HCR™ Pro RNA-ISH Setup Guide for the BOND-III

This Setup Guide demonstrates the use of an HCR™ Pro RNA-ISH kit on the BOND-III platform from Leica Biosystems. Reagent preparation steps, including registering individual BOND Containers for their respective reagents, will be described in further detail. Each BOND-III run takes approximately 13-14.5 hours followed by a short post-processing of stained slides. This time range depends on the number of slides processed in a single run. Processing 5 slides takes around 13 hours, and processing 30 slides takes around 14.5 hours due to the time the BOND-III platform takes 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.

Table of Contents

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 Kit

The HCR™ Pro RNA-ISH kit consists of an HCR™ HiFi Probe and HCR™ Pro 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 A.

HCR™ HiFi Probe

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

[1]*HCR™ HiFi Probes are platform-specific. For example, probes designed for the BOND RX autostainer are not compatible with the BOND-III, and vice versa.*

HCR™ Pro Detect

Please note that all HCR™ Pro 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
Pretreat	5 mL	21 mL	2 to 8 °C
HCR™ Pro Detect A	5 mL	21 mL	2 to 8 °C
HCR™ Pro Detect B	7 mL	29 mL	2 to 8 °C
HCR™ Pro Detect C	5 mL	21 mL	2 to 8 °C
HCR™ Pro Detect D	7 mL	29 mL	2 to 8 °C
HCR™ Pro Detect E	7 mL	29 mL	2 to 8 °C
HCR™ Pro Detect F AP/HRP[1]	7 mL	29 mL	2 to 8 °C
Post-Process A	5 mL	21 mL	2 to 8 °C
Post-Process B[2]	7 mL	29 mL	2 to 8 °C

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

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

Required Materials for BOND-III

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.

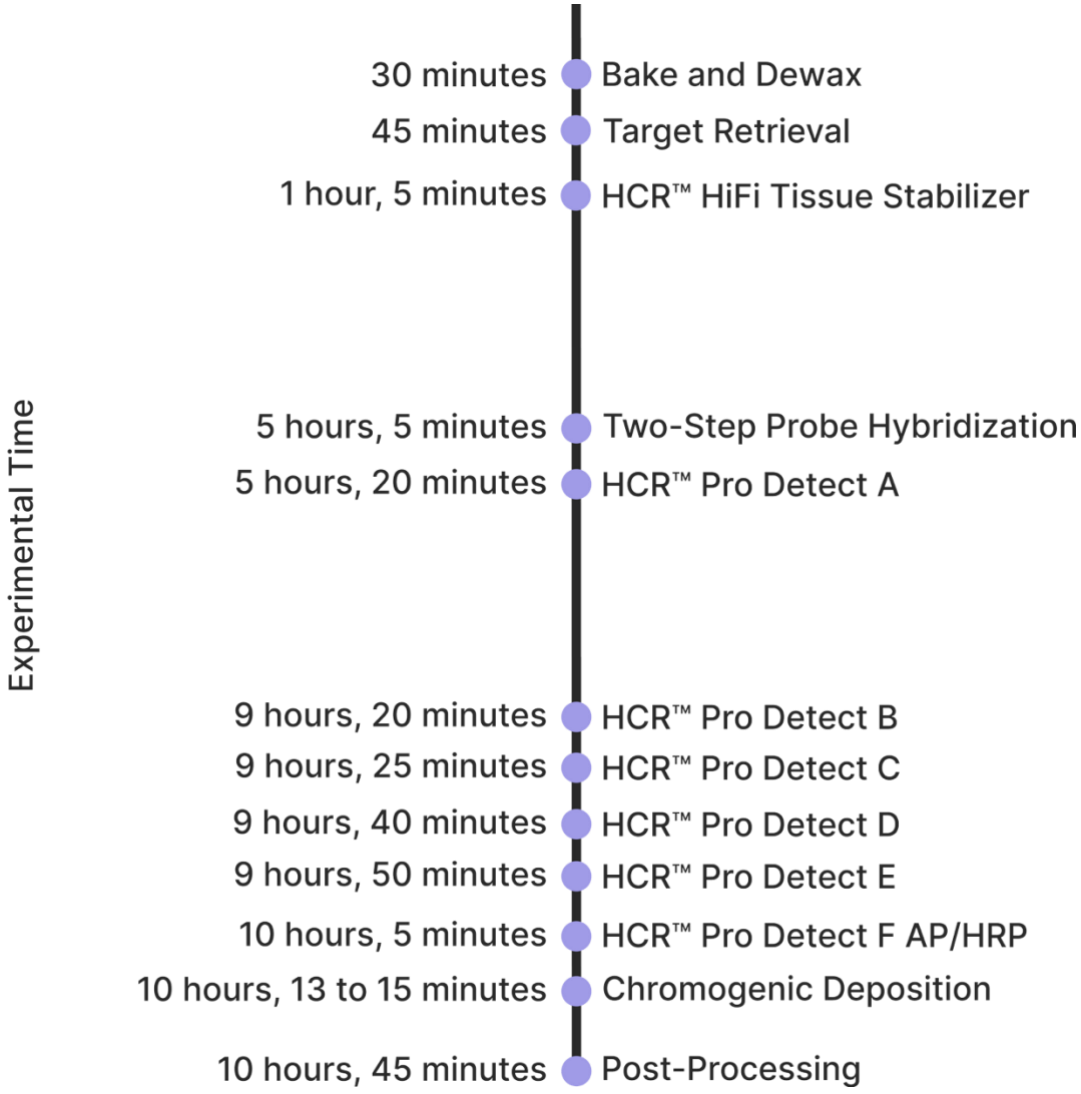
Materials from Leica Biosystems		
	Catalog #	**Storage Temperature**
BOND 6 mL Titration Kit	OPT9049	RT
BOND 7 mL Open Container	OP79193	RT
BOND Universal Covertiles 160 Pack	S21.4611	RT
BOND Epitope Retrieval Solution 2	AR9640	2 to 8 °C
BOND Dewax Solution	AR9222	2 to 26 °C
BOND Wash Solution 10X Concentrate	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 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.*

User-Supplied Materials

Materials from Other Vendors		
	Supplier	**Comment**
FFPE Sample Slides	Any	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
Cover Glass	Any	Dimension depends on the size of the tissue
100% Ethanol	Any	None

Overall Workflow of the HCR™ Pro RNA-ISH Protocol



As mentioned earlier, each BOND-III run takes approximately 13 to 14.5 hours. The timeline above only accounts for 10 hours and 45 minutes of this run, as the remaining time comes from the additional BOND-III washing steps.

Set Up BOND-III Protocols

The HCR™ Pro RNA-ISH assay utilizes an IHC protocol to employ a two-step probe hybridization approach. HCR™ HiFi Probe A and Probe B are registered as the reagent types "primary antibody" and "ancillary," respectively. As a result, HCR™ HiFi Probe A will be denoted as *Marker and HCR™ HiFi Probe B will retain its name in the staining protocol. *Please note that a separate protocol is needed for every target probe (e.g., two separate staining protocols are needed for running DapB and PPIB probes).*

The five main steps in setting up a BOND-III protocol for an HCR™ Pro RNA-ISH run are:

i. Register BOND Containers associated with their respective reagents (if needed)
ii. Create a staining protocol for your custom probe(s) using an existing HCR™ Pro RNA-ISH protocol
iii. Register your custom HCR™ HiFi Probe A(s) as primary antibodies
iv. Set up slides and print labels

Register BOND Containers

In order to create an HCR™ Pro RNA-ISH protocol on the BOND-III, reagent names associated with the BOND Containers must be registered in the BOND-III software. The table below lists all the BOND Containers that need to be registered for the HCR™ Pro RNA-ISH assay.

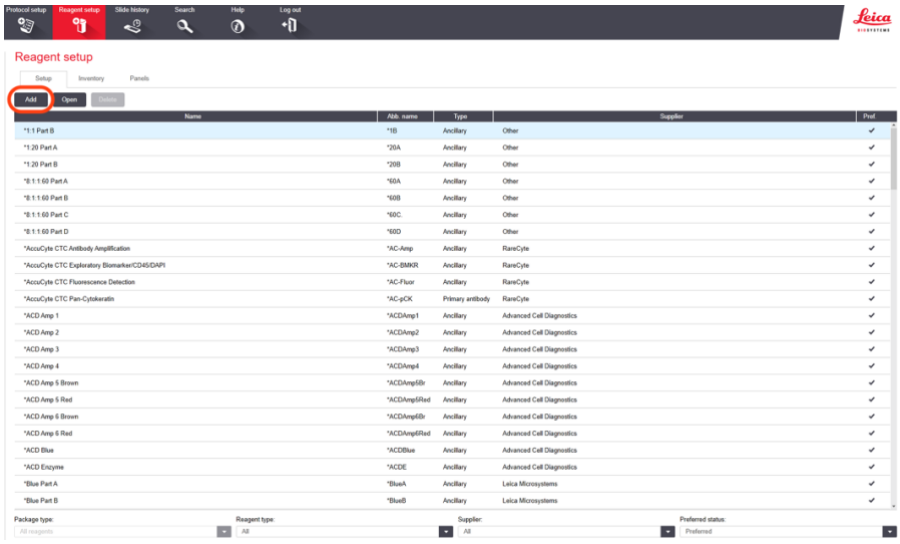
BOND Containers			
Name	**Type of Container[1]**	**Type of Reagent**	**Reagent Registration required?**
*Enzyme 2[2]	7 mL or 30 mL BOND Container	N/A	No
HCR™ HiFi Tissue Stabilizer	7 mL or 30 mL BOND Container	Ancillary	Yes
HCR™ HiFi Probe 1A	6 mL, 7 mL, or 30 mL Container	Primary antibody[3]	Yes
HCR™ HiFi Probe 1B	6 mL, 7 mL, 30 mL Container	Ancillary	Yes
HCR™ Pro Detect A	7 mL or 30 mL BOND Container	Ancillary	Yes
HCR™ Pro Detect B	7 mL or 30 mL BOND Container	Ancillary	Yes
HCR™ Pro Detect C	7 mL or 30 mL BOND Container	Ancillary	Yes
HCR™ Pro Detect D	7 mL or 30 mL BOND Container	Ancillary	Yes
HCR™ Pro Detect E	7 mL or 30 mL BOND Container	Ancillary	Yes
HCR™ Pro Detect F AP/HRP	7 mL or 30 mL BOND Container	Ancillary	Yes

Post-Process A	7 mL or 30 mL BOND Container	Ancillary	Yes
*Enzyme 1[4]	7 mL or 30 mL BOND Container	Ancillary	Yes

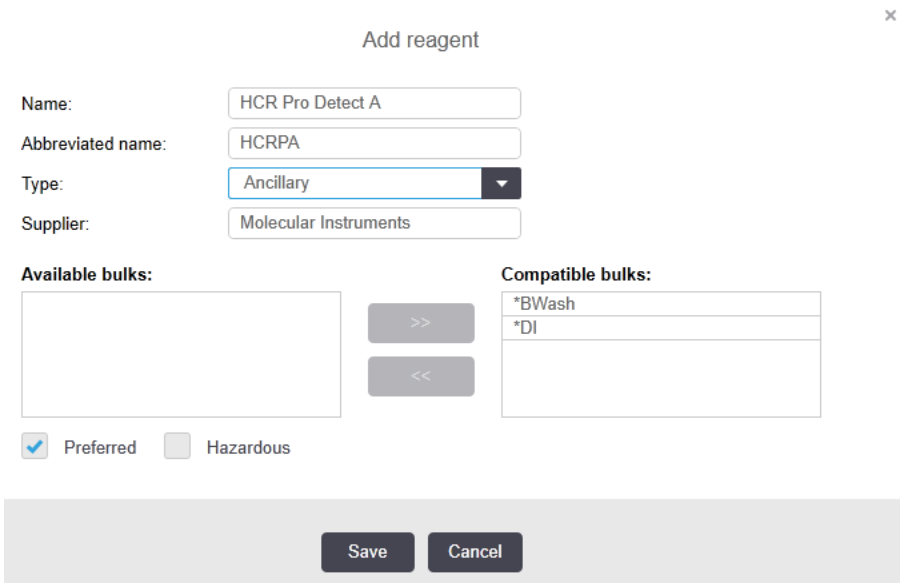
[1]*We recommend using 7 mL and 30 mL BOND Containers for 20-slide and 90-slide kits, respectively.*
[2]*While the *Enzyme 2 reagent does not require registration since it is a pre-existing reagent found in the software, the BOND Container itself does need to be registered. Please note that the BOND Container for *Enzyme 2 will hold the Pretreat reagent (reference page 18).*
[3]*Even though the reagent type, Primary antibody, is used, the Container will need to be filled with HCR™ HiFi Probe 1A Solution.*
[4]*While the *Enzyme 1 reagent does not require registration since it is a pre-existing reagent found in the software, the BOND Container itself does need to be registered. Please note that the BOND Container for *Enzyme 1 will hold the Post-Process B reagent (reference page 18). This reagent is only required for running an HCR™ Pro RNA-ISH + IHC co-detection assay.*

Registering a New Reagent

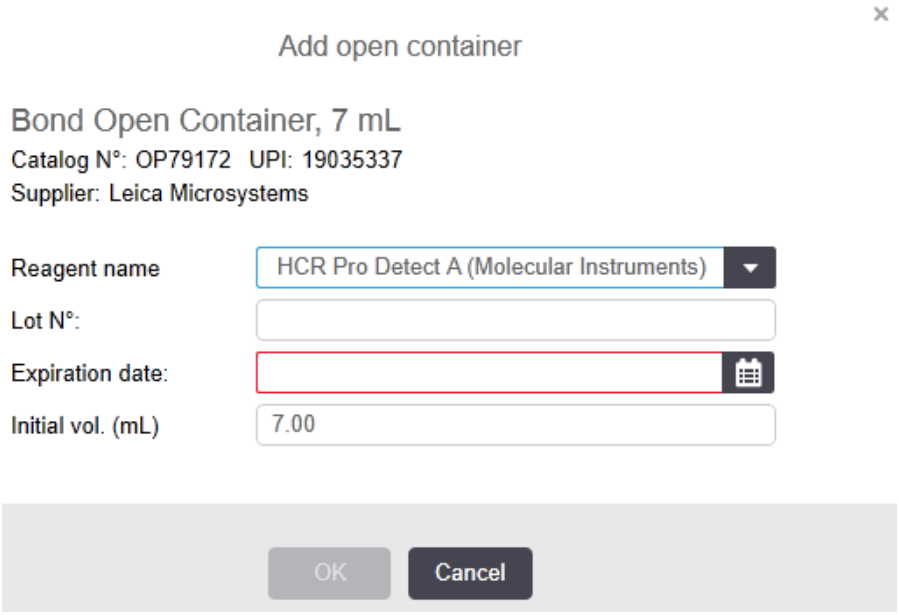
1. From the top navigation bar, click on **Reagent setup** at the top of the screen and select **Add**.



2. The following screenshot is an example of how to add an Ancillary reagent. Check the **Preferred** box and click **Save**.
   Note: Since the default staining cannot be left blank during registration, the HCR™ HiFi Probe 1A reagent can only be added after the ancillary reagents and staining protocols have been added to the BOND-III software.



3. To register a BOND Container (e.g., BOND Open Container) with an associated reagent, scan the barcode on the container, which will prompt the following screen. Select the **reagent name** that was created previously. Add an **expiration date** far into the future as the BOND Container can be reused. Click **OK.**



4. Repeat Steps 1-3 for all the required reagents.

Create Staining Protocols for HCR™ Pro RNA-ISH

1. From the top navigation bar, click on **Protocol setup** on the top of the screen and select **Staining** for the protocol group. Select a previous HCR™ Pro RNA-ISH protocol, and click on the **Copy** button near the top-right corner.

2. Change the name to **HCR™ RNA-ISH AP (or HRP)- custom probe** and enter your preferred **Abbreviated name**. It's important to note that each probe requires a separate staining protocol.

3. Replace the two previous Probe 1B steps with that of your current custom Probe 1B.

4. For AP and HRP-based detection, please select "**BOND Polymer Refine Red Detection**" and "**BOND Polymer Refine Detection**," respectively, as the **Preferred detection system** for the protocol. Click **Save**. Ensure that the **Preferred** box has been checked before saving.

HCR™ HiFi Probe 1A Registration

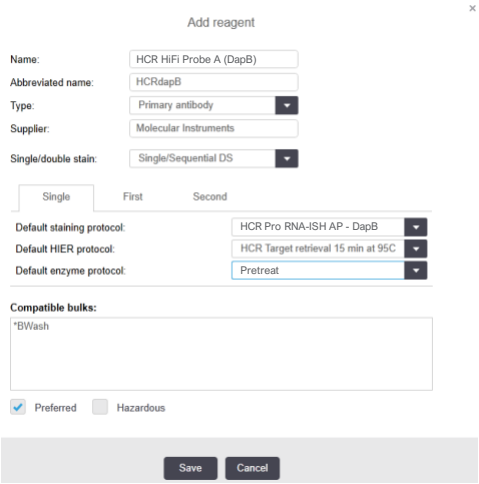
Now that all of the necessary reagents and their respective BOND Containers have been registered and assigned to our protocols, we can register the HCR™ HiFi Probe 1A BOND Container.

1. Similarly to the other reagent registration processes, click on **Reagent setup** from the top navigation bar and select **Add**.

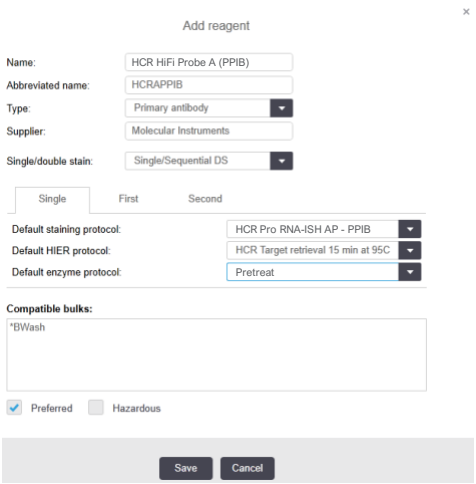
2. Enter the probe **Name**, **Abbreviated name**, **Type** (Primary antibody), and **Supplier** (Molecular Instruments). Then, use the drop-down menus to select the previously created **Default staining**, **HIER**, and **enzyme** protocols. Make sure the **Preferred** box is selected. Two examples of registering HCR™ HiFi Probe A (e.g., DapB and PPIB) are shown below.

   *Note that the probe specified in the default staining protocol must match the target listed under **Name**; mismatches can cause programming errors and result in dispensing the wrong probe reagent.*

DapB:

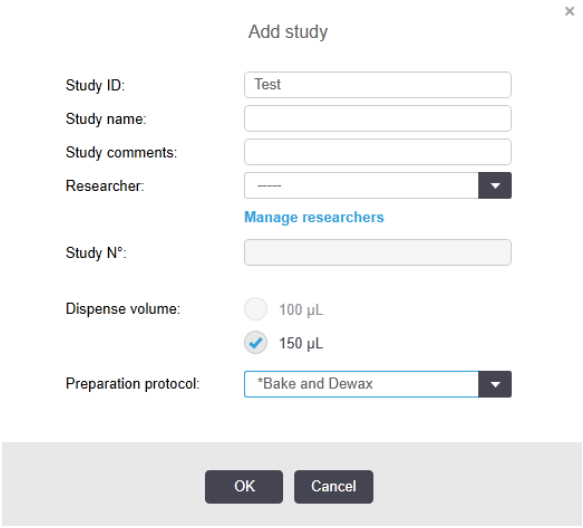


PPIB:

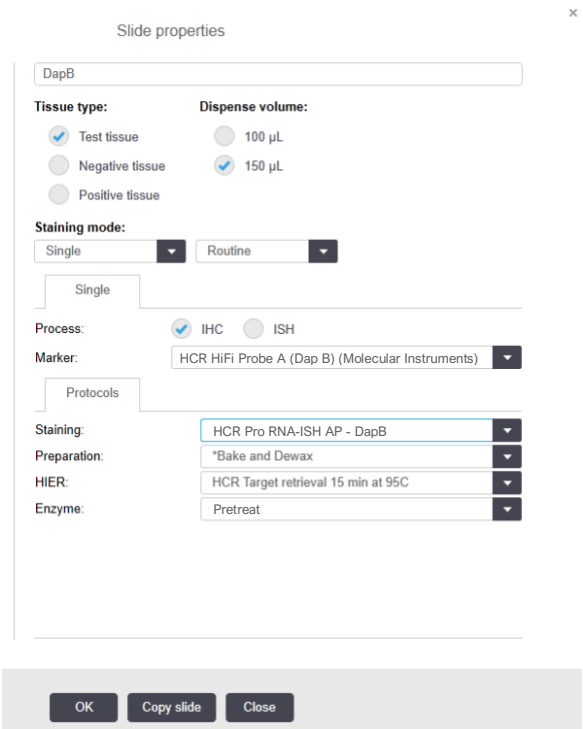


Set Up Slides and Print Labels

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. Click on **Add slide**. In this example, **HCR™ HiFi Probe A (DapB)** is selected as the **Marker**. If you registered the probe as described in the previous section, the associated protocols will self-populate. You can select a different probe as needed based on your RNA target of interest.



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 portion (white region) of the slides.

Post-Processing for AP-Based Detection

After slides are unloaded from the BOND-III, 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-III, 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.

Reagent Preparation

Detailed instructions for preparing probe solutions when probes are supplied at 50× concentration can be found in **Appendix A**. 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 Pretreat to the BOND Container	Store at 4 °C after use
HCR™ HiFi Tissue Stabilizer	Transfer entirety of HCR™ HiFi Tissue Stabilizer to the 7 mL or 30 mL BOND Container	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™ Pro Detect A	Transfer entirety of HCR™ Pro Detect A to the 7 mL or 30 mL BOND Container	Store at 4 °C after use
HCR™ Pro Detect B	Transfer entirety of HCR™ Pro Detect B to the 7 mL or 30 mL BOND Container	Store at 4 °C after use
HCR™ Pro Detect C	Transfer entirety of HCR™ Pro Detect C to the 7 mL or 30 mL BOND Container	Store at 4 °C after use
HCR™ Pro Detect D	Transfer entirety of HCR™ Pro Detect D to the 7 mL or 30 mL BOND Container	Store at 4 °C after use
HCR™ Pro Detect E	Transfer entirety of HCR™ Pro Detect E to the 7 mL or 30 mL BOND Container	Store at 4 °C after use
HCR™ Pro Detect F AP	Transfer entirety of HCR™ Pro Detect F AP to the 7 mL or 30 mL BOND Container	Store at 4 °C after use
HCR™ Pro Detect F HRP	Transfer entirety of HCR™ Pro Detect F HRP to the 7 mL or 30 mL BOND Container	Store at 4 °C after use
Post-Process A	Transfer entirety of Post-Process A to the 7 mL or 30 mL BOND Container	Store at 4 °C after use

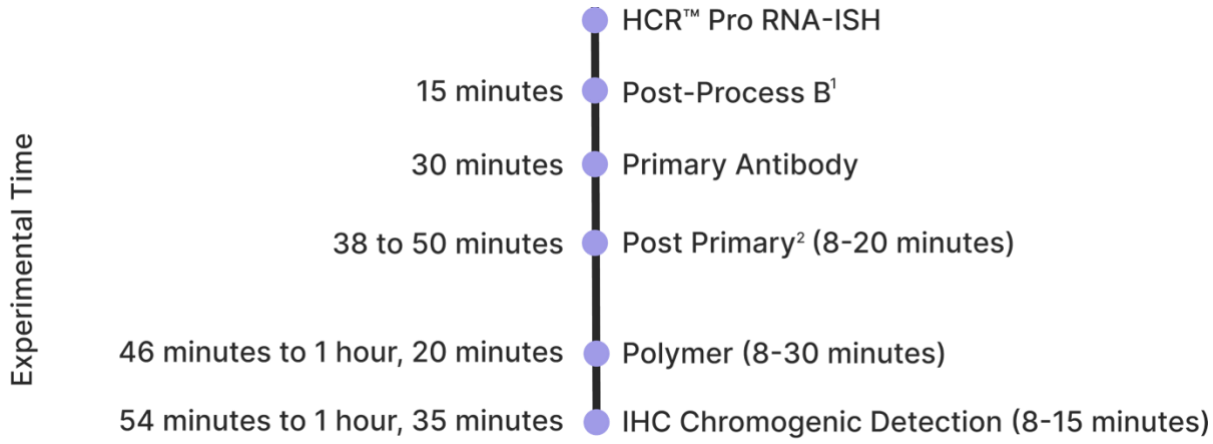
*Enzyme 1[1]	Transfer entirety of Post-Process B to the 7 mL or 30 mL BOND Container	Store at 4 °C after use

[1]*The registration and filling of this reagent is only required for running an HCR™ Pro RNA-ISH + IHC co-detection assay.*

Other important notes:

1. Please avoid excessive bubbles when transferring HCR™ HiFi Probe and HCR™ Pro 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 Protocol

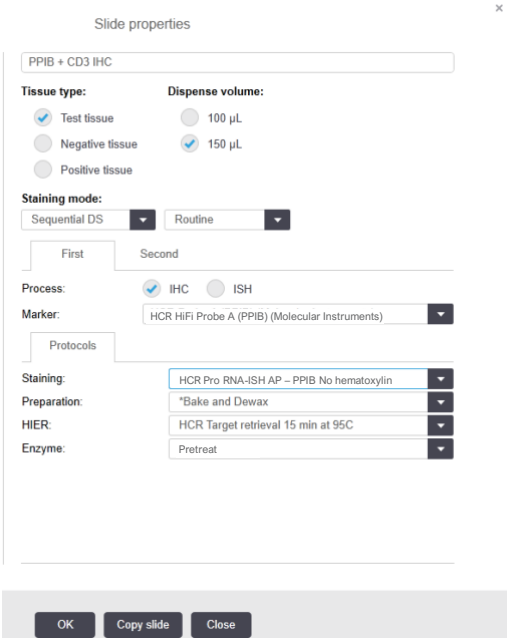


[1]*This step is NOT needed if HCR™ Pro RNA-ISH HRP detection is followed by IHC AP detection.*
[2]*This step is NOT needed if the species of the primary antibody used is rabbit.*

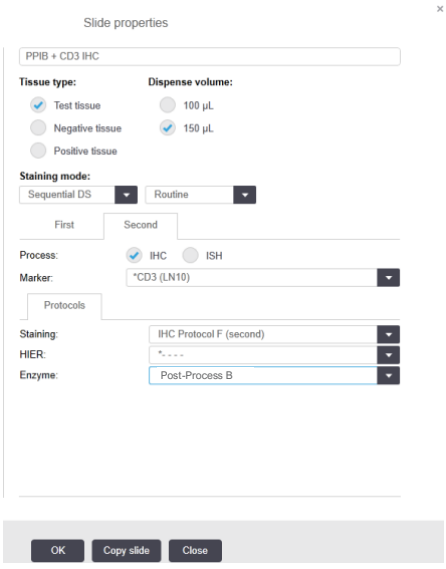
HCR™ Pro RNA-ISH + IHC Co-Detection Protocol

Set Up Slides with Two 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 DS**. Under the **First** tab, select IHC and proceed with the selections for running an HCR™ Pro RNA-ISH protocol with the new hematoxylin-free HCR™ Pro RNA-ISH protocol you created previously.



3. Select the **Second** tab and use the previously modified IHC protocol as the **Staining** protocol. Then, select **Post-Process B** as the **Enzyme** protocol. Click **Add slide**.



Important Notes:

1. ***Enzyme 2** is used for Pretreat in the first sequence, and ***Enzyme 1** is used for Post-Process B in the second sequence.
2. If **HRP-based RNA-ISH** detection is followed by **AP-based IHC**, the Enzyme protocol in the second sequence is **not** required and should be left blank.
3. If a red chromogen is used for either ISH or IHC detection, slides cannot be dehydrated in alcohol. Instead, bake the slides for ~30 minutes or allow them to air-dry overnight prior to mounting.

Appendix

Appendix A: 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.