HCR™ Pro RNA-ISH Setup Guide for the BOND RX/RX^m

This Setup Guide demonstrates the use of an HCR™ Pro RNA-ISH kit on the BOND RX/RX^m platforms from Leica Biosystems. Reagent preparation steps, including registering individual BOND Containers for their respective reagents, will be described in further detail. Each BOND RX/RX^m run takes approximately 7-10.5 hours followed by a short post-processing of stained slides. This time range depends on the number of slides processed in a single run and whether you're using the BOND RX or the BOND RX^m platform. Processing 1 slide on the BOND RX takes around 7 hours, and processing 30 slides on the BOND RX takes around 9 hours due to the time the BOND RX/RX^m platforms take to disperse the reagents onto each slide. The HCR™ Pro RNA-ISH kit can be used to probe and visualize RNA transcripts in FFPE tissue sections. Please read through the Setup Guide for additional information so that you can easily incorporate the HCR™ Pro RNA-ISH kit into your current workflow.

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HCR™ Pro RNA-ISH Kit Information

Upon receiving an HCR™ Pro RNA-ISH kit, please check all reagents and their storage conditions.

HCR™ Pro RNA-ISH Starter Kit

The HCR™ Pro RNA-ISH Starter Kit is provided with enough reagents to perform the assay on 20 slides and includes two HCR™ HiFi Probes and HCR™ Detect reagents. The HCR™ HiFi Probes included in the Starter Kit (*Ppib/dapB*) are provided in volumes sufficient enough to perform the assay on 10 slides each. The Starter Kit also contains an HCR™ Membrane Stain to be used in an HCR™ Pro RNA-ISH + IHC/IF co-detection assay.

HCR™ HiFi Probes

HCR™ Reagents	Amount for an HCR™ Pro RNA-ISH Starter Kit	Storage Temperature
HCR™ HiFi Probe 1A *PPIB/Ppib*[1] – Positive Control	4 mL	2 to 8 °C
HCR™ HiFi Probe 1B *PPIB/Ppib*[1] – Positive Control	4 mL	2 to 8 °C
HCR™ HiFi Probe 1A *dapB* – Negative Control	4 mL	2 to 8 °C
HCR™ HiFi Probe 1B *dapB* – Negative Control	4 mL	2 to 8 °C
HCR™ Membrane Stain[2]	3 mL	2 to 8 °C

[1]*Upper and lower cases are used to denote human and mouse HCR™ HiFi Probes respectively.*

[2]*The HCR™ Membrane Stain's host species is in rabbit and is provided in a volume sufficient to perform the assay on 5 slides. Please reference pages 21-25 for more information on how to perform an HCR™ Pro RNA-ISH + IHC/IF co-detection assay.*

HCR™ Detect

Please note that all HCR™ Detect reagents are provided in amber bottles, as these reagents are sensitive to light.

HCR™ Reagents	Amount for an HCR™ Pro RNA-ISH Starter Kit	Storage Temperature
HCR™ Pretreat	5 mL	2 to 8 °C
HCR™ Detect A	5 mL	2 to 8 °C
HCR™ Detect B	7 mL	2 to 8 °C
HCR™ Detect C	5 mL	2 to 8 °C
HCR™ Detect D	7 mL	2 to 8 °C
HCR™ Detect E	7 mL	2 to 8 °C
HCR™ Detect F AP/HRP[1]	7 mL	2 to 8 °C
HCR™ Post-Process A	5 mL	2 to 8 °C
HCR™ Post-Process B	7 mL	2 to 8 °C

[1]*HCR™ Detect F AP is included in the HCR™ Pro RNA-ISH AP Starter Kit, and HCR™ Detect F HRP is included in the HCR™ Pro RNA-ISH HRP Starter Kit.*

HCR™ Pro RNA-ISH Kit

The HCR™ Pro RNA-ISH kit consists of an HCR™ HiFi Probe and HCR™ Detect reagents. Each HCR™ HiFi Probe includes 2 components: (1) HCR™ HiFi Probe 1A and (2) HCR™ HiFi Probe 1B. The probes are provided ready-to-use and can be transferred directly to open containers for use or storage. If working with a probe supplied at 50× concentration, refer to the HCR™ HiFi Probe Preparation Guide for 50× Concentrated Probes in **Appendix I**.

HCR™ HiFi Probe

HCR™ Reagents	Amount for 20 Slides	Amount for 90 Slides	Storage Temperature
HCR™ HiFi Probe 1A	7 mL	29 mL	2 to 8 °C
HCR™ HiFi Probe 1B	7 mL	29 mL	2 to 8 °C

HCR™ Detect

Please note that all HCR™ Detect reagents are provided in amber bottles, as these reagents are sensitive to light.

HCR™ Reagents	Amount for 20 Slides	Amount for 90 Slides	Storage Temperature
HCR™ Pretreat	5 mL	21 mL	2 to 8 °C
HCR™ Detect A	5 mL	21 mL	2 to 8 °C
HCR™ Detect B	7 mL	29 mL	2 to 8 °C
HCR™ Detect C	5 mL	21 mL	2 to 8 °C
HCR™ Detect D	7 mL	29 mL	2 to 8 °C
HCR™ Detect E	7 mL	29 mL	2 to 8 °C
HCR™ Detect F AP/HRP[1]	7 mL	29 mL	2 to 8 °C
HCR™ Post-Process A	5 mL	21 mL	2 to 8 °C
HCR™ Post-Process B[2]	7 mL	29 mL	2 to 8 °C

[1]*HCR™ Detect F AP is included in the HCR™ Pro RNA-ISH AP kit, and HCR™ Detect F HRP is included in the HCR™ Pro RNA-ISH HRP kit.*

[2]*HCR™ Post-Process B is only needed if you are performing an HCR™ Pro RNA-ISH + IHC/IF co-detection assay.*

Required Materials for the BOND RX/RX^m

The HCR™ Pro RNA-ISH protocol requires specific materials available only from Leica Biosystems (LBS). It is essential to check the availability of these materials prior to setting up an HCR™ Pro RNA-ISH experiment. For more information, please inquire with your Leica Biosystems representative.

Materials from Leica Biosystems		
	Catalog #	**Storage Temperature**
BOND 6 mL Titration Kit	OPT9049	RT
BOND 7 mL Open Container	OP79193	RT
BOND 30 mL Open Container	OP309700	RT
BOND Universal Covertiles 160 Pack	S21.4611	RT
BOND Epitope Retrieval Solution 2	AR9640	2 to 8 °C
BOND Dewax Solution	AR9222	2 to 26 °C
BOND Wash Solution 10X Concentrate	AR9590	2 to 8 °C
BOND Polymer Refine Red Detection[1]	DS9390	2 to 8 °C
BOND Polymer Refine Detection[1]	DS9800	2 to 8 °C
BOND Research Detection	DS9455	RT
BOND Research Detection System 2	DS9777	RT
BOND Mixing Stations	S21.1971	RT

[1]*The HCR™ Pro RNA-ISH protocol uses Fast Red/DAB chromogens and hematoxylin from the BOND Polymer Refine Red Detection Kit or the BOND Polymer Refine Detection Kit for chromogenic deposition and counterstain. The HCR™ Pro RNA-ISH AP kit requires the use of the BOND Polymer Refine Red Detection Kit, and the HCR™ Pro RNA-ISH HRP kit requires the use of the BOND Polymer Refine Detection Kit.*

Required Materials for Running Matisse® Chromogens on BOND RX/RX^m

Matisse® Red Chromogen for AP Detection
For those using our HCR™ Detect reagents for AP detection, you can use our Matisse® Red chromogen for brighter and clearer staining. For further instructions on how to incorporate an HCR™ Pro RNA-ISH assay with Matisse® chromogens, reference **Appendix H** on page 38.

HCR™ Reagents	Amount for 20 Slides	Amount for 90 Slides	Storage Temperature
Matisse® Red	1.5 mL	2.5 mL	2 to 8 °C
Matisse® Red Buffer	7 mL	29 mL	2 to 8 °C
Hematoxylin	7 mL	29 mL	2 to 8 °C

Matisse® Brown and Green Chromogens for HRP Detection
For those using our HCR™ Detect reagents for HRP detection, you can use our Matisse® Brown or Matisse® Green chromogens for brighter and clearer staining. For further instructions on how to incorporate an HCR™ Pro RNA-ISH assay with Matisse® chromogens, reference **Appendix H** on page 38.

Matisse® Brown Chromogen for HRP Detection

HCR™ Reagents	Amount for 20 Slides	Amount for 90 Slides	Storage Temperature
Matisse® Brown	2.5 mL	7 mL	2 to 8 °C
Matisse® Brown Buffer	7 mL	29 mL	2 to 8 °C
Hematoxylin	7 mL	29 mL	2 to 8 °C

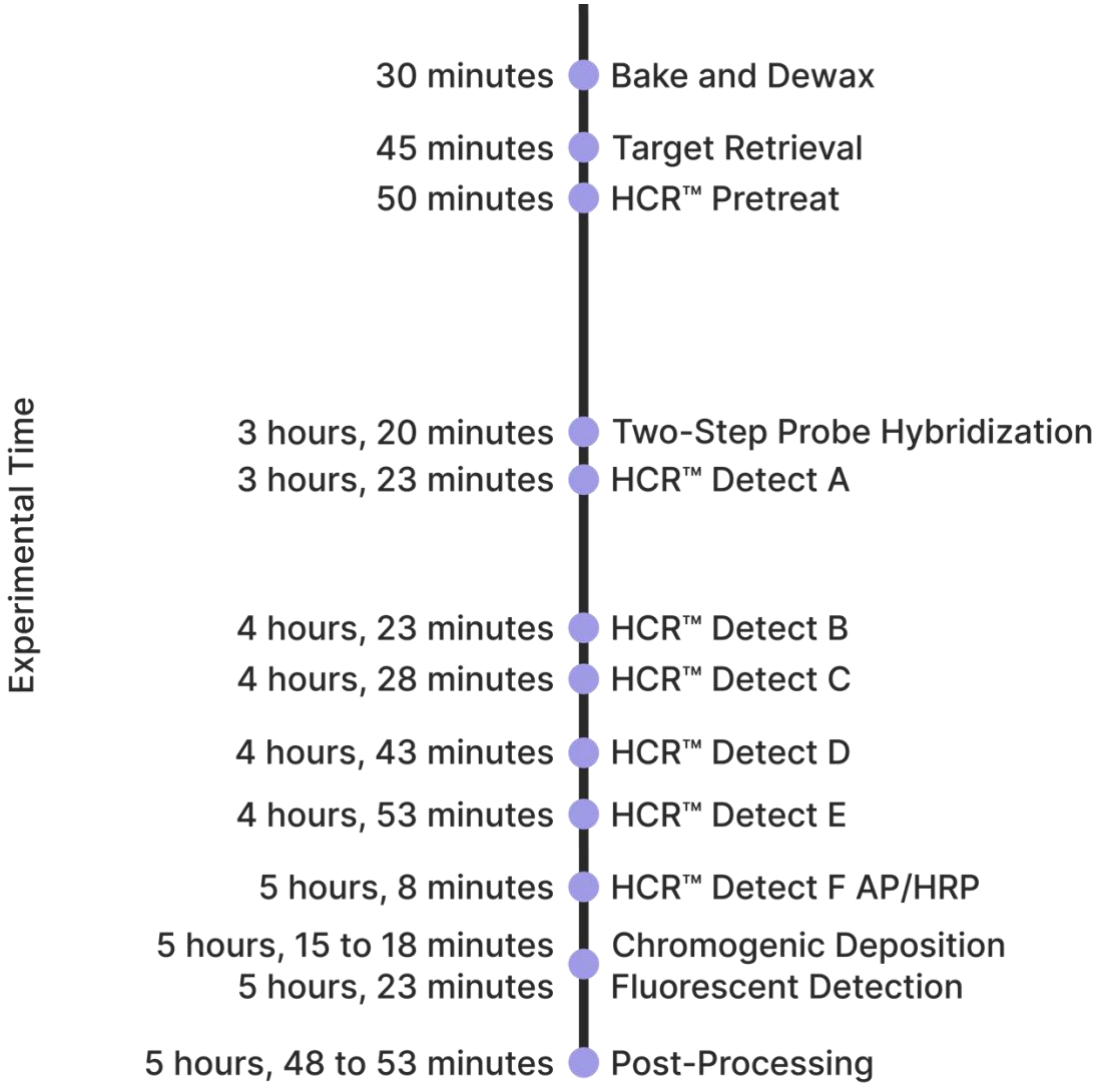
Matisse® Green Chromogen for HRP Detection

HCR™ Reagents	Amount for 20 Slides	Amount for 90 Slides	Storage Temperature
Matisse® Green	4 mL	12.5 mL	2 to 8 °C
Matisse® Green Buffer	7 mL	29 mL	2 to 8 °C
Hematoxylin	7 mL	29 mL	2 to 8 °C

User-Supplied Materials

Materials from Other Vendors		
	Supplier	**Comment**
FFPE Sample Slides	Any, provided they meet the specifications mentioned in the BOND RX User Manual	SuperFrost or SuperFrost® Plus slides are recommended for best results
Propar (xylene substitute)	Fisher Scientific	Xylene may be substituted
Drying Oven	Any	Capable of maintaining temperature at ~60 °C
BioCare EcoMount, Leica CV Ultra Mounting Media, or Vectorlabs VectaMount	BioCare, Leica Biosystems, and Vectorlabs	Mounting medium compatible with all LBS chromogens
Cytoseal	Any	Suitable mounting medium for HRP-driven chromogens
ProLong™ Diamond Antifade Mountant (with or without DAPI) or SlowFade™ Diamond Antifade Mountant (with or without DAPI)	Thermo Fisher	Mounting medium compatible with RNA-FISH and IF staining
Cover Glass	Any	Dimension depends on the size of the tissue
Deionized Water	Any	
100% Ethanol	Any	Used for dehydration of tissues after staining only.
1X PBST	Any	For HCR™ Pro RNA-FISH only. 1X BOND Wash Buffer can be used as an alternative.
Tyramide Dyes	Any	See **Appendix G** for recommendations

Overall Workflow of the HCR™ Pro RNA-ISH Protocol



Each BOND RX/RX^m run takes 8 hours (i.e., 10 slides in one SSA). The timeline above only accounts for 5 hours and 48-53 minutes of this run (depending on whether you're performing the assay for chromogenic or fluorescent detection), as the remaining time comes from the additional BOND RX/RX^m washing steps.

Set Up BOND RX/RX^{m} Protocols

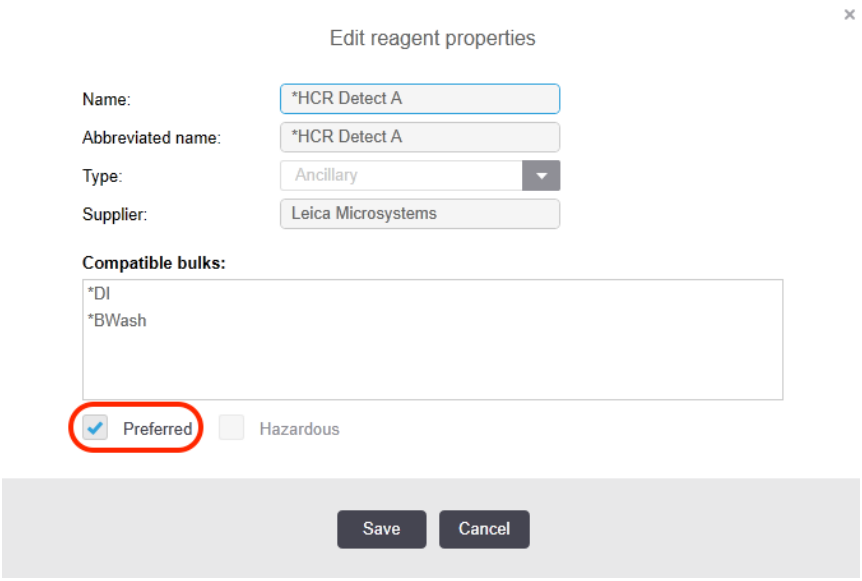
The HCR™ Pro RNA-ISH assay utilizes a sequential staining approach, where the first sequence dispenses HCR™ HiFi Probe A solution, and the second sequence dispenses HCR™ HiFi Probe B solution, followed by the supporting detection reagents. As a result, both the BOND Refine (or Red) kit and the BOND Research Detection System are required to stain each slide.

The five main steps in setting up a BOND RX/RX^{m} protocol for an HCR™ Pro RNA-ISH run are:

i. Register BOND Containers associated with their respective reagents
ii. Register the BOND Research Detection System
iii. Create a staining protocol
iv. Register probe reagents
v. Set up slides and print labels

Register BOND Containers

Reagent names need to be **preferred** before BOND containers can be registered to their respective names. To prefer a reagent name, select the reagent name (e.g., *HCR Detect A) under **Reagent Setup,** and check the **"Preferred"** box.



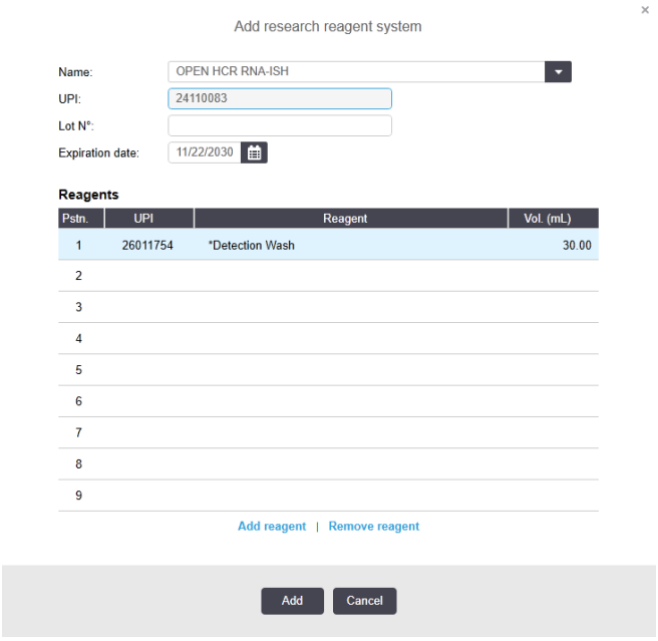
The table below outlines a list of reagents that need to be preferred and registered with individual BOND containers.

BOND Container Registration for HCR™ Pro Reagents	
Reagent[1]	**Type of Container[2]**
*Enzyme 2	7 mL or 30 mL BOND Container
*HCR™ Detect A to E	7 mL or 30 mL BOND Container
*HCR™ Detect F AP/HRP[3]	7 mL or 30 mL BOND Container
*HCR™ Post-Process A	7 mL or 30 mL BOND Container
*HCR™ Post-Process B[4]	7 mL or 30 mL BOND Container

[1]*The reagent names need to be preferred before they can be used for registering BOND Containers.*
[2]*We recommend using 7 mL and 30 mL BOND Containers for 20-slide and 90-slide kits, respectively.*
[3]*Register *HCR™ Detect F AP and *HCR™ Detect F HRP for AP and HRP detection, respectively.*
[4]*HCR™ Post-Process B is only required for running an HCR™ Pro RNA-ISH + IHC/IF co-detection assay.*

Register the BOND Research Detection System 2

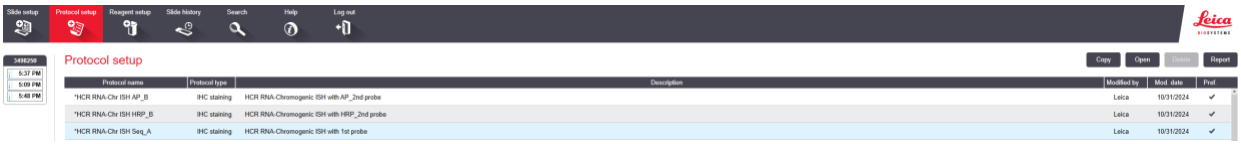
1. Prefer ***Detection Wash** reagent under **Reagent Setup**.
2. Scan the barcode of your BOND Research Detection System 2.
3. Enter the name and expiration date.
4. Select "***Detection Wash**" in the first position, and scan a 30 mL BOND Open Container.
5. Click **Add**.



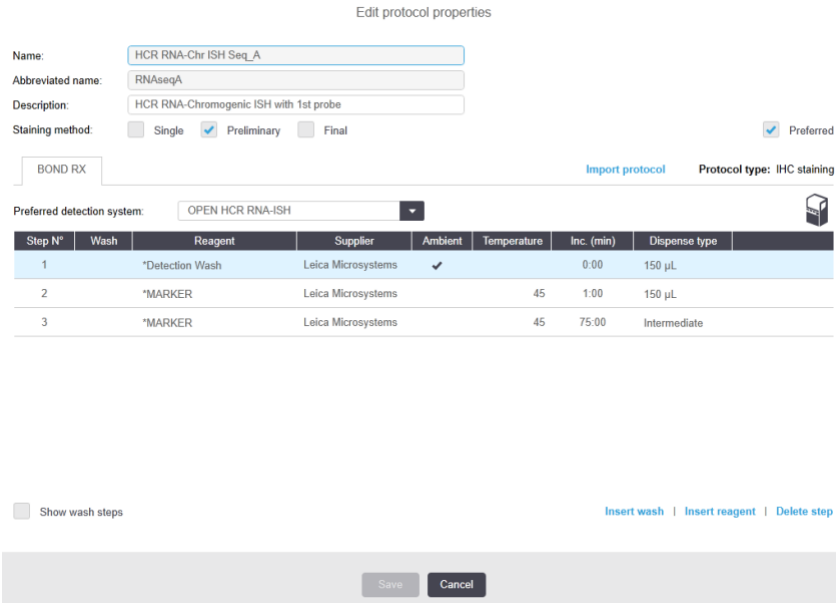
*Note: The ***Detection Wash** Bond Container needs be placed in the first position in the BOND tray to ensure the BOND RX recognizes that the detection system (e.g., OPEN HCR RNA-ISH) is present.*

Create Staining Protocols for HCR™ Pro RNA-CISH

1. First Staining Protocol: From the top navigation bar, click on **Protocol setup** on the top of the screen and select **Staining** for the protocol group and **Leica Microsystem** for **Protocol origin**. Select ***HCR RNA-Chr ISH Seq_A**, and click on the **Copy** button near the top-right corner.



2. Delete the asterisk (*) from the **Name** and **Abbreviated name**, check **Preferred**, and select the name of the previously registered research detection system (e.g., **OPEN HCR RNA-ISH**) as the **Preferred detection system**. Click **Save**.

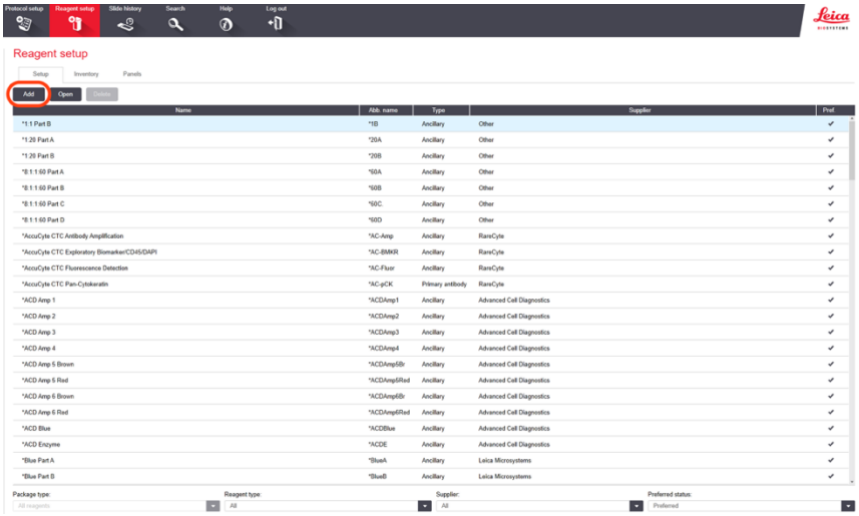


3. Second Staining Protocol: Ensure the ***HCR RNA-Chr ISH HRP_B** and ***HCR RNA-Chr ISH AP_B** protocols have been "**Preferred**" in the protocol setup screen. These protocols will be used in their default form.

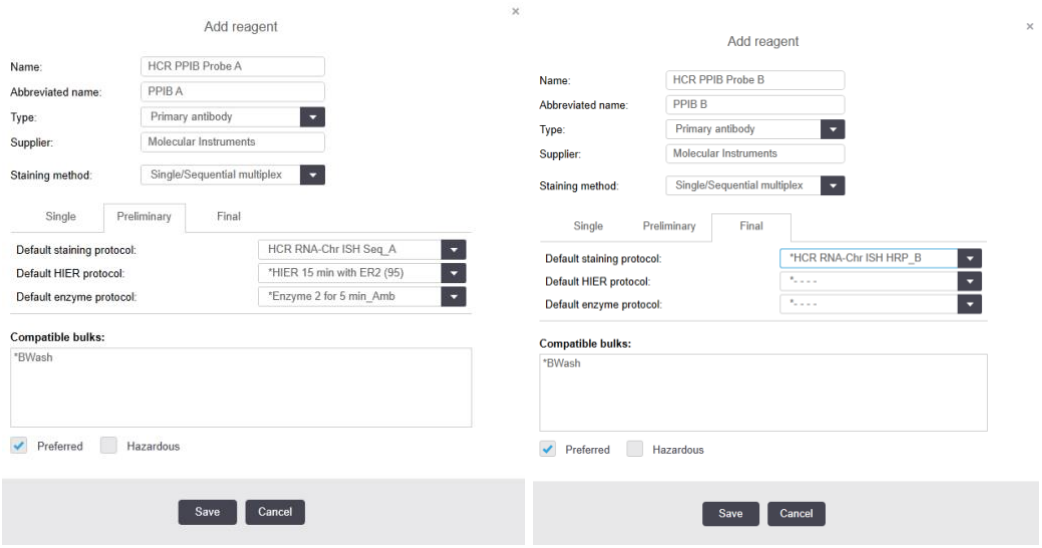
Register HCR™ HiFi Probe Reagents

It is essential to create staining protocols prior to probe registration. Each target probe (e.g., PPIB) requires registration of two probe reagents (e.g., **HCR HiFi Probe 1A PPIB** AND **HCR HiFi Probe 1B PPIB**).

1. Click on **Reagent setup** from the top navigation bar and select **Add**.



2. Check the **Preferred** box, and fill out the selections by referencing the images below.



3. To complete the registration process, default staining protocols for **staining methods** need to be selected as shown below. Click **Save**.
    a. *Note: **default staining protocols** for all **staining methods** need to be defined for the probe to be registered.*

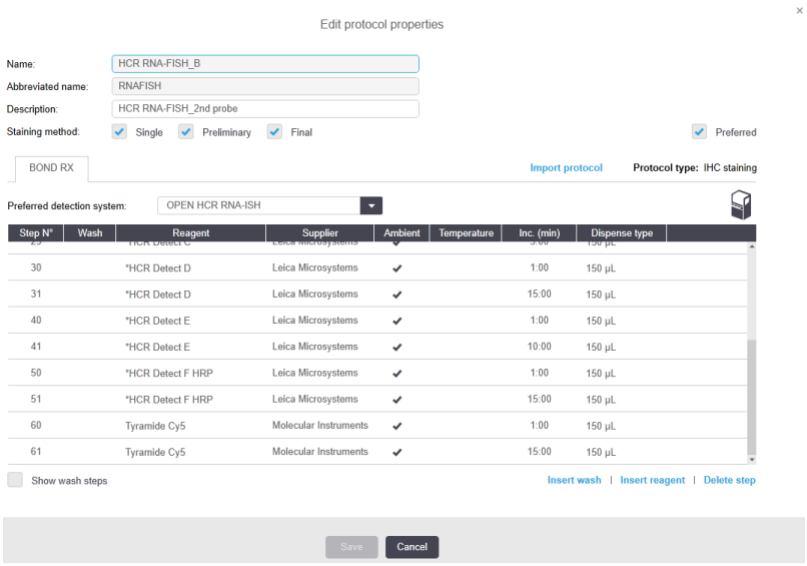
Probe	HCR™ HiFi Probe 1A[1]			HCR™ HiFi Probe 1B[1]		
Type	Primary antibody[2]			Primary antibody[2]		
Staining Methods	Single	Preliminary	Final	Single	Preliminary	Final
Default staining protocol	Any[3]	*HCR RNA-Chr ISH Seq_A	Any[3]	Any[3]	Any[3]	*HCR RNA-Chr ISH HRP_B
Default HIER protocol	*- - - -	*HIER 15 min with ER2 (95)	*- - - -	*- - - -	*- - - -	*- - - -
Default enzyme protocol	*- - - -	*Enzyme 2 for 5 min_Amb[4]	*- - - -	*- - - -	*- - - -	*- - - -

[1]*Each target probe such as PPIB or dapB requires registration of two probe reagents.*
[2]*Although the reagent type is labeled as "Primary antibody," it is specifically used for storing probe reagents.*
[3]*The selected protocol will not be used, so any existing protocol can be selected. However, the default staining protocol field cannot be left blank.*
[4]***Enzyme 2 for 5 min_Amb** needs to be preferred before the selection appears in the dropdown menu.*

4. Register your BOND Containers (e.g., 6 mL titration or 7/30 mL Open Containers) with the associated HCR™ HiFi Probe reagent names by scanning the barcode on the container. Select the **Reagent name** that was created previously and add an expiration date far into the future as the BOND Container can be reused. Click **OK.**

Create a Staining Protocol for HCR™ Pro RNA-FISH

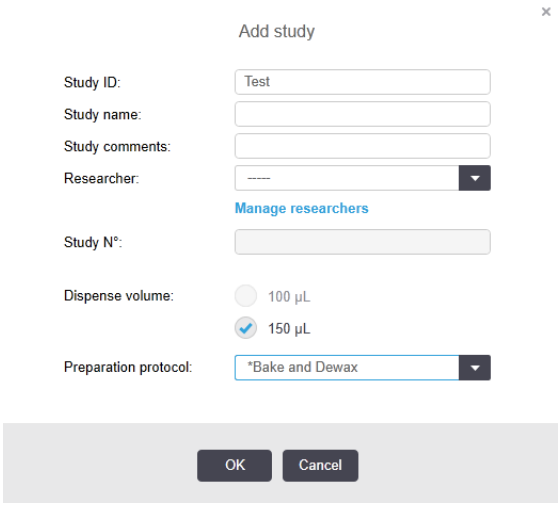
The HCR™ Pro RNA-ISH HRP kit also supports tyramide-based fluorescent detection. The HCR™ Pro RNA-FISH assay follows the same workflow as the HCR™ Pro RNA-CISH assay described above, with a sequential stain that requires 2 staining protocols. To switch from a chromogenic to a fluorescent readout, you only need to modify the second staining protocol (i.e., the first staining protocol remains the same). To create the second staining protocol, copy ***HCR RNA-Chr ISH HRP_B**, change the **Name** to **HCR™ RNA-FISH_B,** and enter your preferred **Abbreviated name**. Select the previously registered research detection system (e.g., OPEN HCR RNA-ISH) as the **Preferred detection system**. Then, modify the protocol according to the detailed staining protocol shown on pages 30-31 (**Appendix C**).



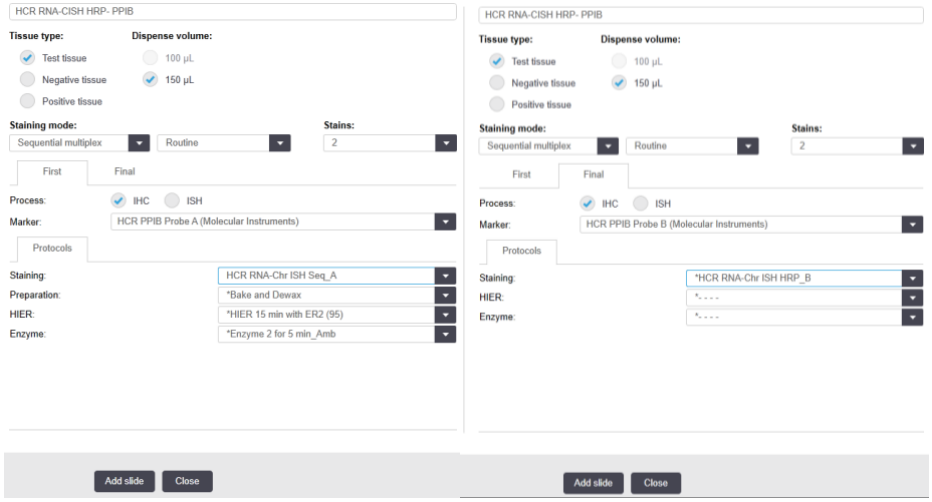
*Note: To run the HCR™ Pro RNA-FISH protocol, you need to use an HCR™ Pro RNA-ISH HRP kit and provide a third-party tyramide dye. Please see **Appendix G** for our recommended dyes and starting concentrations.*

Set Up Slides for HRP, AP, and Fluorescent-Based Protocols and Print Labels

1. From the top navigation bar, click on **Slide setup** on the top of the screen and select **Add study**. Enter **Study ID**, **Study name**, and **Study comments**. Select ***Bake and Dewax** for the **Preparation protocol**. Click **OK**.



2. Follow all the selections shown below. **HCR PPIB Probe A** is selected as the **Marker** for the ***First*** sequence, and **HCR PPIB Probe B** is selected as the **Marker** for the **Final** sequence. Click on **Add slide**.
    a. *Please note:*
        i. *PPIB is used here as an example. Other targets can be selected based on your RNA of interest.*
        ii. *To perform AP-based detection, change the **Staining protocol** in the **Final** sequence to "***HCR RNA-Chr AP_B**." Selections in the **First** sequence remain the same.*
        iii. *To perform HCR™ Pro RNA-CISH with Matisse® chromogens (see **Appendix H**), please select the custom protocol that you created previously (e.g., HCR RNA-Chr ISH AP_B – Matisse Red) in the Final sequence. Selections in the **First** sequence remain the same.*
        iv. *To perform fluorescent-based detection, change the **Staining protocol** in the **Final** sequence to "**HCR RNA-FISH_B**." Selections in the **First** sequence remain the same.*



3. For additional slides, keep this window open, change the parameters as needed, and then click **Add slide**. Continue until all slides have been added, then click **Close**.
4. After all the slides have been added, click on **Print labels**, select **All slide labels not yet printed for the current study**, and click **Print**.
5. Peel off the labels and attach them individually to the top frosted area of the slides.

Post-Processing for AP-Based Detection

After slides are unloaded from the BOND RX/RX^m, we recommend rinsing the slides with Deionized water. Bake the slides for 15 minutes (or until dry) at 60 °C. We recommend using either the EcoMount (Biocare), VectaMount (Vectorlabs), or Leica CV Ultra mounting media for cover-slipping.

Post-Processing for HRP-Based Detection

After slides are unloaded from the BOND RX/RX^m, we recommend rinsing the slides with Milli-Q water. Dehydrate by immersing the slides in 95% ethanol for 3 minutes twice followed by 100% ethanol for 3 minutes. Then, immerse the slides in a xylene (or xylene substitute) solution for 5 minutes and mount one slide at a time with Cytoseal (or any other xylene-based mounting medium). Allow slides to air dry for 5 minutes before imaging.

Post-Processing for Fluorescent Detection

After slides are unloaded from the BOND RX/RX^m, we recommend immersing the slides in 1X PBST (or 1X BOND Wash Solution) for 3 minutes twice. Mount one slide at a time with any suitable mounting medium that has worked for you. For mounting medium and counterstain, we recommend using the ProLong™ Diamond Antifade Mountant (with or without DAPI) or the SlowFade™ Diamond Antifade Mountant (with or without DAPI).

Reagent Preparation

Detailed instructions for preparing probe solutions when probes are supplied at 50× concentration can be found in **Appendix I**. Please refer to this section before setting up your experiment to ensure your probe solutions have been prepared properly.

Reagent Transfer and Storage

Instructions for reagent transfer can be found in the table below. Please transfer all HCR™ Pro RNA-ISH reagents to their name-associated containers. Please store all BOND Containers in a 4 °C fridge after use.

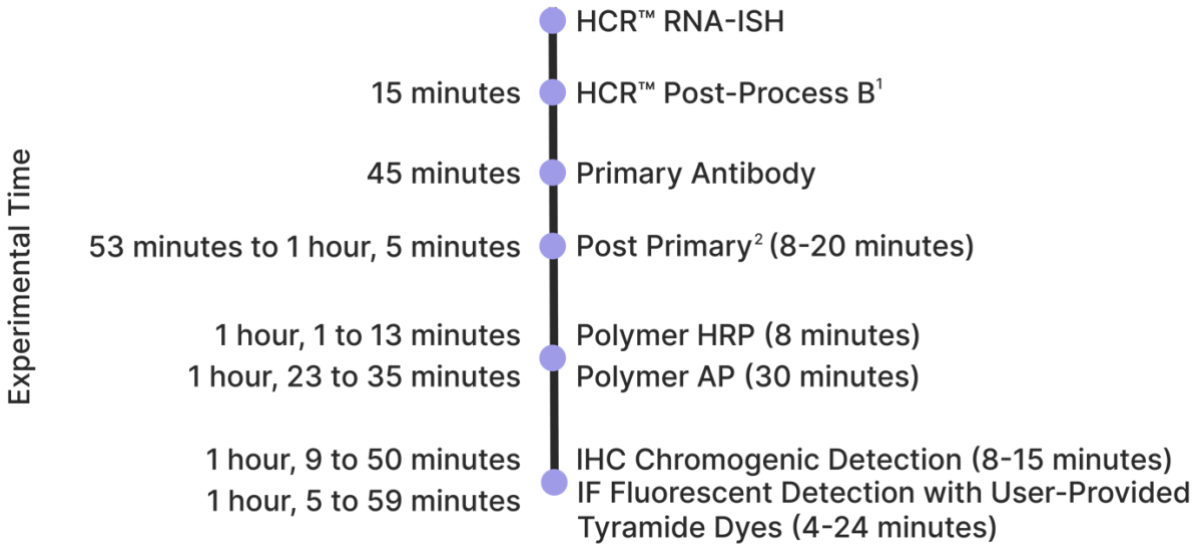
BOND Container	Instructions	Storage Conditions
*Enzyme 2	Transfer entirety of HCR™ Pretreat to the BOND Container	Store at 4 °C after use
*Detection Wash	Fill the 30 mL BOND container with 1X BOND Wash Buffer	Store at 4 °C after use
HCR™ HiFi Probe 1A	Transfer the entirety of HCR™ HiFi Probe 1A Solution to the 6 mL, 7 mL, or 30 mL BOND Container	Store at 4 °C after use
HCR™ HiFi Probe 1B	Transfer the entirety of HCR™ HiFi Probe 1B Solution to the 6 mL, 7 mL, or 30 mL BOND Container	Store at 4 °C after use
*HCR™ Detect A	Transfer entirety of HCR™ Detect A to the 7 mL or 30 mL BOND Container	Store at 4 °C after use
*HCR™ Detect B	Transfer entirety of HCR™ Detect B to the 7 mL or 30 mL BOND Container	Store at 4 °C after use
*HCR™ Detect C	Transfer entirety of HCR™ Detect C to the 7 mL or 30 mL BOND Container	Store at 4 °C after use
*HCR™ Detect D	Transfer entirety of HCR™ Detect D to the 7 mL or 30 mL BOND Container	Store at 4 °C after use
*HCR™ Detect E	Transfer entirety of HCR™ Detect E to the 7 mL or 30 mL BOND Container	Store at 4 °C after use
*HCR™ Detect F AP	Transfer entirety of HCR™ Detect F AP to the 7 mL or 30 mL BOND Container	Store at 4 °C after use
*HCR™ Detect F HRP	Transfer entirety of HCR™ Detect F HRP to the 7 mL or 30 mL BOND Container	Store at 4 °C after use
*HCR™ Post-Process A	Transfer entirety of HCR™ Post-Process A to the 7 mL or 30 mL BOND Container	Store at 4 °C after use
*HCR™ Post-Process B	Transfer entirety of HCR™ Post-Process B to the 7 mL or 30 mL BOND Container	Store at 4 °C after use

HCR™ Membrane Stain	Transfer the entirety of HCR™ Membrane Stain to the 6 mL, 7 mL, or 30 mL BOND Container	Store at 4 °C after use

Other important notes:

1. Please avoid excessive bubbles when transferring HCR™ HiFi Probe and HCR™ Detect Solutions to the 7 mL or 30 mL BOND Open Containers.
2. If the reagent-filled BOND Containers have not been used for more than a week, it is highly recommended to gently invert the BOND Containers several times with the lid closed to ensure solution uniformity before next use.

Overall Workflow of the HCR™ Pro RNA-ISH + IHC/IF Protocol



[1]*This step is NOT needed if HCR™ RNA-CISH HRP detection is followed by IHC AP detection.*
[2]*This step is NOT needed if the species of the primary antibody used is rabbit.*

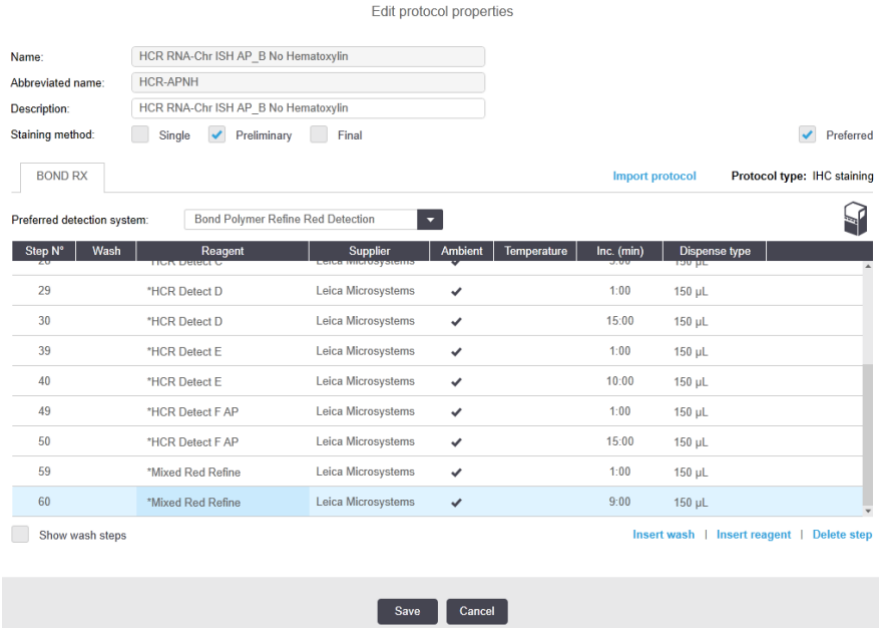
Creating an HCR™ Pro RNA-ISH + IHC/IF Co-Detection Protocol

The HCR™ Pro RNA-ISH + IHC/IF Co-Detection Protocols comprises a 3-plex sequential multiplex stain. The first protocol is the same as described previously. The second protocol is a modified version of the HCR™ Pro RNA-ISH protocol, excluding the counterstain reagents. The third protocol involves the IHC/IF assay, incorporating an alternative chromogen or fluorophore. The list below outlines the main steps required to perform an HCR™ Pro RNA-ISH + IHC/IF co-detection assay:

i. Remove the hematoxylin step from the HCR™ Pro RNA-CISH protocol for chromogenic detection (not applicable for fluorescent detection)
ii. Create an IHC/IF protocol from an existing IHC protocol
iii. Set up slides with three sequential staining protocols

Remove Hematoxylin from the HCR™ Pro RNA-CISH Protocol

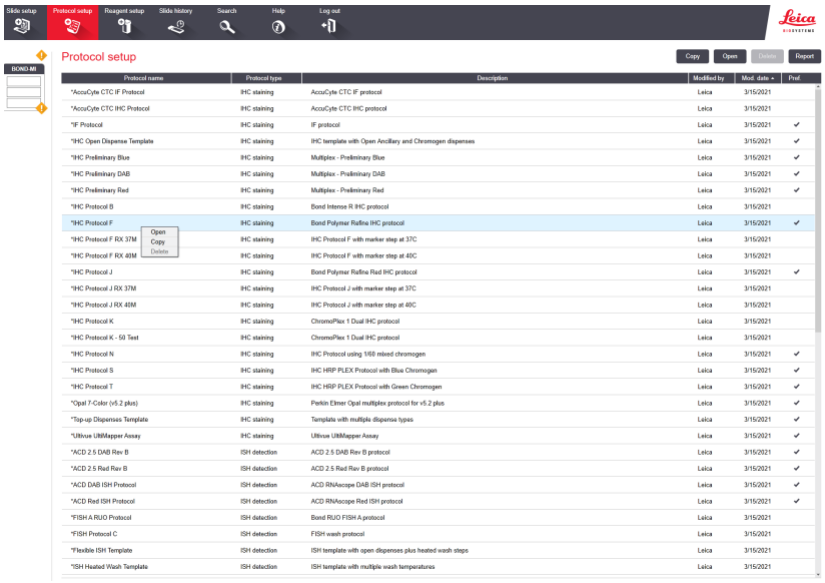
Since HCR™ Pro RNA-CISH is performed first, it is essential that you remove the hematoxylin and bluing steps at the end of the HCR™ Pro RNA-ISH protocol for chromogenic detection. Start by copying the previously created *HCR™ RNA-Chr ISH HRP/AP_B protocol and editing the **Name** to **HCR RNA-Chr ISH AP** (or HRP) **No Hematoxylin**. Enter your preferred **Abbreviated name**. Highlight the ***Hematoxylin** step and select **Delete step** in the lower right corner of the window. Do the same for all steps thereafter, including the **HCR™ Post-Process A** step and all of the ***Deionized Water** washing steps. For the **Staining method**, check **Preliminary** and click **Save**.



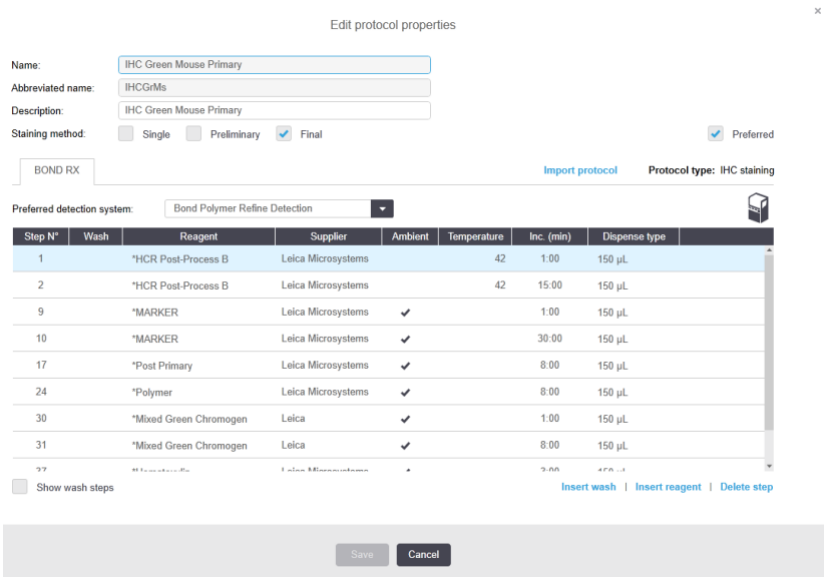
Create an IHC/IF Protocol from an Existing IHC Protocol

IHC Protocol

1. To create an IHC protocol, you can use ***IHC Protocol F** for HRP detection and ***IHC Protocol J** for AP detection.
    a. *Note: Be sure to select the protocol that uses the alternative chromogen corresponding to your second protocol.*

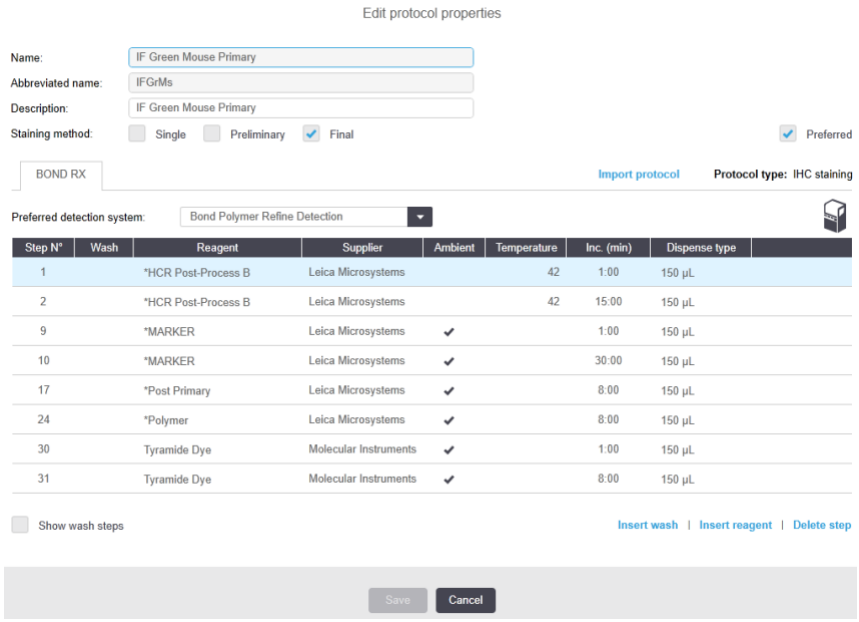


2. Click on the required protocol and select **copy**. Change the **Name**, **Abbreviated Name,** and **Description**. Modify the protocol by inserting ***HCR™ Post-Process B** steps, one extra ***MARKER** step, additional washing steps, and ***HCR™ Post-Process A** after the ***Hematoxylin** step. Check the **Final** box and click **Save**. Please see pages 32-35 (**Appendix D** and **Appendix E**) for the detailed staining protocols for IHC HRP and AP detections.



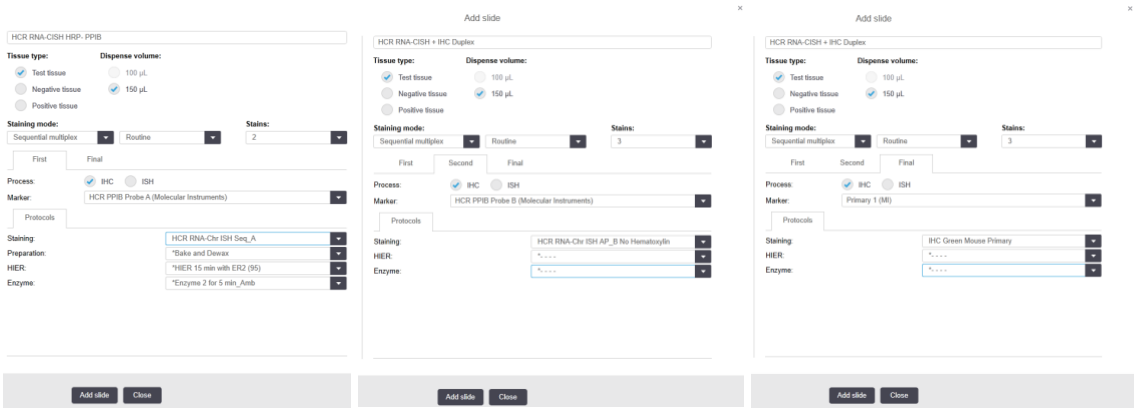
IF Protocol

1. To create an IF protocol:
    a. Repeat Steps 1-4 in creating the IHC protocol
    b. Replace chromogen deposition steps with third-party Tyramide dyes
    c. Remove **HCR™ Post-Process A**
2. Please see page 36 (**Appendix F**) for the detailed IF staining protocol.
    a. *Note: The Tyramide incubation time (step 31) is a parameter that you need to optimize based on the primary antibody and abundance level of the target.*



Set Up Slides with Three Sequential Staining Protocols

1. From the top navigation bar, click on **Slide setup** on the top of the screen and select **Add study**. Enter **Study ID**, **Study name**, and **Study comments**. Select ***Bake and Dewax** for the **Preparation protocol**. Click **OK.**
2. Click on **Add slide**, change the **Staining mode** to **Sequential multiplex** and choose **3** for **stains**. Under the **First** tab, the protocol selections are identical to performing HCR™ Pro RNA-CISH. Select the new hematoxylin-free HCR™ Pro RNA-ISH protocol that you created previously as the staining protocol under the **Second** tab. Under the **Final** tab, select the previously modified IHC/IF protocol as the staining protocol. Please reference the images down below for proper selections.



*Note: Leave **HIER** and **Enzyme** blank for Second and Final, unless the IHC targets require additional HIER or enzyme digestion for optimal staining. Click **Add slide**.*

Important Note: If a red chromogen is used for either ISH or IHC detection, slides cannot be dehydrated in alcohol. The slides need to be baked (~15 mins) or air-dried overnight prior to mounting.

Appendix

Appendix A: Detailed Staining Protocol for HCR™ RNA-Chr ISH Seq_A [Chromogenic Detection]

Step No.	Reagent	Step Type	Incubation Time	Temperature	Dispense Type
1	*Detection Wash	Reagent	0 min	Ambient	150 µL
2	*MARKER[1]	Reagent	1 min	45 °C	150 µL
3	*MARKER[1]	Reagent	75 min	45 °C	Intermediate
4	*BOND Wash Solution	Wash	0 min	45 °C	150 µL
5	*BOND Wash Solution	Wash	1 min	45 °C	150 µL
6	*BOND Wash Solution	Wash	1 min	45 °C	150 µL
7	*BOND Wash Solution	Wash	5 min	45 °C	150 µL
8	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
9	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
10	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
11	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
12	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
13	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
14	*BOND Wash Solution	Wash	0 min	Ambient	150 µL

[1]**MARKER refers to the HCR™ HiFi Probe 1A solution.*

Appendix B: Detailed Staining Protocol for HCR™ RNA-Chr ISH HRP_B and HCR™ RNA-Chr ISH AP_B [Chromogenic Detection]

Step No.	Reagent	Step Type	Incubation Time	Temperature	Dispense Type
1	*MARKER[1]	Reagent	1 min	45 °C	150 µL
2	*MARKER[1]	Reagent	75 min	45 °C	Intermediate
3	*BOND Wash Solution	Wash	0 min	45 °C	150 µL
4	*BOND Wash Solution	Wash	1 min	45 °C	150 µL
5	*BOND Wash Solution	Wash	1 min	45 °C	150 µL
6	*BOND Wash Solution	Wash	5 min	45 °C	150 µL
7	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
8	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
9	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
10	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
11	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
12	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
13	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
14	*HCR™ Detect A	Reagent	3 min	42 °C	150 µL
15	*HCR™ Detect B	Reagent	1 min	42 °C	150 µL
16	*HCR™ Detect B	Reagent	60 min	42 °C	Intermediate
17	*BOND Wash Solution	Wash	0 min	42 °C	150 µL
18	*BOND Wash Solution	Wash	1 min	42 °C	150 µL
19	*BOND Wash Solution	Wash	5 min	42 °C	150 µL
20	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
21	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
22	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
23	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
24	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
25	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
26	*BOND Wash Solution	Wash	1 min	Ambient	Open
27	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
28	*HCR™ Detect C	Reagent	5 min	Ambient	150 µL
29	*HCR™ Detect D	Reagent	1 min	Ambient	150 µL
30	*HCR™ Detect D	Reagent	15 min	Ambient	150 µL
31	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
32	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
33	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
34	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
35	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
36	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
37	*BOND Wash Solution	Wash	1 min	Ambient	Open
38	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
39	*HCR™ Detect E	Reagent	1 min	Ambient	150 µL

40	*HCR™ Detect E	Reagent	10 min	Ambient	150 µL
41	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
42	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
43	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
44	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
45	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
46	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
47	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
48	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
49	*HCR™ Detect F HRP#	Reagent	1 min	Ambient	150 µL
50	*HCR™ Detect F HRP#	Reagent	15 min	Ambient	150 µL
51	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
52	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
53	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
54	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
55	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
56	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
57	*BOND Wash Solution	Wash	1 min	Ambient	Open
58	*Deionized Water	Wash	1 min	Ambient	150 µL
59	*Mixed DAB Refine#	Reagent	1 min	Ambient	150 µL
60	*Mixed DAB Refine#	Reagent	7 min	Ambient	150 µL
61	*Deionized Water	Wash	0 min	Ambient	150 µL
62	*Deionized Water	Wash	0 min	Ambient	150 µL
63	*Deionized Water	Wash	0 min	Ambient	150 µL
64	*Deionized Water	Wash	0 min	Ambient	150 µL
65	*Deionized Water	Wash	0 min	Ambient	150 µL
66	*Deionized Water	Wash	0 min	Ambient	150 µL
67	*Hematoxylin#	Reagent	3 min	Ambient	150 µL
68	*Deionized Water	Wash	0 min	Ambient	150 µL
69	*Deionized Water	Wash	0 min	Ambient	150 µL
70	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
71	*Deionized Water	Wash	0 min	Ambient	150 µL
72	*Deionized Water	Wash	0 min	Ambient	150 µL
73	*Deionized Water	Wash	0 min	Ambient	150 µL
74	*HCR™ Post-Process A	Reagent	5 min	Ambient	150 µL
75	*Deionized Water	Wash	0 min	Ambient	150 µL
76	*Deionized Water	Wash	0 min	Ambient	150 µL
77	*Deionized Water	Wash	0 min	Ambient	150 µL
78	*Deionized Water	Wash	0 min	Ambient	150 µL
79	*Deionized Water	Wash	0 min	Ambient	150 µL

[1] **MARKER refers to the HCR™ HiFi Probe 1B solution.*

#For AP-based detection, please make changes to steps 49, 50, 59, and 60 as follows:

49	*HCR™ Detect F AP#	Reagent	1 min	Ambient	150 µL
50	*HCR™ Detect F AP#	Reagent	15 min	Ambient	150 µL

59	*Mixed Red Refine	Reagent	1 min	Ambient	150 µL
60	*Mixed Red Refine	Reagent	9 min	Ambient	150 µL

Appendix C: Detailed Staining Protocol for HCR™ RNA-FISH_B [Fluorescent Detection]

Step No.	Reagent	Step Type	Incubation Time	Temperature	Dispense Type
1	*Detection Wash	Wash	0 min	Ambient	150 µL
2	*MARKER[1]	Reagent	1 min	45 °C	150 µL
3	*MARKER[1]	Reagent	75 min	45 °C	Intermediate
4	*BOND Wash Solution	Wash	0 min	45 °C	150 µL
5	*BOND Wash Solution	Wash	1 min	45 °C	150 µL
6	*BOND Wash Solution	Wash	1 min	45 °C	150 µL
7	*BOND Wash Solution	Wash	5 min	45 °C	150 µL
8	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
9	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
10	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
11	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
12	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
13	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
14	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
15	*HCR™ Detect A	Reagent	3 min	42 °C	150 µL
16	*HCR™ Detect B	Reagent	1 min	42 °C	150 µL
17	*HCR™ Detect B	Reagent	60 min	42 °C	Intermediate
18	*BOND Wash Solution	Wash	0 min	42 °C	150 µL
19	*BOND Wash Solution	Wash	1 min	42 °C	150 µL
20	*BOND Wash Solution	Wash	5 min	42 °C	150 µL
21	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
22	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
23	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
24	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
25	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
26	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
27	*BOND Wash Solution	Wash	1 min	Ambient	Open
28	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
29	*HCR™ Detect C	Reagent	5 min	Ambient	150 µL
30	*HCR™ Detect D	Reagent	1 min	Ambient	150 µL
31	*HCR™ Detect D	Reagent	15 min	Ambient	150 µL
32	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
33	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
34	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
35	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
36	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
37	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
38	*BOND Wash Solution	Wash	1 min	Ambient	Open
39	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
40	*HCR™ Detect E	Reagent	1 min	Ambient	150 µL

41	*HCR™ Detect E	Reagent	10 min	Ambient	150 µL
42	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
43	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
44	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
45	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
46	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
47	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
48	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
49	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
50	*HCR™ Detect F HRP#	Reagent	1 min	Ambient	150 µL
51	*HCR™ Detect F HRP#	Reagent	15 min	Ambient	150 µL
52	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
53	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
54	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
55	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
56	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
57	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
58	*BOND Wash Solution	Wash	1 min	Ambient	Open
59	*Deionized Water	Wash	1 min	Ambient	150 µL
60	Tyramide	Reagent	1 min	Ambient	150 µL
61	Tyramide	Reagent	15 min	Ambient	150 µL
62	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
63	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
64	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
65	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
66	*BOND Wash Solution	Wash	0 min	Ambient	150 µL

[1]**MARKER refers to the HCR™ HiFi Probe 1B solution.*

Appendix D: Detailed IHC HRP Detection Staining Protocol for HCR™ Pro RNA-CISH + IHC Co-Detection

Step No.	Reagent	Step Type	Incubation Time	Temperature	Dispense Type
1	*HCR™ Post-Process B	Reagent	1 min	42 °C	150 µL
2	*HCR™ Post-Process B	Reagent	15 min	42 °C	150 µL
3	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
4	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
5	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
6	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
7	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
8	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
9	*MARKER[1]	Reagent	1 min	Ambient	150 µL
10	*MARKER[1]	Reagent	30 min	Ambient	150 µL
11	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
12	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
13	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
14	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
15	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
16	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
17	*Post Primary[2]	Reagent	8 min	Ambient	150 µL
18	*BOND Wash Solution[2]	Wash	0 min	Ambient	150 µL
19	*BOND Wash Solution[2]	Wash	0 min	Ambient	150 µL
20	*BOND Wash Solution[2]	Wash	0 min	Ambient	150 µL
21	*BOND Wash Solution[2]	Wash	2 min	Ambient	150 µL
22	*BOND Wash Solution[2]	Wash	2 min	Ambient	150 µL
23	*BOND Wash Solution[2]	Wash	2 min	Ambient	150 µL
24	*Polymer	Reagent	8 min	Ambient	150 µL
25	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
26	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
27	*BOND Wash Solution	Wash	2 min	Ambient	150 µL
28	*BOND Wash Solution	Wash	2 min	Ambient	150 µL
29	*Deionized Water	Wash	0 min	Ambient	150 µL
30	*Mixed Green Chromogen[#]	Reagent	1 min	Ambient	150 µL
31	*Mixed Green Chromogen[#]	Reagent	8 min	Ambient	150 µL
32	*Deionized Water	Wash	0 min	Ambient	150 µL
33	*Deionized Water	Wash	0 min	Ambient	150 µL
34	*Deionized Water	Wash	0 min	Ambient	150 µL
35	*Deionized Water	Wash	0 min	Ambient	150 µL
36	*Deionized Water	Wash	0 min	Ambient	150 µL
37	*Hematoxylin	Reagent	3 min	Ambient	150 µL
38	*Deionized Water	Wash	0 min	Ambient	150 µL

39	*Deionized Water	Wash	0 min	Ambient	150 µL
40	*Deionized Water	Wash	0 min	Ambient	150 µL
41	*Deionized Water	Wash	0 min	Ambient	150 µL
42	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
43	*Deionized Water	Wash	0 min	Ambient	150 µL
44	*HCR™ Post-Process A	Reagent	5 min	Ambient	150 µL
45	*Deionized Water	Wash	0 min	Ambient	150 µL
46	*Deionized Water	Wash	0 min	Ambient	150 µL
47	*Deionized Water	Wash	0 min	Ambient	150 µL
48	*Deionized Water	Wash	0 min	Ambient	150 µL
49	*Deionized Water	Wash	0 min	Ambient	150 µL

[1]**MARKER refers to the primary antibody.*

[2]*These steps may be deleted if you are using a primary antibody that is already cloned from rabbit.*

[#]You can switch to using the DAB chromogen by making changes to steps 30 and 31 as follows:

30	*Mixed DAB Refine	Reagent	0 min	Ambient	150 µL
31	*Mixed DAB Refine	Reagent	10 min	Ambient	150 µL

Appendix E: Detailed IHC AP Detection Staining Protocol for HCR™ Pro RNA-CISH + IHC Co-Detection

Step No.	Reagent	Step Type	Incubation Time	Temperature	Dispense Type
1	HCR™ Post-Process B	Reagent	1 min	42 °C	150 µL
2	HCR™ Post-Process B	Reagent	15 min	42 °C	150 µL
3	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
4	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
5	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
6	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
7	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
8	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
9	*MARKER[1]	Reagent	1 min	Ambient	150 µL
10	*MARKER[1]	Reagent	30 min	Ambient	150 µL
11	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
12	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
13	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
14	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
15	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
16	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
17	*Post Primary AP	Reagent	20 min	Ambient	150 µL
18	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
19	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
20	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
21	*BOND Wash Solution	Wash	2 min	Ambient	150 µL
22	*BOND Wash Solution	Wash	2 min	Ambient	150 µL
23	*BOND Wash Solution	Wash	2 min	Ambient	150 µL
24	*Polymer AP	Reagent	30 min	Ambient	150 µL
25	*BOND Wash Solution	Wash	2 min	Ambient	150 µL
26	*BOND Wash Solution	Wash	2 min	Ambient	150 µL
27	*BOND Wash Solution	Wash	5 min	Ambient	Intermediate
28	*BOND Wash Solution	Wash	2 min	Ambient	150 µL
29	*BOND Wash Solution	Wash	0min	Ambient	150 µL
30	*Deionized Water	Wash	0 min	Ambient	150 µL
31	*Mixed Red Refine	Reagent	10 min	Ambient	150 µL
32	*Mixed Red Refine	Reagent	5 min	Ambient	150 µL
33	*Deionized Water	Wash	0 min	Ambient	150 µL
34	*Deionized Water	Wash	0 min	Ambient	150 µL
35	*Deionized Water	Wash	0 min	Ambient	150 µL
36	*Deionized Water	Wash	0 min	Ambient	150 µL
37	*Deionized Water	Wash	0 min	Ambient	150 µL
38	*Hematoxylin	Reagent	3 min	Ambient	150 µL
39	*Deionized Water	Wash	0 min	Ambient	150 µL

40	*Deionized Water	Wash	0 min	Ambient	150 µL
41	*Deionized Water	Wash	0 min	Ambient	150 µL
42	*Deionized Water	Wash	0 min	Ambient	150 µL
43	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
44	*Deionized Water	Wash	0 min	Ambient	150 µL
44	*HCR™ Post-Process A	Reagent	5 min	Ambient	150 µL
45	*Deionized Water	Wash	0 min	Ambient	150 µL
46	*Deionized Water	Wash	0 min	Ambient	150 µL
47	*Deionized Water	Wash	0 min	Ambient	150 µL
48	*Deionized Water	Wash	0 min	Ambient	150 µL
49	*Deionized Water	Wash	0 min	Ambient	150 µL

[1] **MARKER refers to the primary antibody.*

Appendix F: Detailed IF Detection Staining Protocol for HCR™ Pro RNA-FISH + IF Co-Detection

Step No.	Reagent	Step Type	Incubation Time	Temperature	Dispense Type
1	*HCR™ Post-Process B	Reagent	1 min	42 °C	150 µL
2	*HCR™ Post-Process B	Reagent	15 min	42 °C	150 µL
3	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
4	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
5	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
6	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
7	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
8	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
9	*MARKER[1]	Reagent	1 min	Ambient	150 µL
10	*MARKER[1]	Reagent	30 min	Ambient	150 µL
11	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
12	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
13	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
14	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
15	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
16	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
17	*Post Primary	Reagent	8 min	Ambient	150 µL
18	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
19	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
20	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
21	*BOND Wash Solution	Wash	2 min	Ambient	150 µL
22	*BOND Wash Solution	Wash	2 min	Ambient	150 µL
23	*BOND Wash Solution	Wash	2 min	Ambient	150 µL
24	*Polymer	Reagent	8 min	Ambient	150 µL
25	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
26	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
27	*BOND Wash Solution	Wash	2 min	Ambient	150 µL
28	*BOND Wash Solution	Wash	2 min	Ambient	150 µL
29	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
30	Tyramide Dye	Reagent	1 min	Ambient	150 µL
31	Tyramide Dye	Reagent	8 min	Ambient	150 µL
32	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
33	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
34	*BOND Wash Solution	Wash	0 min	Ambient	150 µL
35	*BOND Wash Solution	Wash	1 min	Ambient	150 µL
36	*BOND Wash Solution	Wash	0 min	Ambient	150 µL

[1]**MARKER refers to the primary antibody.*

Appendix G: Third-Party Recommended Tyramide Dyes

Validated Tyramide Dyes	Incubation Time	Recommended Starting Concentration	Vendor	Catalog #
CF488A	8-24 min	5 µM	Biotium	92171
CF550R	8-24 min	5 µM	Biotium	96077
CF555	8-24 min	5 µM	Biotium	96021
CF583R	8-24 min	5 µM	Biotium	96085
CF594	8-24 min	5 µM	Biotium	92174
CF640R	8-24 min	5 µM	Biotium	92175
CF754	8-24 min	5 µM	Biotium	96090
Alexa Fluor 488 - Tyramide	8-24 min	1X	ThermoFisher	B40953
Alexa Fluor 546 - Tyramide	8-24 min	1X	ThermoFisher	B40954
Alexa Fluor 647 - Tyramide	8-24 min	1X	ThermoFisher	B40958
Alexa Fluor 750 - Tyramide	8-24 min	1X	ThermoFisher	B56131

Appendix H: Create a Staining Protocol using Matisse® Chromogens

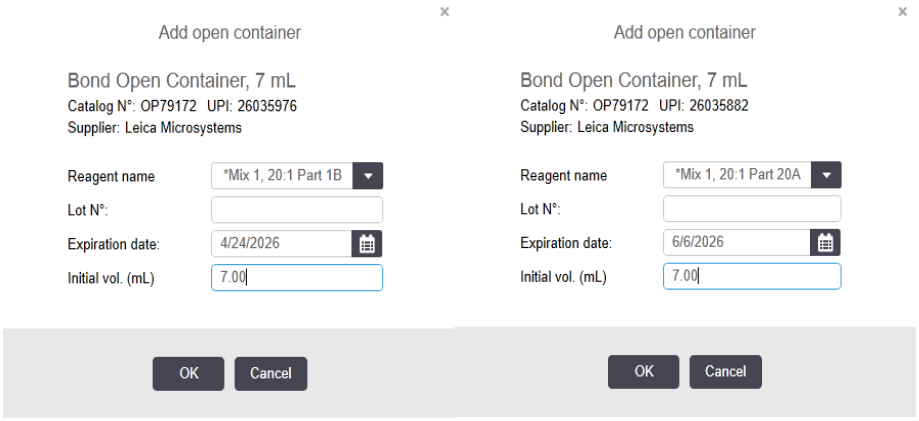
The two main steps in setting up an HCR™ Pro RNA-ISH with the Matisse® chromogens are:

1. Register and fill two 7 mL (or 30 mL) Open BOND Containers for the Matisse® chromogen and one 7 mL (or 30 mL) Open BOND Container for the hematoxylin counterstain
2. Create a custom staining protocol with the Matisse® chromogen

Register the Matisse® Chromogen and Hematoxylin Counterstain

Online mixing of chromogens requires registering and filling two 7 mL BOND Open Containers. The following example demonstrates the registration process for the Matisse® Red chromogen; however, these steps can be similarly applied to register other Matisse® chromogens.

1. Register two 7 mL BOND Containers for mixing at a ratio of 20:1 as shown below:



2. Transfer 1.5 mL of the Matisse® Red Chromogen Substrate and 7 mL of the Matisse® Red Chromogen Buffer to **Mix 1, 20:1 Part 1B** and **Mix 1, 20:1 Part 20A** BOND Containers, respectively. The table below outlines the required volumes for both 20-slide and 90-slide scales across our range of Matisse® chromogens. Make sure you input the correct mixing ratios specific to the Matisse® chromogen used in your HCR™ Pro RNA-ISH assay.

Matisse® Chromogens	Reagents	Volume Transferred to BOND Containers (20-Slide Kit)	Volume Transferred to BOND Containers (90-Slide Kit)	Name of Registered BOND Containers
Matisse® Red	Matisse® Red	1.5 mL[1]	2.5 mL[1]	***Mix 1, 20:1 Part 1B**
	Matisse® Red Buffer	7 mL[1]	29 mL[2]	***Mix 1, 20:1 Part 20A**
Matisse® DAB (Brown)	Matisse® DAB	2.5 mL[1]	7 mL[1]	***Mix 9, 6:1 Part 1B**
	Matisse® DAB Buffer	7 mL[1]	29 mL[2]	***Mix 9, 6:1 Part 6A**
Matisse® Green	Matisse® Green	4 mL[1]	12.5 mL[2]	***Mix 2, 2:1 Part 1B**
	Matisse® Green Buffer	7 mL[1]	29 mL[2]	***Mix 2, 2:1 Part 2A**

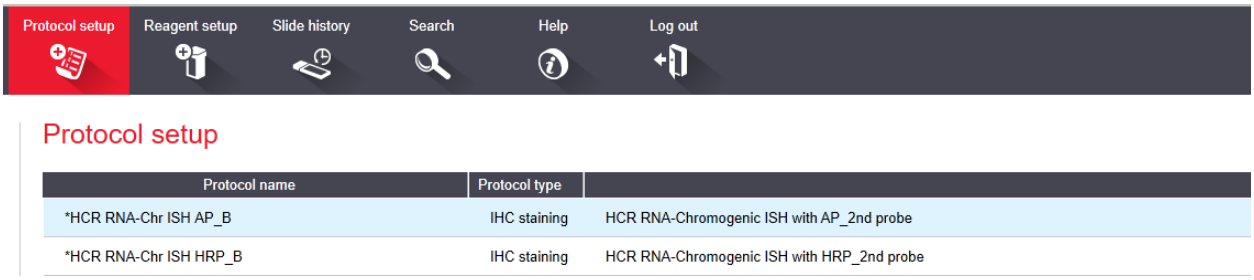
[1]*Use of 7 mL BOND Open Container is recommended.*
[2]*Use of 30 mL BOND Open Container is recommended. If 7 mL Containers are used, you can refill with the chromogen and chromogen buffers on an as-need basis.*

3. Register another 7 mL (or 30 mL) BOND Open Container and name it **Hematoxylin**. Transfer the entirety of the provided Hematoxylin into this container.

Create a Custom Staining Protocol with the Matisse® Chromogen

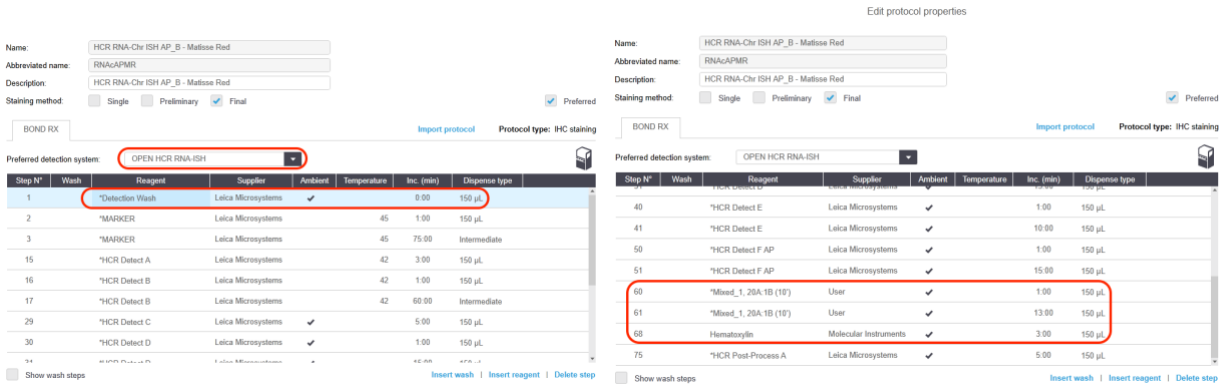
To add Matisse® chromogens to the staining protocol, you need to create a custom staining protocol by using a pre-existing HCR™ Pro RNA-ISH protocol (e.g., *HCR RNA-Chr ISH AP_B) as a template:

1. From the top navigation bar, click on **Protocol setup** on the top of the screen and select **Staining** for the protocol group and **Leica Microsystem** for **Protocol origin**. Select ***HCR RNA-Chr ISH AP_B (or *HCR RNA-Chr ISH HRP_B if you plan to use HRP-based chromogens)**, and click on the **Copy** button near the top-right corner.



2. Modify the protocol:
   a. Select the same preferred detection system (e.g., OPEN HCR RNA-ISH) as you created previously under the “Create Staining Protocols for HCR™ Pro RNA-CISH” section on page 13.
   b. Add one “*Detection Wash” step as the first step.
   c. Replace Leica Chromogens (e.g., *Mixed Red Refine) with user-defined mixing steps (e.g., *Mixed_1, 20A:1B (10’)).
      i. **Note**: Make sure mixing steps such as *Mixed_1, 20A:1B (10’) are preferred before they can be used in the staining protocol.
   d. Replace *Hematoxylin from LBS with Hematoxylin from Molecular Instruments.

The staining protocol should resemble the images shown below:



Please note that Matisse® Red chromogens are typically only stable within 30 minutes after mixing. Thus, it is important to select Mixed_1, 20A:1B **(10’)** as opposed to Mixed_1, 20A:1B **(300’)**. Refer to the

table below for detailed information on incubation times and names of the chromogenic deposition steps.

Chromogen	Recommended Chromogen Incubation Time (2 steps)	Name of the Chromogenic Deposition Steps
Matisse® Red	1 min	**Mixed_1 20A:1B (10')**
	13 min	
Matisse® DAB (Brown)	1 min	**Mixed_1 6A:1B (10') or Mixed_1 6A:1B (300')**
	13 min	
Matisse® Green	1 min	**Mixed_2 2A:1B (10')**

Appendix I: HCR™ HiFi Probe Preparation Guide for 50× Concentrated Probes

In some experiments (e.g., when both probes target short transcripts), HCR™ HiFi Probe 1A and 1B are supplied at 50× concentration. In this case, your kit will include additional Probe Diluent reagents to bring HiFi Probe 1A/1B to working concentration. This section provides a detailed breakdown of how to calculate the required volumes of HCR™ HiFi Probe 1A, 1B, and their respective diluents when the probes are supplied at 50× concentration.

Important: For short target applications, HiFi Probes must be freshly prepared (within 48 hours of each experiment). Please note that a unique probe diluent reagent is provided specifically for use with short targets. This short target-specific diluent must be used when preparing probe solutions for short targets.

HCR™ HiFi Probe 1A/1B = 50×

When both probes are at 50x concentration, HCR™ HiFi Probe A/B Diluents are required to prepare your probe solution.

1. **Calculate the Volume of HCR™ HiFi Probe A/B Diluents**: To determine the total volume (V_D) of HCR™ HiFi Probe A/B Diluent required for your selected number of slides (N_S), use the following equation:

$$\mathbf{V_D = 300 * N_S + V_C}$$

Where V_C is the recommended dead volume for the BOND RX/RX^m as listed below:

i. 580 µL for a 6 mL Titration Container
ii. 835 µL for a 7 mL Open Container
iii. 1898 µL for a 30 mL Open Container

2. **Calculate the Volume of 50× Concentrated HCR™ HiFi Probe A/B:** To determine the volume of concentrated probe (V_P) needed, divide the previously calculated diluent volume (V_D) by 50:

$$\mathbf{V_P = V_D / 50}$$

After thoroughly mixing the 50× HiFi Probe concentrate and probe diluent, transfer the HCR™ HiFi Probe A/B Solutions to BOND Containers. For optimal performance, use the prepared probe solutions within 48 hours.

The table below shows an example of how to calculate the individual component volumes required to stain **4** slides using a 7 mL Open Container:

Container Type	HCR™ HiFi Probe A & B Diluent Volume	50× HCR™ HiFi Probe A & B Volume	Component Volumes of HCR™ HiFi Probe A and B Solution
Dispenser	$V_D = 300 * \mathbf{4} + 835$ $V_D = 2035\ \mu L$	$V_P = 2035 / 50$ $V_P = 41\ \mu L$	**HCR™ HiFi Probe A Solution** 41 µL HiFi Probe 1A 2035 µL HiFi Probe A Diluent **HCR™ HiFi Probe B Solution** 41 µL HiFi Probe 1B 2035 µL HiFi Probe B Diluent

The table below outlines the individual component volumes required to stain 3, 5, 10, and 20 slides using a 7 mL Open Container:

# of Slides	HCR™ HiFi Probe A/B Diluent Volume	HCR™ HiFi Probe 1A/1B Volume
3	1735 µL	35 µL
5	2335 µL	47 µL
10	3835 µL	77 µL
20	6835 µL	137 µL

After preparing the HCR™ HiFi Probe A and B solutions, proceed with the HCR™ Pro RNA-ISH assay as described above.

Appendix J: Tips & Tricks

Adjusting Signal Intensity with HCR™ Pro RNA-ISH

The HCR™ Pro RNA-ISH signal intensity can be adjusted using various parameters to either decrease or increase the strength of the signal.

If you would like to tune down the signal intensity to facilitate data interpretation (e.g., dot counting), we recommend the following options:

Level of Tuning	Guidelines
Coarse-tuning[1]	To significantly reduce signal intensity, you can reduce the incubation time of **HCR™ Detect E** (Step #53 as written in Appendix A and B) from 10 minutes to **5 minutes**.
Fine-tuning[2]	To finely reduce signal intensity, you can reduce the incubation time of **HCR™ Detect B** (Step #29 as written in Appendix A and B) from 60 minutes to **45 minutes**.

Note: If further signal reduction is desired, you can also try reducing the **chromogen deposition** (Step #73 as written in Appendix A) time from 9 minutes to **7 minutes**. (This only applies if you are using a red chromogen.)

If you would like to increase the signal intensity, you can try the following:

Level of Tuning	Guidelines
Coarse-tuning[1]	Increase the **Antigen Retrieval** time from 15 minutes to up to **30 minutes** or increase the incubation time of **HCR™ Detect E** (Step #53 as written in Appendix A and B) from 10 minutes to **15-20 minutes**.
Fine-tuning[2]	Increase the incubation time of **HCR™ Detect B** (Step #29 as written in Appendix A and B) from 60 minutes to **120 minutes**.

Note: If further signal enhancement is desired, you can increase the incubation time of **HCR™ Detect D** and **HCR™ Detect F** from 15 minutes to **20 minutes** (Steps #43 and #63, respectively, as written in Appendix A and B).

[1]*Use coarse-tuning if the signal needs to be adjusted significantly.*
[1]*Use fine-tuning if the signal only needs to be tuned up/down slightly.*

Increasing RNA Signal Performance

We have three recommendations for increasing the performance of RNA signal with your HCR™ HiFi Probe:

1. To open up the tissue more (i.e., break the cross-linkage caused by formalin fixation) and help accessibility of the HCR™ HiFi Probe and Detect reagents to the target, increase the **Antigen Retrieval** time from 15 to up to **30 minutes**.
2. Though we recommend a 1.25-hour *Marker incubation (the probe hybridization time), this may not be optimal across all tissue types. The incubation time for this step (Steps #3 and #16 as written in Appendix A and B) can be increased from 1.25 hours to **2 hours** to allow sufficient time for probes to diffuse in and bind to their targets.
3. A hallmark feature of the HCR™ Pro RNA-ISH assay is that it has been optimized to not require protease digestion. However, there are rare cases in which protease can help increase the accessibility of the HCR™ HiFi Probe to the target molecule. In these cases, incorporating a mild protease step can result in improvements, but could negatively impact co-detection capabilities. We suggest using the BOND Enzyme Pretreatment kit [AR9551] with the following parameters: **0.5 µg/mL, 1:34000 at 37 °C for 15 minutes.**

Appendix K: FAQ

1. **How do I incorporate HCR™ Pro RNA-ISH into existing IHC/IF assays?**
    a. Please check out this blog post for a general overview of HCR™ Pro RNA-ISH and IHC/IF co-detection. For the IHC/IF portion of the protocol, you can use any off-the-shelf primary antibody and detection kit you have already validated, such as Leica's BOND Polymer Refine Detection Kit, from Leica Biosystems. There are no additional reagents that are required from MI to perform an IHC/IF co-detection assay with HCR™ Pro RNA-ISH.
2. **Which chromogens do you recommend for an HCR™ Pro RNA-ISH + IHC co-detection assay?**
    a. We recommend using a red chromogen for RNA-ISH and a green chromogen for IHC (e.g., BOND Green Chromogen, from Leica Biosystems [DS9914]). This combination provides a nice contrast between targets, but you are not limited to these colors in any technical way.
3. **Which detection systems do you recommend using with the HCR™ Membrane Stain?**
    a. For ready-to-use amplified detection, we suggest using the BOND Polymers (either HRP or AP conjugated). For direct-labeled detection, we recommend using either the Donkey Anti Rabbit (IgG) secondary antibody (Alexa Fluor 647) or the Donkey Anti Rabbit (IgG) secondary antibody (Alexa Fluor 555).
4. **I notice bubbles on my slides that have little to no staining. How do I resolve this?**
    a. In general, we advise that you follow these steps:
        i. Check that you programmed each step of our protocol correctly (reference Appendix A-E). Double-check that **Open** is selected as the Dispense Type for **Steps #26**, **#37**, and **#57** (as written in Appendix B).
            1. The Open Wash dispense type serves to eliminate bubbles trapped beneath the covertile, so it is essential that you ensure that the correct dispense type is selected.
        ii. Contact your Leica FAS/FSE.
    b. Skip to this section if you noticed bubbles with HCR™ Pro RNA-ISH + IHC/IF co-detection and observed no impact to your ISH signal.
        i. In this case, we suggest that you implement an open wash step as the bubbles may have originated from either the HCR™ Post-Process B reagent or the primary antibody. Since the primary diluent is slightly more viscous, it can lead to additional bubbles during the transfer of the reagent to the BOND Container.
        ii. First, adjust the dispense type to **Open Wash** for **Steps #7** and **#15** (as written in Appendix D and E).
        iii. Ensure there are no excessive bubbles inside the BOND Container filled with the primary antibody.
        iv. Rescan and electronically refill BOND Containers to allow the autostainer to remeasure the volumes.

5. **How do I lighten the hematoxylin staining on my tissues?**
    a. You can try either of the following two options outlined below to lighten hematoxylin staining:
        i. Decrease the **Hematoxylin** incubation time from 3 minutes to **1 or 2 minutes**.
        ii. Dilute 1/3 of the **Hematoxylin** reagent with DI water and use this as a counterstain. For this option, please note that you will need to purchase a separate Hematoxylin reagent from Leica Biosystems and do a 3-fold dilution. You will also need to register a separate BOND Container to use this reagent in the assay.
6. **Can I skip post-process A(bluing step) after hematoxylin counterstain step?**
    a. Yes, you can! The BOND washes towards the end of the protocol can help with tissue bluing, so if you are happy with your results, feel free to omit bluing entirely.
7. **Do you use ER1 or ER2?**
    a. We use ER2 for HCR™ Pro RNA-ISH. If your protein target requires ER1, then you can perform the ISH staining first and incorporate a separate ER1 step when conducting the IHC/IF staining.
8. **I'd like to incorporate protease in my sample. What are your suggestions?**
    a. Our HCR™ Pro RNA-ISH protocol does not require any protease digestion, thereby supporting native compatibility with IHC/IF and preserving tissue morphology. However, we understand that there are certain situations where you may want to incorporate protease in your sample like over-fixed tissue samples or the need to unmask epitopes for certain IHC/IF targets (e.g., for an ISH + IHC/IF co-detection assay).
    b. In such cases, we recommend that you use a light protease digestion at a diluted level and/or increase both *Marker incubation (**probe hybridization step)** and **amplification** (Step #16 as written in Appendix B) times to **2 hours**. As a starting point, you can choose to dilute your protease to **0.5 µg/mL at 37 °C**, and treat your sample for **5-10 minutes**. The digestion step (e.g., followed by 3-4 BOND Washing steps) can be added in the beginning of the "HCR™ RNA-Chr ISH Seq_A" protocol (see Appendix A) before the *Detection Wash step. Please feel free to adjust the concentration later on as needed.