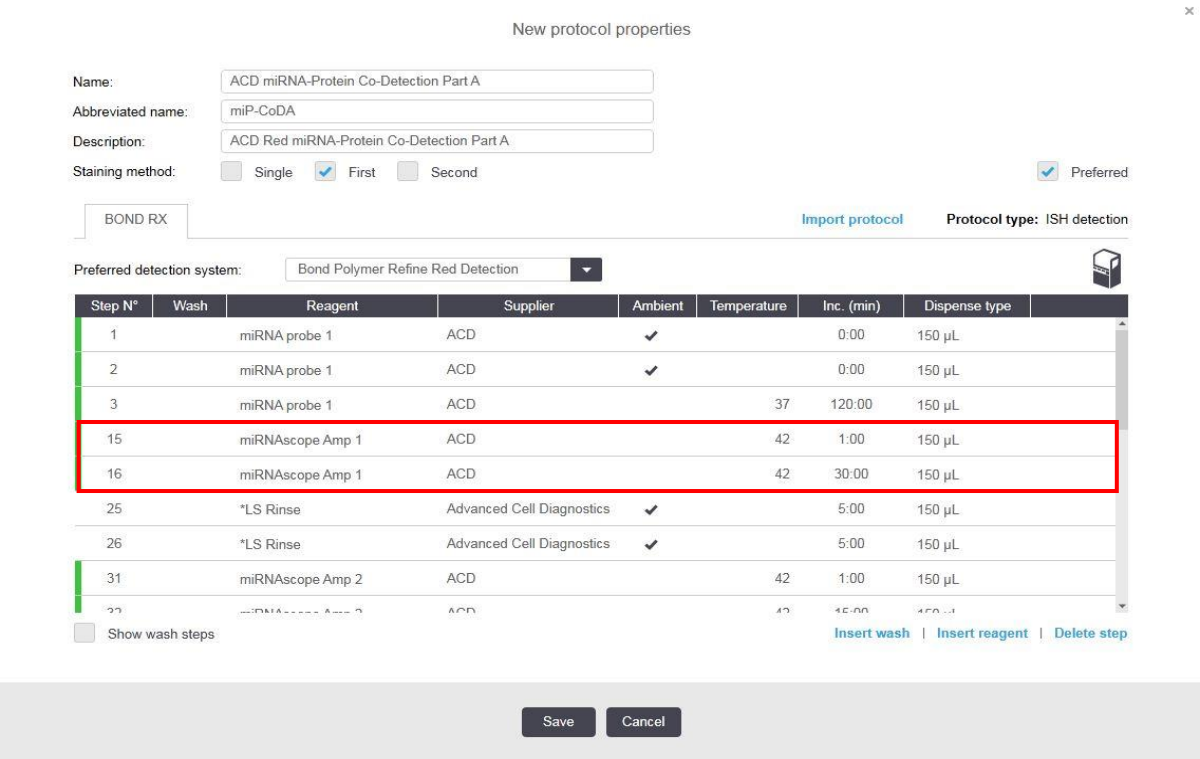
This section provides instructions for creating two staining protocols for ICW. Use the protocols together in a sequential dual stain procedure on the Leica BOND RX System. Part A applies primary antibody followed by crosslinking, RNAscope Pretreatment, and ISH staining. Part B applies and detects secondary 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

1. The figures and table from the following procedure display the steps for the Leica miRNAscope protocol. 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 miRNA-Protein Co-Detection Part A** in the Name text box, **miP-CoDA** in the Abbreviated name text box, and **ACD Red miRNA-Protein Co-Detection Part A** in the Description text box.
4. For Staining method, select **First**.
5. From the reagent drop-down menu select the appropriate miRNA probe on steps 1, 2, and 3.
6. For step 3, change the probe hybridization temperature to 37^{0}C.



7. From the appropriate drop-down menus for the Amp steps, change the reagents *ACD Amp 1, *ACD Amp 2,…, *ACD Amp 6 to miRNAscope Amp 1, miRNAscope Amp 2, …, miRNAscope Amp 6.
8. If using 5.2 software, select the appropriate tab for your instrument (BOND RXm or BOND RX).



9. Select **Show wash** steps, and add steps 1–35 from the following table before the probe step. Once additional steps have been added, verify that probe application miRNA probe 1 begins at step 36.

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

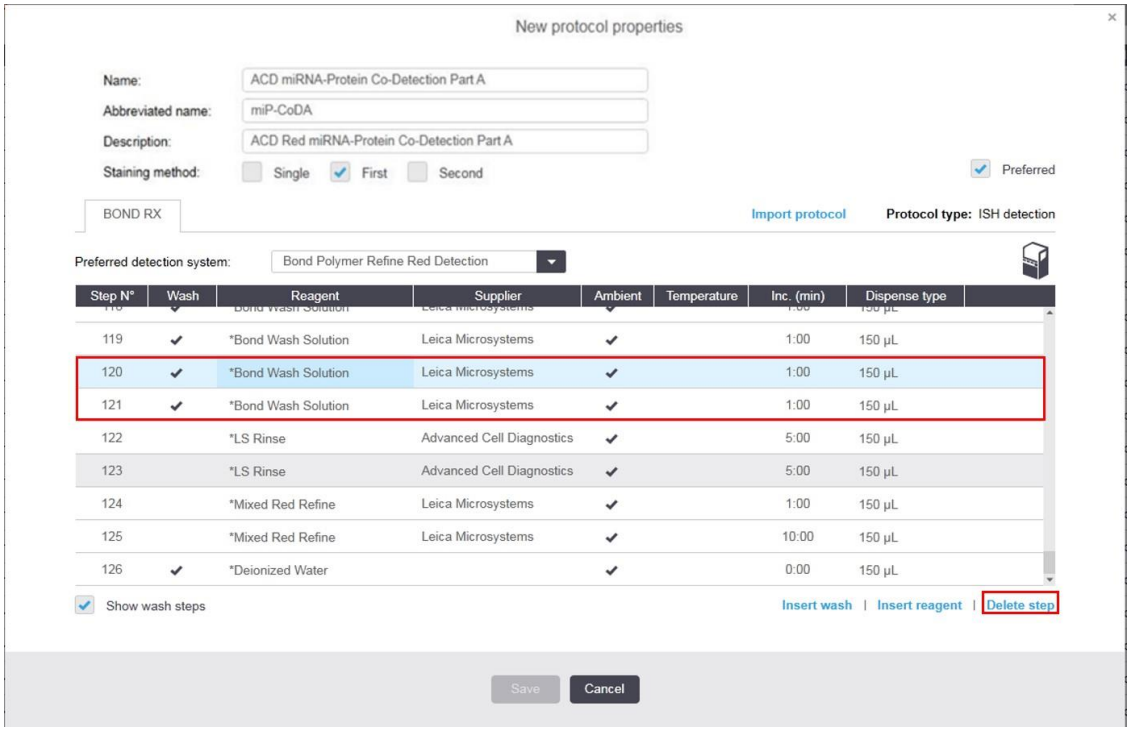
18	*ACD Enzyme	Reagent	30 MIN	40°C
19	*Bond Wash Solution	Wash	0 MIN	Ambient
20	*Bond Wash Solution	Wash	0 MIN	Ambient
21	*Bond Wash Solution	Wash	0 MIN	Ambient
22	*Open 0 Haz	Reagent	10 MIN	Ambient
23	*Bond Wash Solution	Wash	0 MIN	Ambient
24	*Bond Wash Solution	Wash	0 MIN	Ambient
25	*Bond Wash Solution	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	miRNA probe 1	Reagent	0 MIN	Ambient
37	miRNA probe 1	Reagent	0 MIN	Ambient
38	miRNA probe 1	Reagent	120 MIN	37°C

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

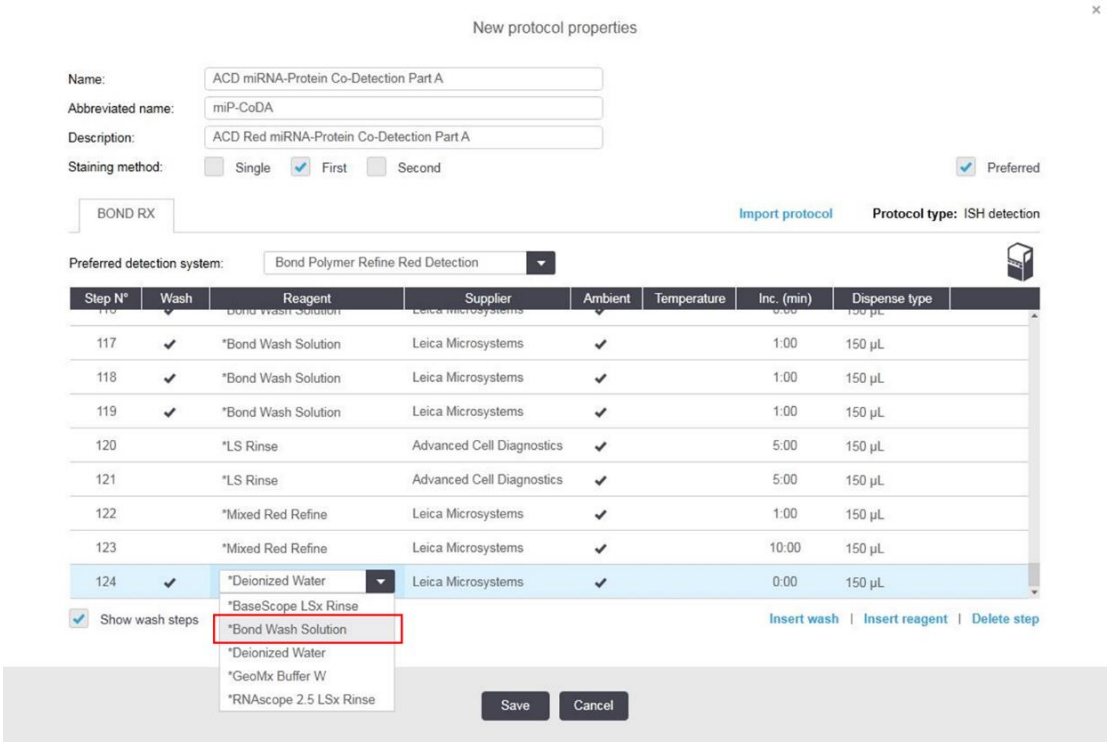
Note: For steps 17–18, select ***ACD Enzyme**, which is the appropriate protease for the miRNAscope assay.

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

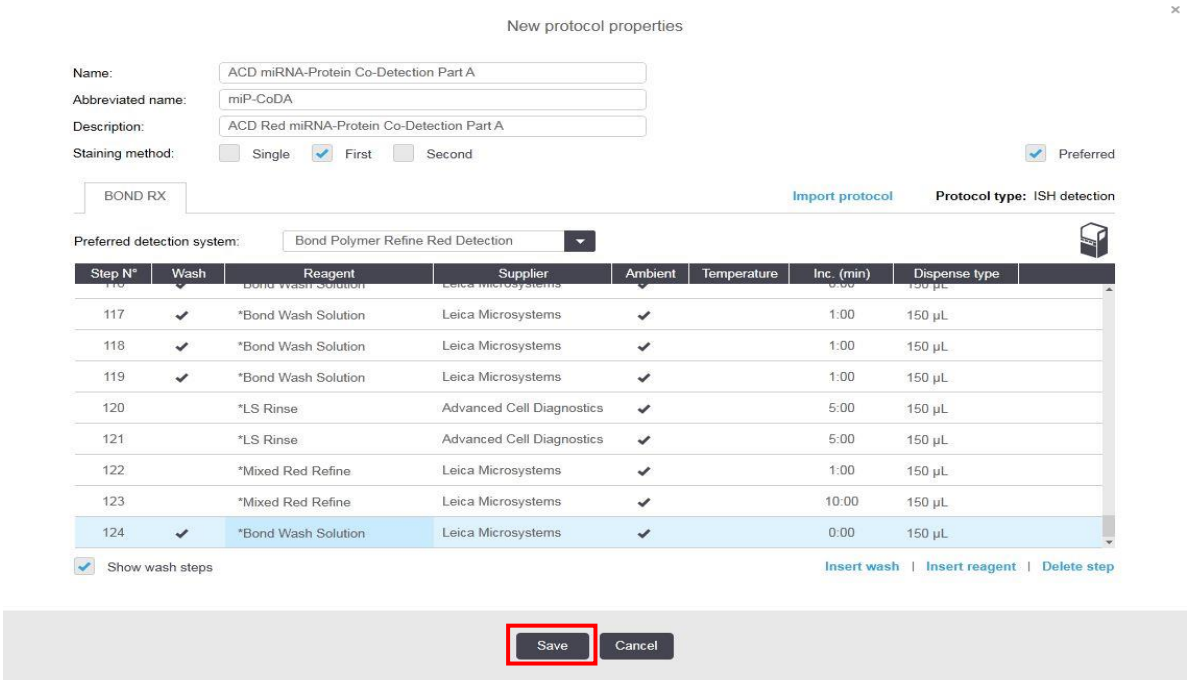
10. Delete two ***Bond Wash Solution 1 MIN** steps directly before the ***LS Rinse** steps that precede ***Mixed Red Refine**. For the miRNAscope assay, these are steps 120–121.



11. The Wash step after ***Mixed Red Refine** is the final step of the Part A protocol. For the miRNAscope assay, delete all steps following step124 ***Deionized Water**.



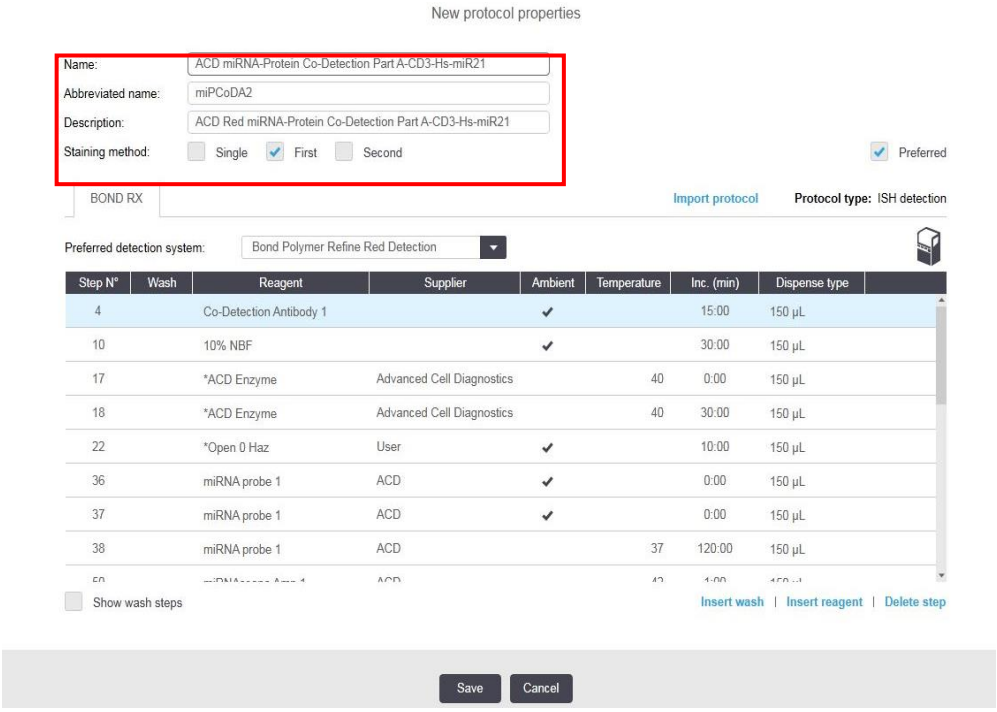
12. Change the Wash step after ***Mixed Red Refine** from ***Deionized Water** to ***Bond Wash Solution**.
13. Select **Save**.



14. Select **Yes** to proceed.

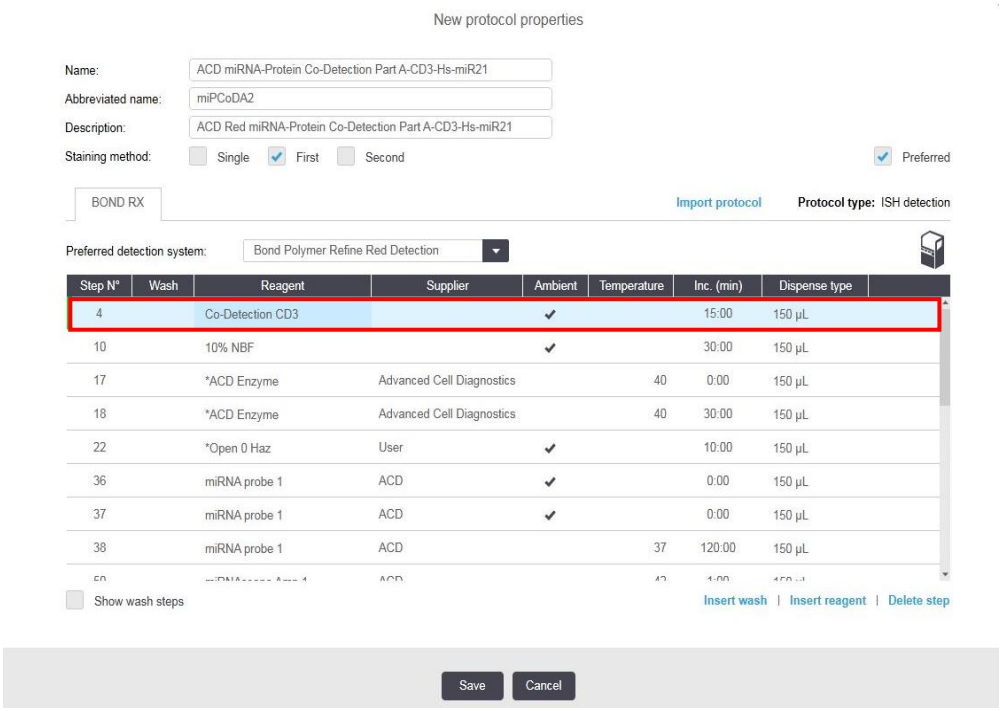
Note: Additional Part A protocols must be created for each new probe and primary antibody combination.

15. To create a protocol for each additional probe and primary antibody, follow these steps:
    a. Highlight the **ACD miRNA-Protein Co-Detection Part A** protocol. Select **Copy**.
    b. Change the protocol name by adding your antibody and probe name (for example, **ACD miRNA-Protein Co-Detection Part A – CD3 Hs-miR-21**) in the Name text box. Change the Abbreviated name text and Description text box accordingly.
    c. Under Staining Method, select **First**.



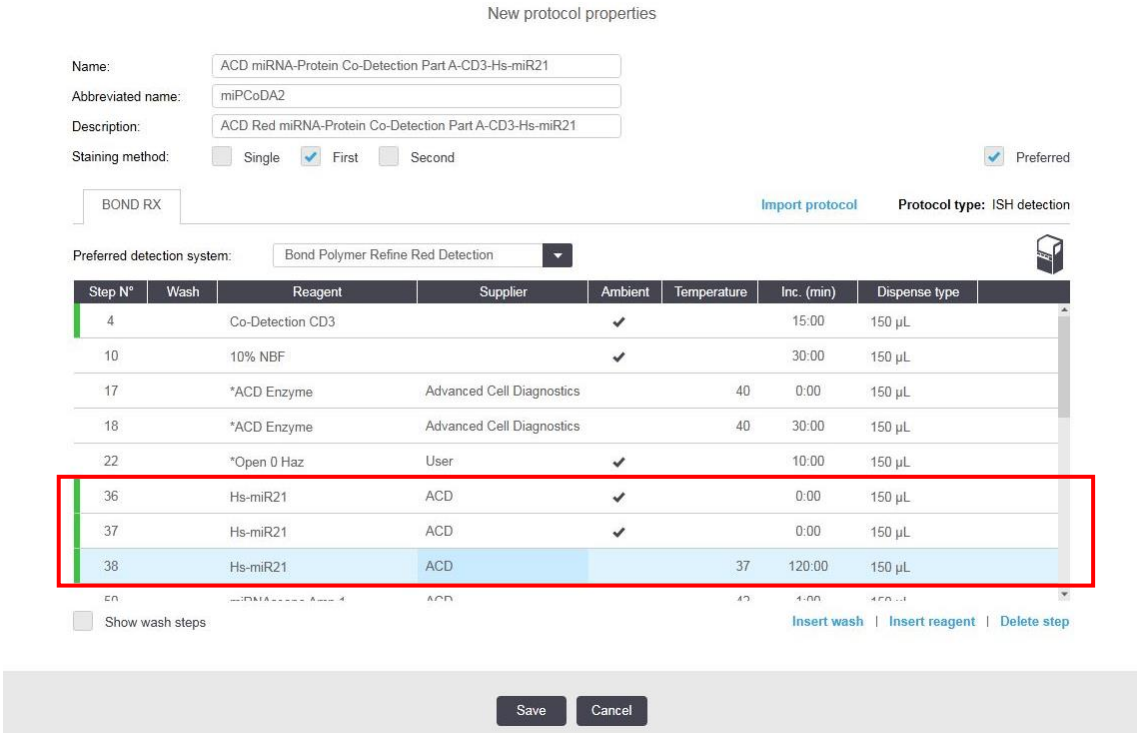
d. If using 5.2 software, select the appropriate tab for your instrument (BOND RXm or BOND RX).

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



f. Select **miRNA probe 1**. Change the Reagent to your registered ancillary probe (for example, **Hs-miR-21**).

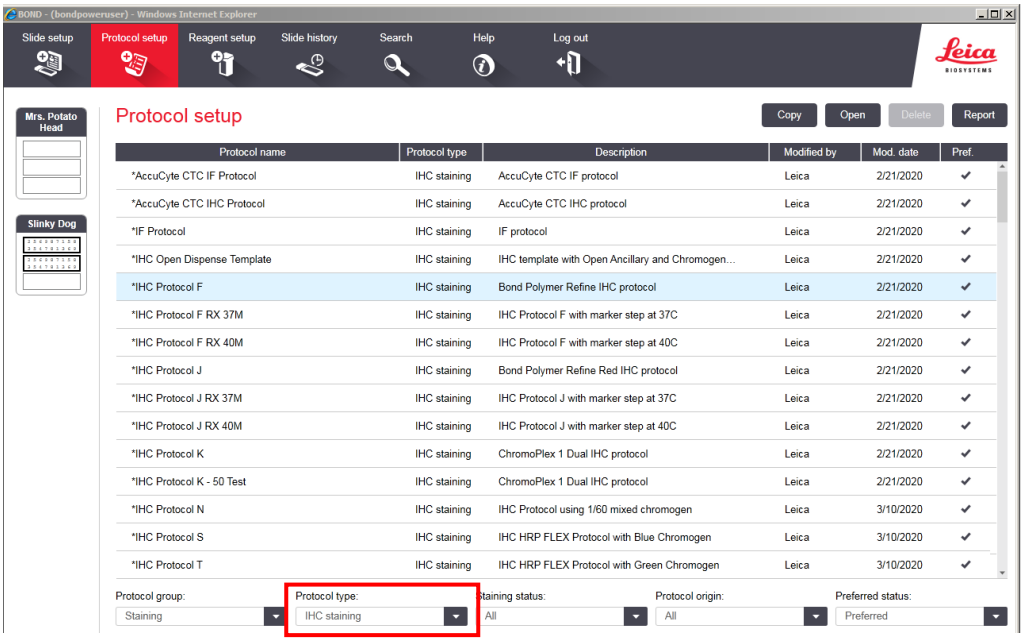
IMPORTANT! Make sure to change all three probesteps.

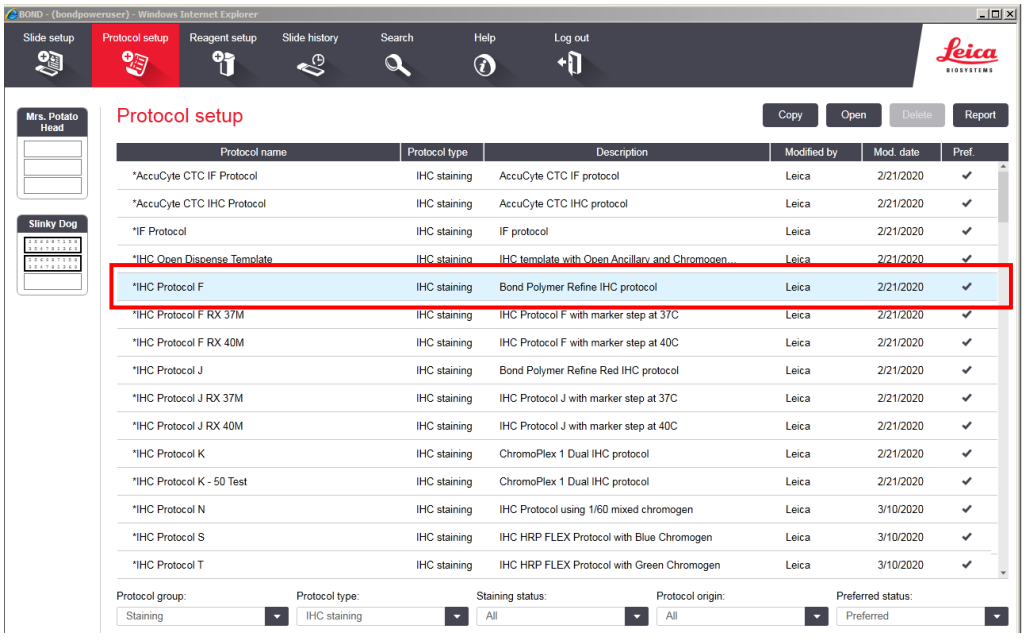


g. Select **Save**.

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

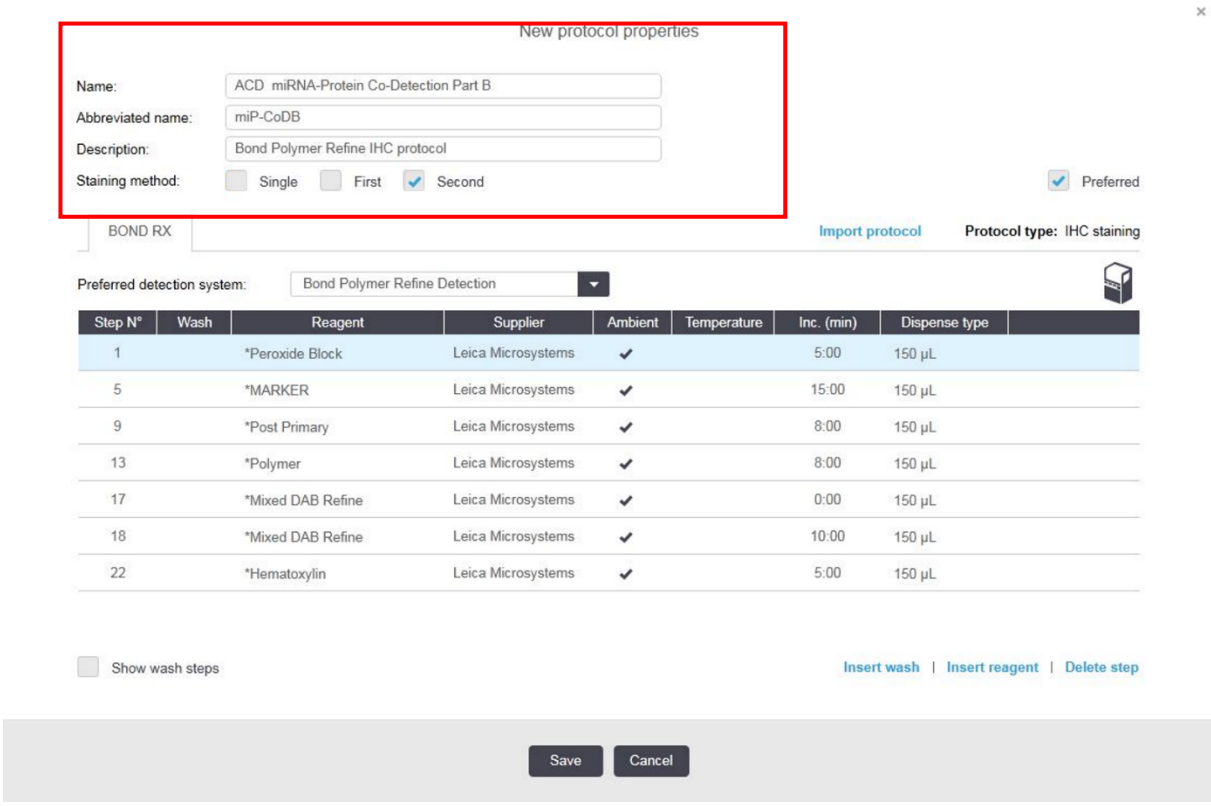
1. On the Protocol setup screen, select **IHC** Staining under the Protocol type menu.



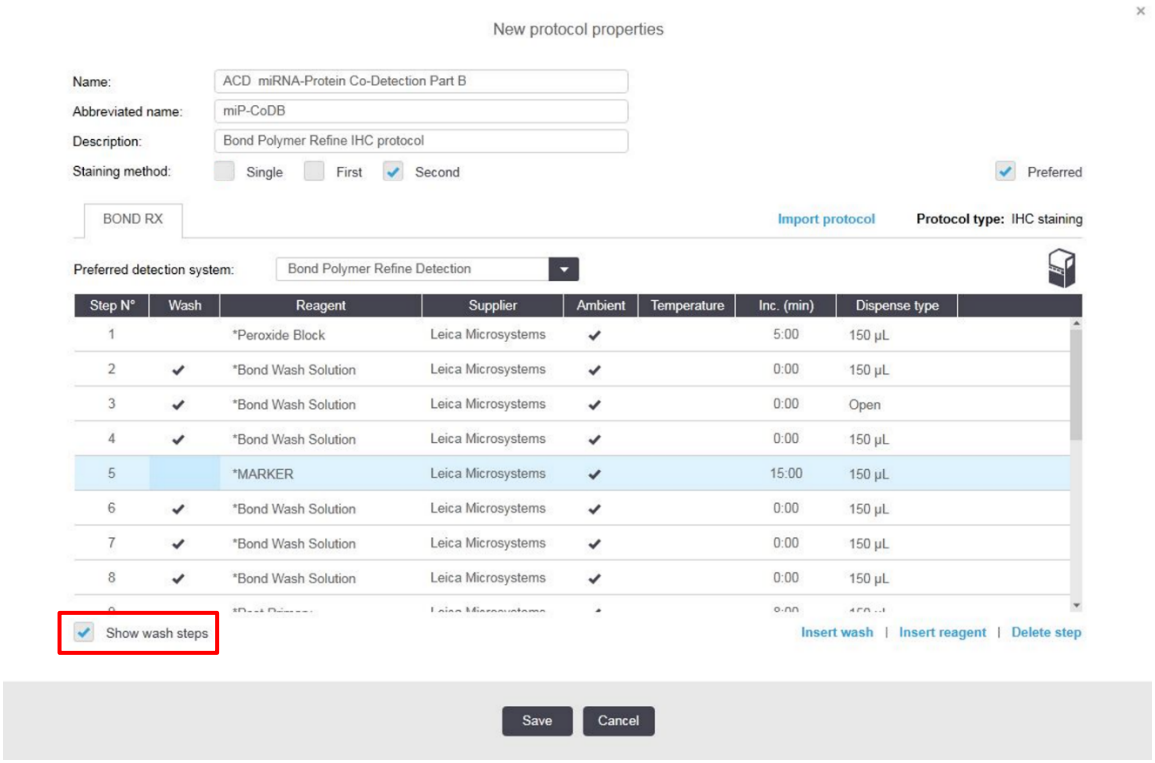


2. To create an IHC protocol, highlight ***IHC Protocol F** in the protocol set up page and select **Copy**.
3. Change the protocol name to **ACDmiRNA-Protein Co-Detection Part B** in the Name text box and **miP-CoD B** in the Abbreviated name text box.
4. For Staining Method, select **Second**.

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



6. Select **Show wash steps**.



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, and 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, and 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**.

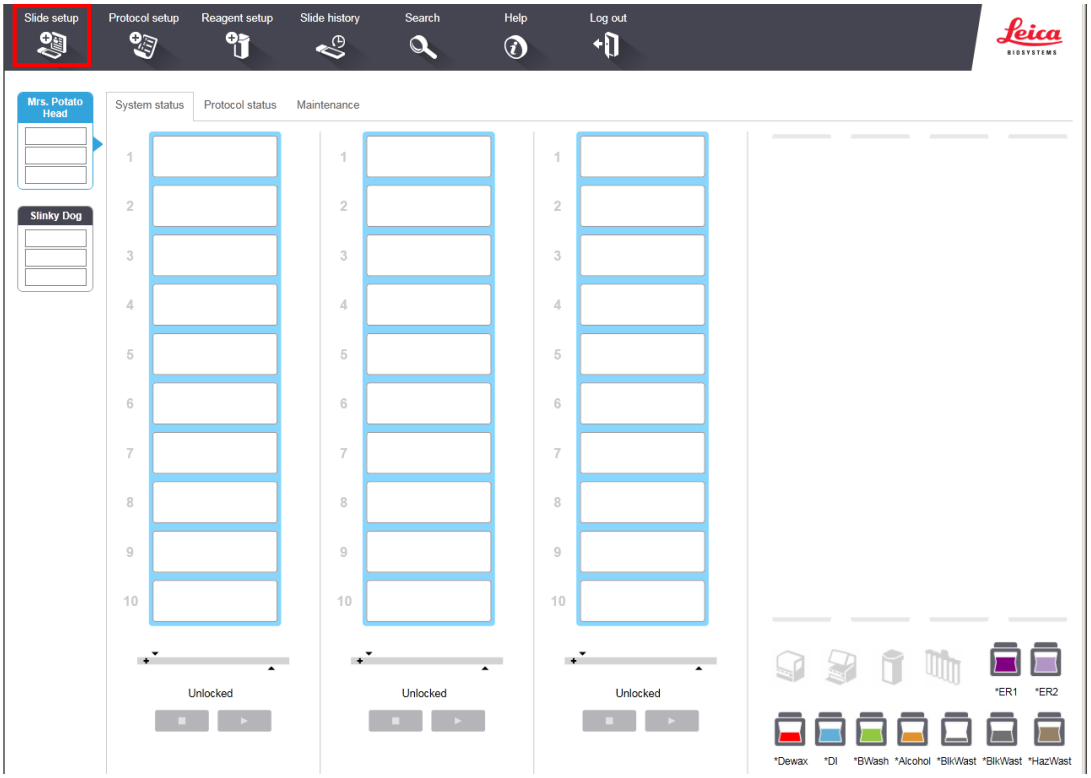
Create an HIER protocol for use with Co-Detection protocols

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.

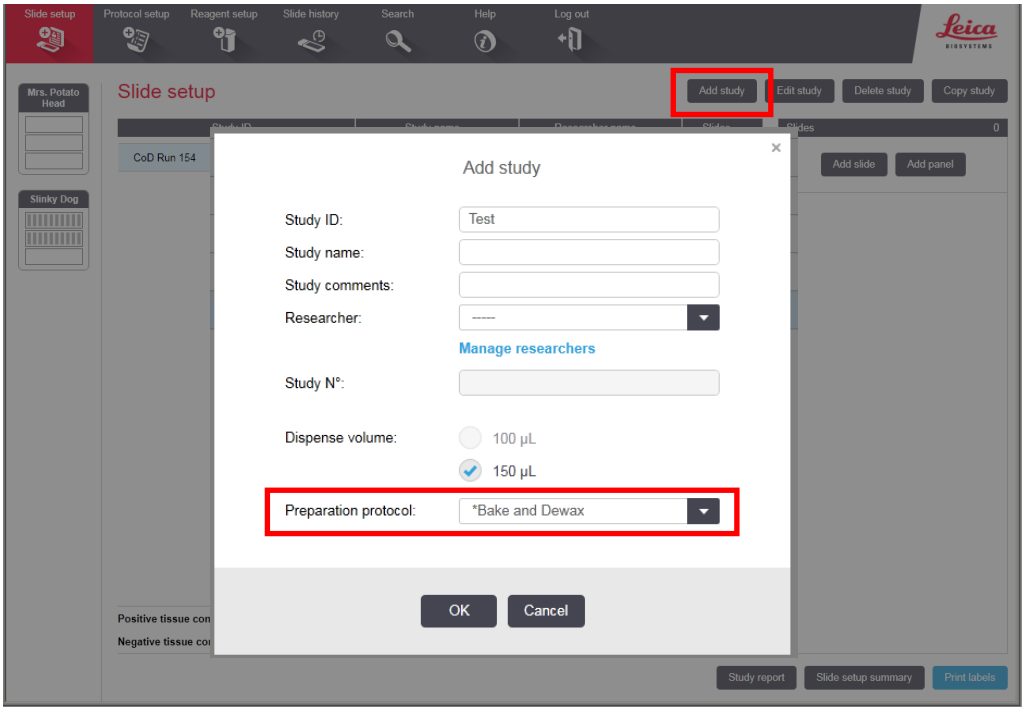
Part 3: Set up a Study for Co-Detection

Build a study

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



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).

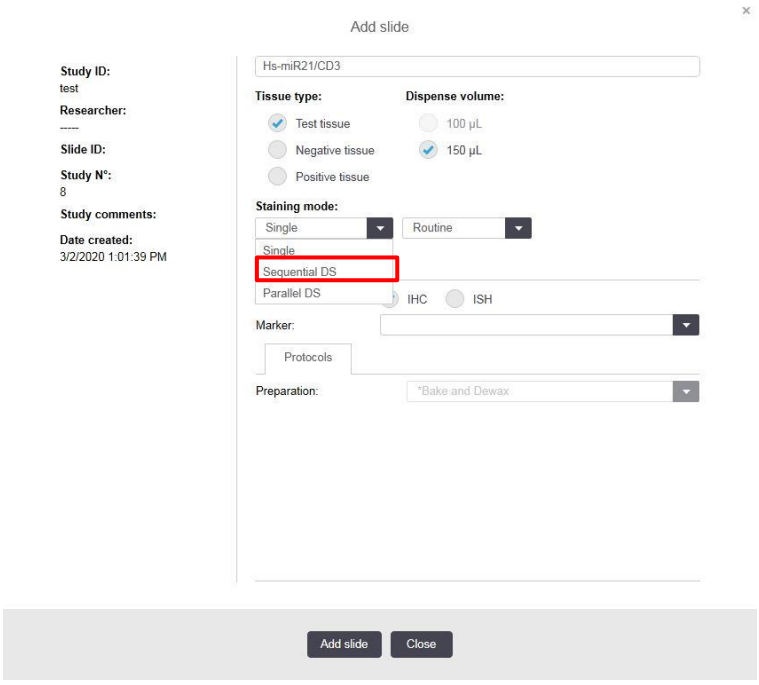


4. Select **OK**.

Add a slide for ICW in FFPE samples

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.

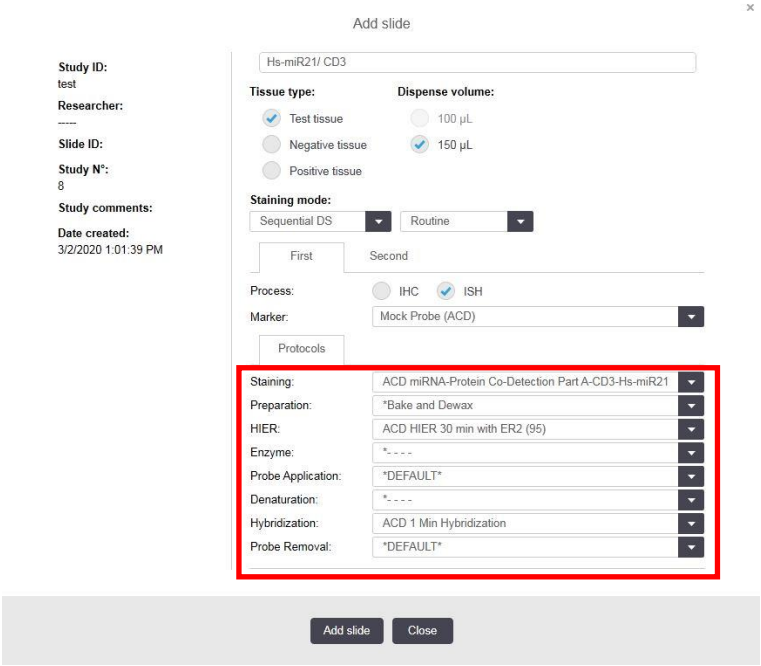


4. Under the First tab, select **ISH**

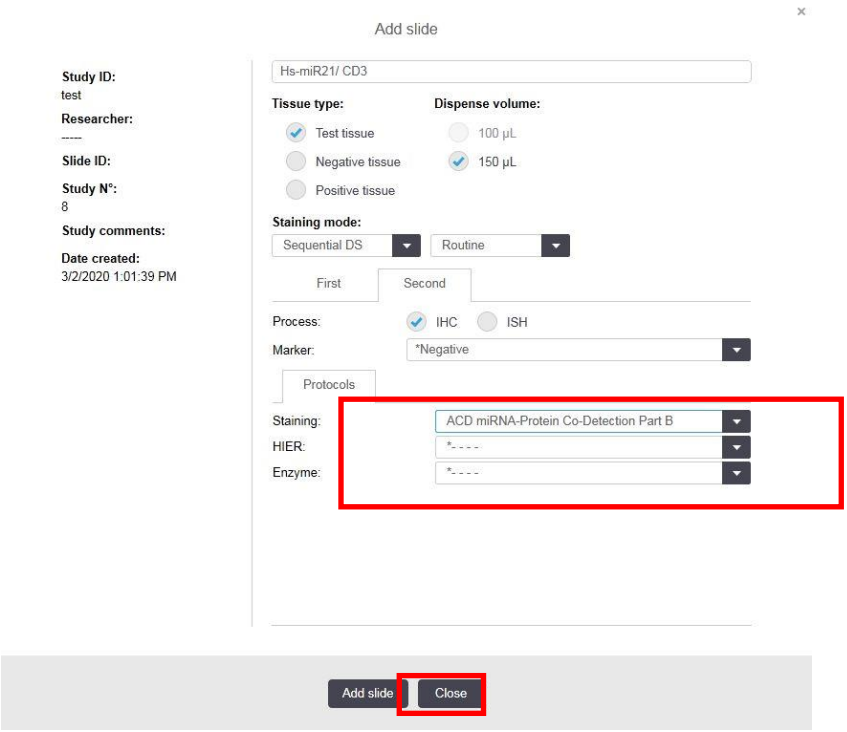
5. Select **Mock Probe** as Marker.
6. Under Protocols:
7. For each probe, select a protocol from the Staining drop down menu. Make sure that each probe and primary antibody combination is associated with a different Part A protocol (for example, **ACD miRNA-Protein Co-Detection Part A – Hs-miR-21 CD3**).
8. For HIER protocol, select **ACD HIER 30 min with ER2 (95)** as the HIER protocol.
9. Select ***---** for Enzyme.

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

10. Select ***DEFAULT*** for Probe Application and Probe Removal.
11. Select ***---** for Denaturation and **ACD 1 min Hybridization** for Hybridization.



12. Under the Second tab, select **IHC**.
13. Select ***Negative** as Marker.
14. Under Protocols:
15. Select **ACD miRNA-Protein Co-Detection Part B** from the Staining drop down menu.
16. For HIER, select ***---**.
17. For Enzyme, select ***---**.
18. Select **Add Slide**.



19. Repeat steps 1–18 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 miRNAscope assay, you can run up to three SSAs simultaneously using ICW.

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

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

- 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 µL 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 µL GREEN solution onto each tissue section. Ensure sections are covered.
8. Incubate the slides for **15–30 MIN** at **RT** to achieve the desired level of chromogen intensity.
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 look 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.

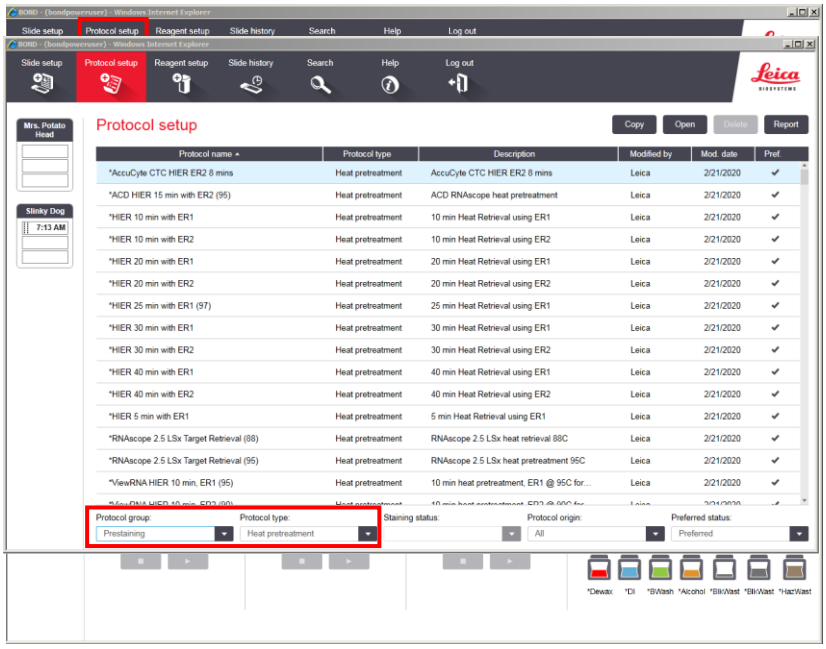
2. Cool the slides for **5 MIN** at **RT**.
3. Briefly dip one slide into fresh pure xylene and immediately place 1–2 drops of VectaMount® Mounting Medium on the slide before the xylene dries.
4. Carefully place a 24 mm x 50 mm coverslip over the tissue section. Avoid trapping air bubbles.
5. Repeat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

Appendix A. Creating a new HIER Protocol

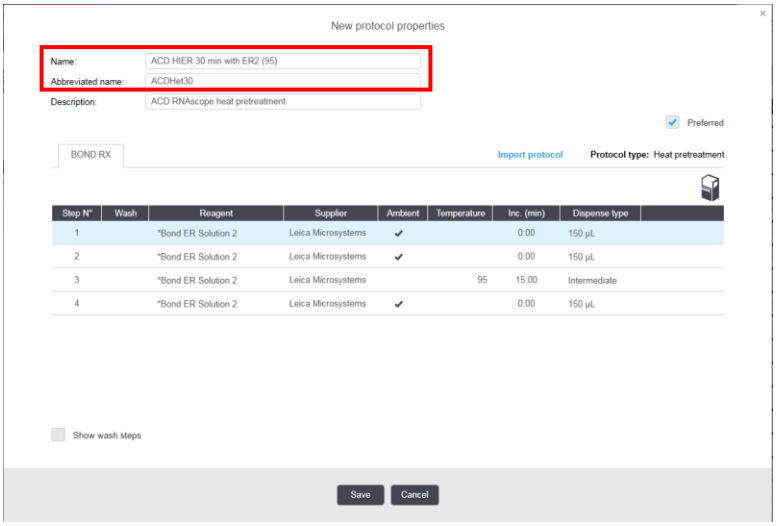
For optimal RNA and protein detection, we recommend using an extended heat-induced epitope retrieval (HIER) step. Follow the steps to edit the Epitope retrieval procedure in the software.

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



ACDHet30.

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

Appendix B. LS Chromogenic ICW using Fresh Frozen Samples

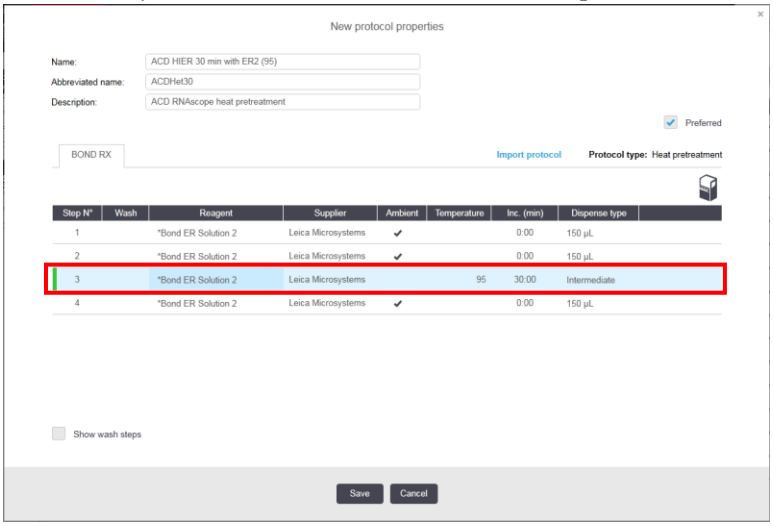
Part 1. Prepare Fresh Frozen Samples

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 for **1 HR** at **–20°C**.
8. Store in air-tight slide boxes at **–80°C** until use.

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. 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 one week.

Part 2. Run the Assay

Setup a Protocol

1. Refer to **Part 1: Add and Register Reagents for Co-Detection** on page 4 to add and register new reagents.
2. Refer to **Part 2: Create Co-Detection Software Protocols** on page 7 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
miRNAscope LS Red	Protease IV	17	*Enzyme 1	Reagent	0 MIN	Ambient
	Protease IV	18	*Enzyme 1	Reagent	30 MIN	Ambient

† The table lists the standard Protease conditions for miRNAScope 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 MIN** 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 could 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:
8. Select a protocol from the Staining drop down menu for each probe. Make sure that each probe and primary antibody combination is associated with a different Part A protocol (for example, **ACD miRNA-Protein Co-Detection Part A – CD3 Hs-miR-21**).
   a. For HIER and Enzyme, select ***---**.
9. 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.
10. Select ***---** for Denaturation and **ACD 1 min Hybridization** for Hybridization.
11. Under the Second tab, select **IHC**. Select ***Negative** as Marker.
12. Under Protocols:
13. Select **ACD miRNA-Protein Co-Detection Part B** from the Staining drop down menu.
    a. For HIER and Enzyme, select ***---**.
14. Select **Add Slide**.
15. Repeat steps 1–14 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 miRNAscope assay, you can run up to three SSAs simultaneously using ICW.

1. 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 for **30–60 MIN** at **60°C**.
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: Add and Register Reagents for Co-Detection on page 4 to add and register new reagents.
2. Refer to Part 2: Create Co-Detection Software Protocols on page 7 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:

8. Select a protocol from the Staining drop down menu for each probe. Make sure that each probe and primary antibody combination is associated with a different Part A protocol (for example, **ACD miRNA-Protein Co-Detection Part A – CD3 Hs-miR-21**.
9. For HIER protocol, select **ACD HIER 5 min with ER2 (95)**.
10. For Enzyme, Select ***- - -**.
11. 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.
12. Select ***---** for Denaturation and **ACD 1 min Hybridization** for Hybridization.
13. Under the Second tab, select **IHC**, and select ***Negative for Marker**.
14. Under Protocols:
15. Select **ACD miRNA-Protein Co-Detection Part B** from the Staining drop down menu.
16. For HIER and Enzyme, select ***---**.
17. Select **Add Slide**.
18. Repeat steps 3–17 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 theslides.
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 miRNAscope assay, you can run up to three SSAs simultaneously using ICW.

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 ICW workflow than you would normally use for IHC alone. To optimize protein detection, we recommend optimizing the antibody concentration.

The crosslinking and pretreatment conditions in this Tech Note provide optimal miRNA 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 at 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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