HCR™ Pro RNA-ISH Setup Guide for the ONCORE Pro X

This Setup Guide demonstrates the use of an HCR™ Pro RNA-ISH kit on the ONCORE Pro X platform from Biocare Medical. Reagent preparation steps, including registering individual ONCORE Pro vials for their respective reagents, will be described in further detail. Each ONCORE Pro X run takes approximately 10 hours followed by a short post-processing of stained slides. 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 assay 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 an HCR™ HiFi Probe kit and an HCR™ Detect kit. 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 includes an HCR™ Membrane Stain to be used in an HCR™ Pro RNA-ISH + IHC/IF co-detection assay.

HCR™ HiFi Probe Kit

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 page 17 for more information on how to perform an HCR™ Pro RNA-ISH + IHC/IF co-detection assay.*

HCR™ Detect Kit

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 kit and an HCR™ Detect kit. Each HCR™ HiFi Probe includes 2 components: (1) HCR™ HiFi Probe 1A and (2) HCR™ HiFi Probe 1B.

HCR™ HiFi Probe Kit

HCR™ Reagents	Amount for a 20 Slide Kit	Amount for a 90 Slide Kit	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 Kit

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

HCR™ Reagents	Amount for a 20 Slide Kit	Amount for a 90 Slide Kit	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.*

Matisse® Red Chromogen Kit for AP Detection

For those using our HCR™ Detect kit 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, reach out to your MI representative.

HCR™ Reagents	Amount for a 20 Slide Kit	Amount for a 90 Slide Kit	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 Chromogen Kits for HRP Detection

For those using our HCR™ Detect kit 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, reach out to your MI representative.

Matisse® Brown Chromogen Kit for HRP Detection

HCR™ Reagents	Amount for a 20 Slide Kit	Amount for a 90 Slide Kit	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 Kit for HRP Detection

HCR™ Reagents	Amount for a 20 Slide Kit	Amount for a 90 Slide Kit	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

Required Materials for the ONCORE Pro X

The HCR™ Pro RNA-ISH protocol requires specific materials available only from Biocare Medical. 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 Biocare Medical representative.

Materials from Biocare Medical		
	Catalog #	**Storage Temperature**
ONCORE Pro 7 mL Improv Reagent Vials	ONCPR101JJ	RT
ONCORE Pro 15 mL Improv Reagent Vials	ONCPR102JJ	RT
Dewax Solution 1, DS1 Kit	OPRI6001KT60	2 to 8 °C
ONCORE Pro Antigen Retrieval 1 (AR1), high pH	OPRI6006T60	2 to 8 °C
ONCORE Pro Hematoxylin	OPRI6087T60	2 to 8 °C
ONCORE Pro DAB Chromogen Kit[1]	OPRI6056KT180	2 to 8 °C
ONCORE Pro Warp Red Chromogen Kit[1]	OPRI6083KT60	2 to 8 °C
ONCORE Pro Rabbit HRP Detection[2]	OPRI6008T60	2 to 8 °C
ONCORE Pro Rabbit AP Detection[2]	OPRI6043T60	2 to 8 °C
ONCORE Pro Mouse HRP Detection[2]	OPRI6007T60	2 to 8 °C
ONCORE Pro Mouse AP Detection[2]	OPRI6044T60	2 to 8 °C

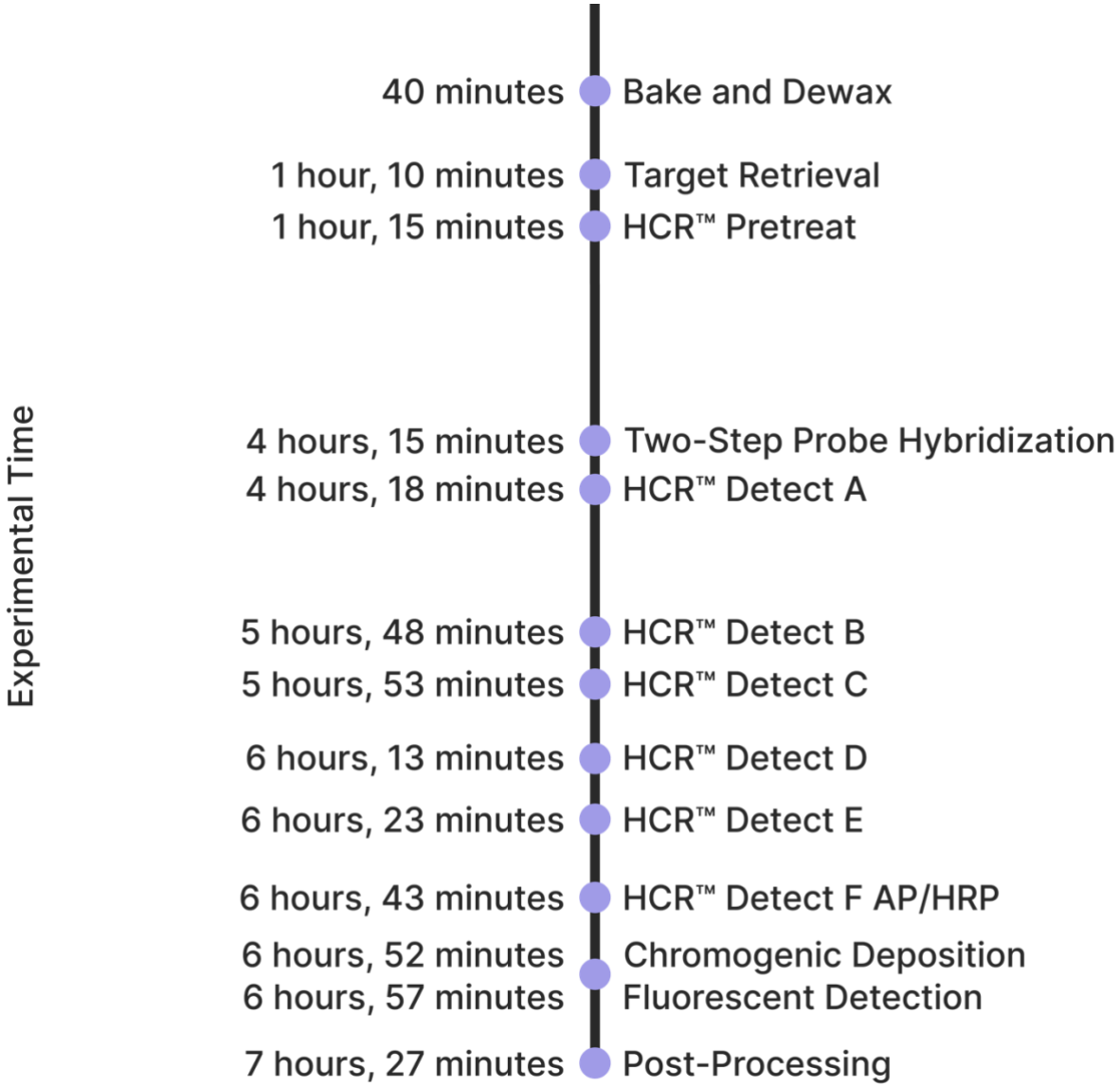
[1]*The HCR™ Pro RNA-ISH AP assay requires the use of MI's Matisse® Red Chromogen kit, and the HCR™ Pro RNA-ISH HRP assay requires the use of MI's Matisse® Brown or Green Chromogen kit. Alternatively, you can use the DAB and Warp Red chromogens for HRP and AP detection, respectively, or with third-party chromogens as well.*

[2]*The Rabbit or Mouse Polymer Detection Kits are only needed for ISH + IHC/IF co-detection assays.*

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	Biocare Medical	Mounting medium compatible with all Biocare chromogens
Cytoseal	Any	Suitable mounting medium for HRP-driven chromogens
ProLong™ Gold Antifade Mountant with DAPI	Thermo Fisher	Mounting medium compatible with RNA-FISH and IF staining
Cover Glass	Any	Dimension depends on the size of the tissue
100% Ethanol	Any	None
Tyramide Dyes	Any	See **Appendix E** for Recommendations

Overall Workflow of the HCR™ Pro RNA-ISH Protocol



As mentioned earlier, each ONCORE Pro X run takes approximately 10 hours. The timeline above only accounts for 7 hours and 27 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 ONCORE Pro X washing steps.

Set Up ONCORE Pro X Protocols

The four main steps in setting up an ONCORE Pro X protocol for an HCR™ Pro RNA-ISH run are:

i. Register HCR™ Pro RNA-ISH reagents in the ONCORE Pro X software
ii. Create an HCR™ Pro RNA-ISH protocol using an existing IHC staining protocol as a template
iii. Select protocols for individual slide modules
iv. Scan and map reagents and initiate the staining process

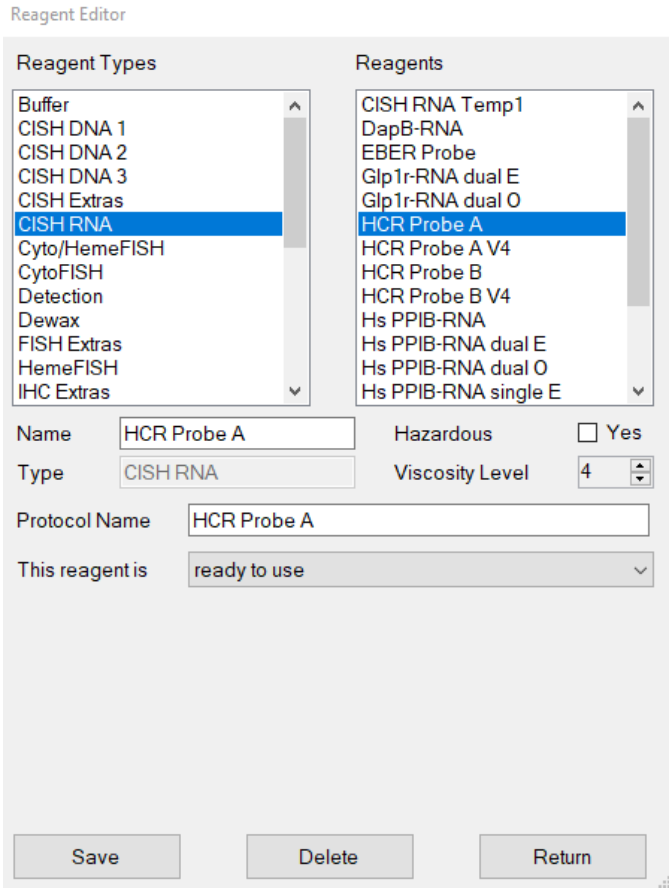
Register HCR™ Pro RNA-ISH Reagents in the ONCORE Pro X Software

In order to create ONCORE Pro X staining protocols, reagent names associated with the HCR™ reagents need to be registered in the ONCORE Pro X System Software. The table below lists all the reagents that need to be registered for the HCR™ Pro RNA-ISH assay.

Oncore Pro Reagent Vials			
Name	**Type of Container[1]**	**Reagent Type**	**Viscosity Level**
HCR™ Pretreat	7 mL or 15 mL ONCORE Pro Reagent Vial	Special	1
HiFi Probe 1A	7 mL or 15 mL ONCORE Pro Reagent Vial	CISH RNA	4
HiFi Probe 1B	7 mL or 15 mL ONCORE Pro Reagent Vial	CISH RNA	4
HCR™ Detect A	7 mL or 15 mL ONCORE Pro Reagent Vial	Special	1
HCR™ Detect B	7 mL or 15 mL ONCORE Pro Reagent Vial	Special	1
HCR™ Detect C	7 mL or 15 mL ONCORE Pro Reagent Vial	Special	1
HCR™ Detect D	7 mL or 15 mL ONCORE Pro Reagent Vial	Special	1
HCR™ Detect E	7 mL or 15 mL ONCORE Pro Reagent Vial	Special	1
HCR™ Detect F HRP/AP	7 mL or 15 mL ONCORE Pro Reagent Vial	Special	1
HCR™ Post-Process A	7 mL or 15 mL ONCORE Pro Reagent Vial	Special	1
HCR™ Post-Process B[2]	7 mL or 15 mL ONCORE Pro Reagent Vial	Special	1
Primary Antibody[2]	7 mL ONCORE Pro Reagent Vial	Special	1

[1]*We recommend using 7 mL and 15 mL Oncore Pro Reagent Vials for 20-slide and 90-slide kits, respectively.*
[2]*These reagents are only required for running an HCR™ Pro RNA-ISH + IHC/IF co-detection assay.*

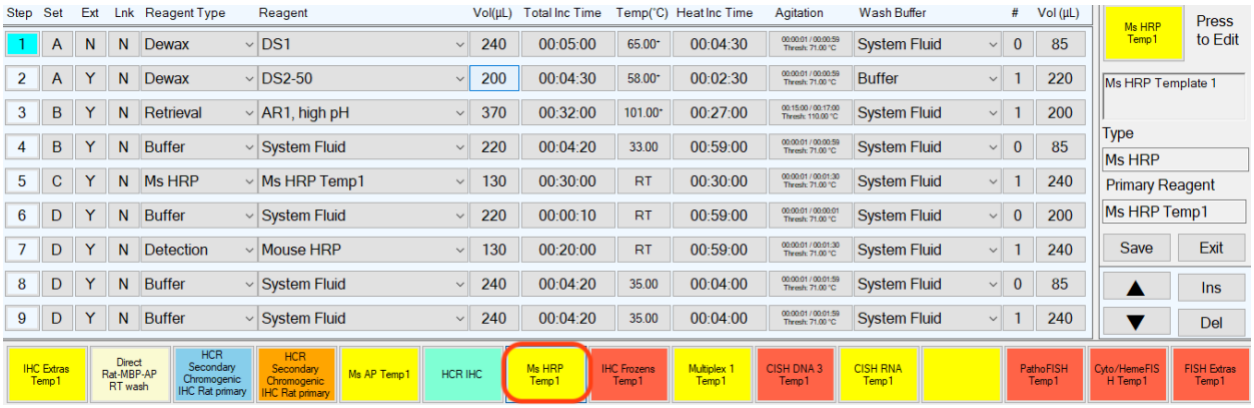
1. To register HCR™ Pro RNA-ISH reagents, open the ONCORE Pro X System Software and proceed with the following steps: System utilities → Reagent Editor → Select Reagent Type (e.g., CISH RNA) → Add New → Enter the HCR™ reagent names → Check/change the viscosity level → Save.



2. Repeat Step 1 for all other HCR™ reagents listed in the table above.

Create an HCR™ Pro RNA-ISH Protocol Using an Existing IHC Staining Protocol as a Template

The Advanced Protocol Editor is a software that enables you to create custom protocols with the help of a protocol template. After opening the Advanced Protocol Editor, select one of the pre-existing IHC protocols such as **Ms HRP Temp1** and use it as a template.



*Note: This template already includes the dewax and target retrieval steps used in the HCR™ Pro RNA-ISH protocols. The target retrieval temperature should be set to **95 ℃**.*

There are three single-plex RNA-ISH protocols:

1. HCR™ Pro RNA-CISH HRP (see pages 18-20)
2. HCR™ Pro RNA-CISH AP (see pages 18-20)
3. HCR™ Pro RNA-FISH (see pages 21-23)

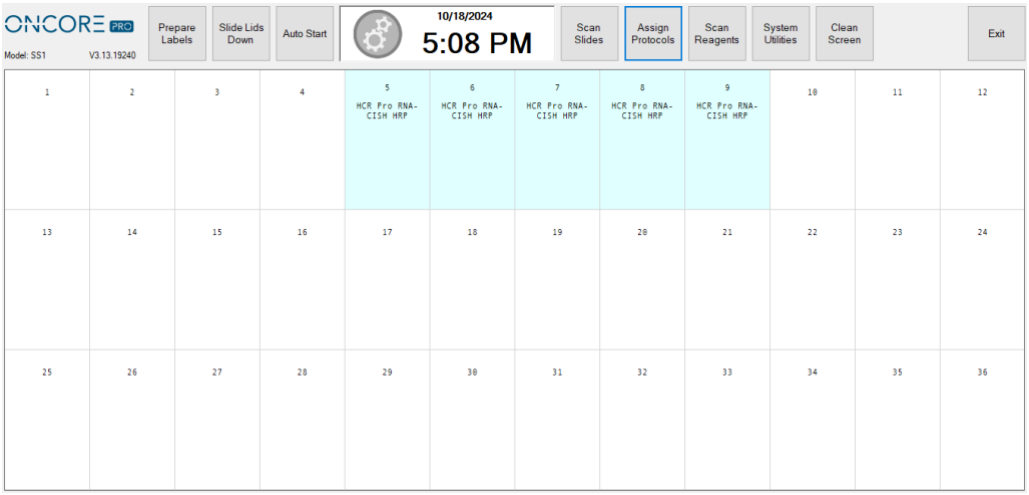
	HCR™ Pro RNA-CISH HRP	**HCR™ Pro RNA-CISH AP**	**HCR™ Pro RNA-FISH**
Main Differences:	Uses HCR™ Detect F HRP and an HRP-driven chromogen (e.g., Matisse® Brown).	Uses HCR™ Detect F AP and an AP-driven chromogen (e.g., Matisse® Red).	Uses third-party Tyramide dyes. Hematoxylin and HCR™ Post-Process A should be omitted.

*Note: Please see the **Appendix** for detailed staining protocols for each assay type (**Appendices A** and **B**) and third-party recommended Tyramide dyes (**Appendix E**).*

Select Protocols for Individual Slide Modules

1. Slides could be labeled by either (1) preparing label options (see the ONCORE User Manual for more information) or (2) handwriting directly on the slides.

2. Click on **Slide Modules**. This is where the slides will be stained after assigning protocols by clicking on **Assign Protocols**. Staining protocols created from the Advanced Protocol Editor will show up from the drop-down menu. Select the appropriate protocols (HCR Pro RNA-CISH HRP is selected in the following figure).

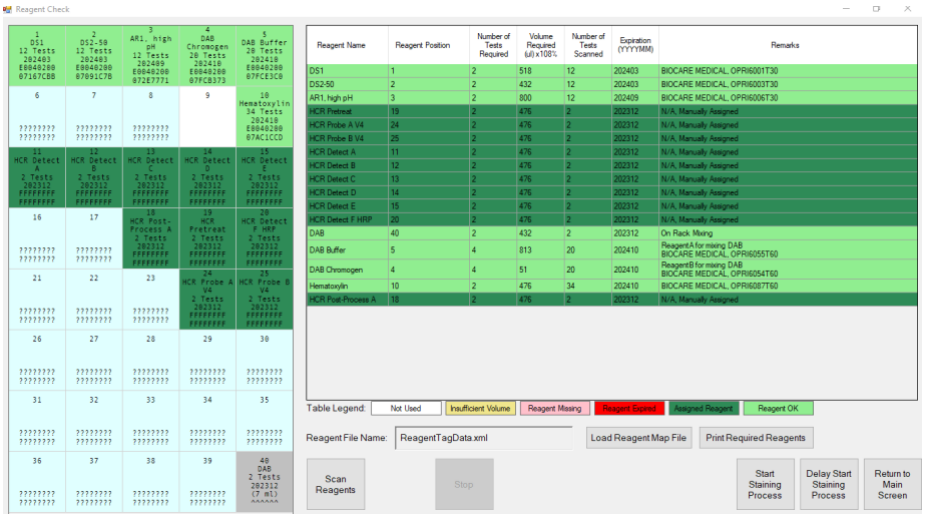


Scan and Map Reagents and Initiate the Staining Process

Before initiating a run, all reagents used by the previously programmed protocols need to be either (1) scanned or (2) manually mapped. We recommend placing ready-to-use (RTU) Biocare reagents in the first two rows (i.e., positions 1-10), and the rest of the HCR™ reagents in the other rows (i.e., positions 11-39).

1. Click on **Scan Reagents** to allow the instrument to scan all the Biocare reagents. Once all the Biocare reagents have been scanned, click on **Stop** to conclude the scanning process. The unscanned HCR™ reagents will appear in red.

2. Map the HCR™ reagents to the proper positions by clicking on the **Reagent Name First** in the table. Then, click **reagent position** in the left panel. Following the manual assignment, the outcome should resemble the image provided below.



3. Initiate the HCR™ Pro RNA-ISH run immediately by selecting **Start Staining Process**. Alternatively, **Delay Start Staining Process** allows you to schedule the run to finish at a specific time.

Post-Processing for HRP-Based Detection

After slides are unloaded from the ONCORE Pro X, we recommend washing the slides thoroughly with tap water. Dehydrate by immersing the slides in 95% ethanol for 3 minutes twice followed by 100% ethanol for 3 minutes twice. 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) or EcoMount. Allow slides to air dry for 5 minutes before imaging.

Post-Processing for AP-Based Detection

After slides are unloaded from the ONCORE Pro X, we recommend washing the slides thoroughly with tap water. Bake the slides for at least 15 minutes (or until dry) at 60 °C. Dip the slide in xylene (or xylene substitute) for 1-3 seconds and immediately apply EcoMount for cover-slipping.

Post-Processing for Fluorescent Detection

After slides are unloaded from the ONCORE Pro X, we recommend immersing the slides in 1 x PBST/TBST for 3 minutes. Mount one slide at a time with the ProLong™ Gold Antifade mounting medium (or any other suitable mounting medium that you are familiar with).

Reagent Preparation

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 vials. Store all ONCORE Pro X Vials in a 4 °C fridge after use.

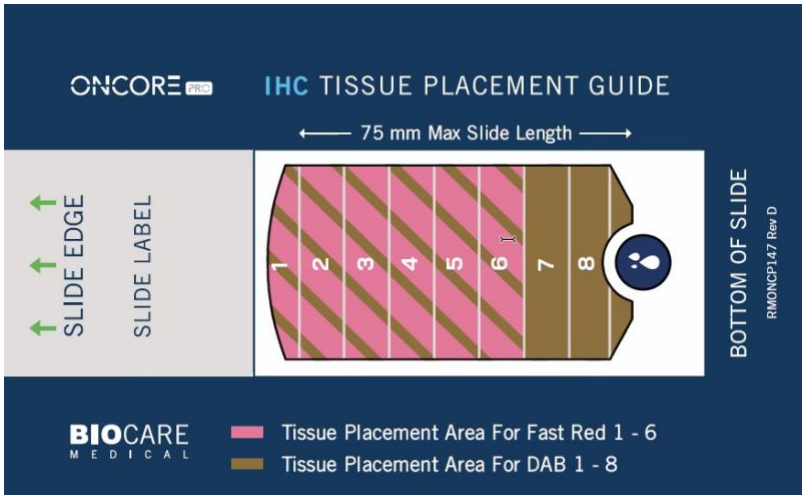
Oncore Vials	Instructions[1]	Storage Conditions
HCR™ Pretreat	Transfer the entirety of HCR™ Pretreat to the 7 mL or 15 mL Vial	Store at 4 °C after use
HCR™ HiFi Probe 1A	Transfer the entirety of HCR™ HiFi Probe 1A Solution to the 7 mL or 15 mL Vial	Store at 4 °C after use
HCR™ HiFi Probe 1B	Transfer the entirety of HCR™ HiFi Probe 1B Solution to the 7 mL or 15 mL Vial	Store at 4 °C after use
HCR™ Detect A	Transfer entirety of HCR™ Detect A to the 7 mL or 15 mL Vial	Store at 4 °C after use
HCR™ Detect B	Transfer entirety of HCR™ Detect B to the 7 mL or 15 mL Vial	Store at 4 °C after use
HCR™ Detect C	Transfer entirety of HCR™ Detect C to the 7 mL or 15 mL Vial	Store at 4 °C after use
HCR™ Detect D	Transfer entirety of HCR™ Detect D to the 7 mL or 15 mL Vial	Store at 4 °C after use
HCR™ Detect E	Transfer entirety of HCR™ Detect E to the 7 mL or 15 mL Vial	Store at 4 °C after use
HCR™ Detect F AP	Transfer entirety of HCR™ Detect F AP to the 7 mL or 15 mL Vial	Store at 4 °C after use
HCR™ Detect F HRP	Transfer entirety of HCR™ Detect F HRP to the 7 mL or 15 mL Vial	Store at 4 °C after use
HCR™ Post-Process A	Transfer entirety of HCR™ Post-Process A to the 7 mL or 15 mL Vial	Store at 4 °C after use
HCR™ Post-Process B	Transfer entirety of HCR™ Post-Process B to the 7 mL or 15 mL Vial	Store at 4 °C after use
Primary Antibody	Transfer entirety of HCR™ Membrane Stain[2] to the 7 mL Vial	Store at 4 °C after use

[1]*Use 7 mL and 15 mL vials for 20-slide and 90-slide kits respectively. Please note that the HCR™ reagent volumes for the 90-slide kits will exceed 15 mL. You can easily refill the 15 mL vials as needed after each run.*

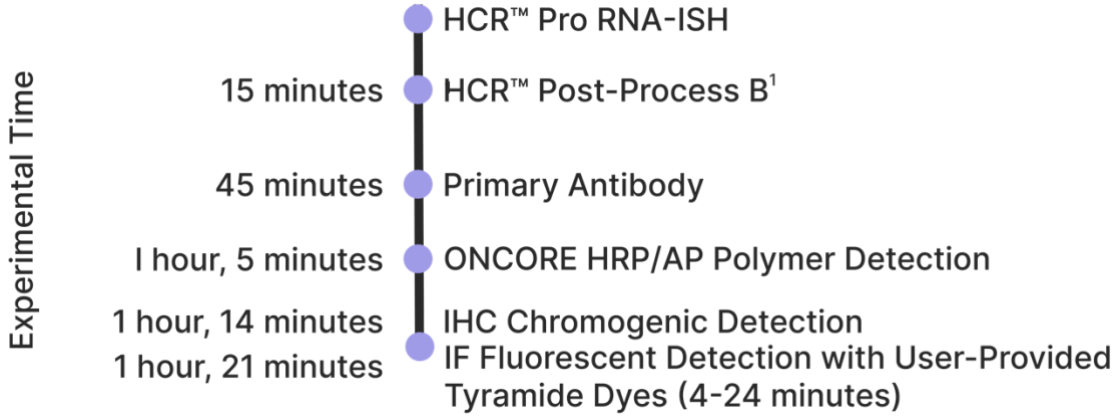
[2]*The HCR™ Membrane Stain is only provided in the HCR™ Pro RNA-ISH Starter Kit.*

Other important notes:

1. Please avoid excessive bubbles when transferring HCR™ HiFi Probe and HCR™ Detect Solutions to the 7 mL or 15 mL ONCORE Vials.
2. If the reagent-filled ONCORE Vials have not been used for more than a week, it is highly recommended to gently invert the vials several times with the lid closed to ensure solution uniformity before next use.
3. Please note that the use of Warp Red and Fast Red Biocare chromogens can sometimes result in non-uniform staining for tissues placed close to the injection port. To mitigate the risk of non-uniform staining, Biocare suggests placing your tissue samples in a designated area, as illustrated in the image below.



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



[1]*This step is NOT needed if HCR™ Pro RNA-CISH HRP detection is followed by IHC AP detection.*

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

The additional reagents needed to run an HCR™ Pro RNA-ISH + IHC/IF co-detection assay include:

i. HCR™ Post-Process B
ii. User-provided primary antibody (or the HCR™ Membrane Stain from the HCR™ Pro RNA-ISH Starter Kit)
iii. Anti-Ms, Rb AP-, or HRP-conjugated polymer detection kits from Biocare Medical
iv. HRP- or AP-driven chromogens for IHC or third-party Tyramide-conjugated fluorophores for IF

Create an HCR™ Pro RNA-CISH + IHC Co-Detection Protocol

This co-detection protocol can be created using the previously programmed HCR™ Pro RNA-CISH HRP/AP protocol as a template. Please follow the detailed HCR™ Pro RNA-CISH + IHC co-detection protocol (see pages 24 to 27) in **Appendix C** for additional steps.

Note: HCR™ Post-Process B (steps 33 to 34) is not needed if HCR™ Pro RNA-CISH HRP detection is followed by AP-driven IHC detection.

Create an HCR™ Pro RNA-FISH + IF Co-Detection Protocol

This co-detection protocol can be created using the previously programmed HCR™ Pro RNA-FISH protocol as a template. Please follow the detailed HCR™ Pro RNA-FISH + IF co-detection protocol (see pages 28-30) in **Appendix D** for additional steps.

*Note: You will need to provide another Tyramide dye for IF that is spectrally different from the Tyramide dye used for HCR™ Pro RNA-FISH. See **Appendix E** for a list of recommended third-party Tyramide dyes and their suggested starting concentrations.*

Appendix

Appendix A: Detailed Staining Protocol for HCR™ Pro RNA-CISH [Chromogenic Detection]

Step No.	Reagent Type	Reagent name	Reagent Vol. (µL)	Total Inc. Time	Temp (°C)	Heat Inc. Time	Agitation	#	Wash Buffer Vol. (µL)
1	Dewax	DS1 (No extraction)	240	00:05:00	65.00	00:04:30	Lid up: 1 sec (injection) Lid down: 59 sec (A4)	0	85
2	Dewax	DS2-50	200	00:04:30	58.00	00:02:30	Lid up: 1 sec (injection) Lid down: 59 sec (A4)	1	220
3	Retrieval	AR1, high pH	370	00:32:00	95.00	00:27:00	Lid up: 15 min (A3) Lid down: 17 min (A4)	1	200
4	Buffer	System Fluid	220	00:04:20	33.00	00:59:00	Lid up: 1 sec (injection) Lid down: 59 sec (A4)	1	85
5	Buffer	System Fluid	220	00:00:10	RT	00:00:00	Lid up: 1 sec (injection) Lid down: 1 sec (A4)	0	220
6	Special	HCR™ Pretreat	220	00:05:00	RT	00:05:00	Lid up: 1 sec (injection) Lid down: 1 min (A4)	1	240
7	Buffer	System Fluid	220	00:00:10	RT	00:00:10	Lid up: 1 sec (injection) Lid down: 1 sec (A4)	0	200
8	CISH RNA	HiFi Probe A	220	01:30:00	45.00	01:30:00	Lid up: 1 sec (injection) Lid down: 30 min (A4)	1	240
9	Buffer	System Fluid	240	00:01:00	45.00	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85
10	Buffer	System Fluid	240	00:01:00	45.00	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85
11	Buffer	System Fluid	240	00:05:00	45.00	00:05:00	Lid up: 1 sec (injection) Lid down: 85 sec (A4)	0	85
12	CISH RNA	HiFi Probe B	220	01:30:00	45.00	01:30:00	Lid up: 1 sec (injection) Lid down: 30 min (A4)	1	240
13	Buffer	System Fluid	240	00:01:00	45.00	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85

14	Buffer	System Fluid	240	00:01:00	45.00	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85
15	Buffer	System Fluid	240	00:05:00	45.00	00:05:00	Lid up: 1 sec (injection) Lid down: 85 sec (A4)	0	85
16	Special	HCR™ Detect A	220	00:03:00	42.00	00:03:00	Lid up: 1 sec (injection) Lid down: 1 min (A4)	0	240
17	Special	HCR™ Detect B	220	01:30:00	42.00	01:30:00	Lid up: 1 sec (injection) Lid down: 30 min (A4)	1	240
18	Buffer	System Fluid	240	00:01:00	42.00	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85
19	Buffer	System Fluid	240	00:05:00	42.00	00:05:00	Lid up: 1 sec (injection) Lid down: 90 sec (A4)	1	240
20	Special	HCR™ Detect C	220	00:05:00	RT	00:05:00	Lid up: 1 sec (injection) Lid down: 90 sec (A4)	0	85
21	Special	HCR™ Detect D	220	00:20:00	RT	00:20:00	Lid up: 1 sec (injection) Lid down: 3 min (A4)	1	240
22	Buffer	System Fluid	240	00:01:00	RT	00:01:00	Lid up: 1 sec (injection) Lid down: 15 sec (A4)	0	85
23	Buffer	System Fluid	240	00:03:00	RT	00:03:00	Lid up: 1 sec (injection) Lid down: 1 min (A4)	0	85
24	Special	HCR™ Detect E	220	00:12:00	RT	00:12:00	Lid up: 1 sec (injection) Lid down: 3 min (A4)	1	240
25	Buffer	System Fluid	240	00:01:00	RT	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85
26	Buffer	System Fluid	240	00:00:10	RT	00:00:10	Lid up: 1 sec (injection) Lid down: 1 sec (A4)	0	85
27*	Special	HCR™ Detect F HRP*	220	00:20:00	RT	00:20:00	Lid up: 1 sec (injection) Lid down: 3 min (A4)	1	240
28	Buffer	System Fluid	240	00:01:00	RT	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85

29	Buffer	System Fluid	240	00:01:00	RT	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85
30*	Detection	DAB*	220	00:08:40	37.00	00:08:00	Lid up: 1 sec (injection) Lid down: 90 sec (A4)	1	240
31	Buffer	System Fluid	240	00:01:00	RT	00:01:00	Lid up: 1 sec (injection) Lid down: 15 sec (A4)	0	85
32	Others	Hematoxylin	220	00:01:00	RT	00:01:00	Lid up: 1 sec (injection) Lid down: 30 sec (A4)	2	240
33	Buffer	System Fluid	220	00:01:00	RT	00:01:00	Lid up: 1 sec (injection) Lid down: 15 sec (A4)	0	85
34	Special	HCR™ Post-Process A	220	00:03:00	RT	00:03:00	Lid up: 1 sec (injection) Lid down: 1 min (A4)	1	240
35	Buffer	System Fluid	130	00:01:00	RT	00:01:00	Lid up: 1 sec (injection) Lid down: 15 sec (Down)	0	85

** For AP-based detection, please make changes to steps 27 and 30 as follows:*

27*	Special	HCR™ Detect F AP*	220	00:20:00	RT	00:20:00	Lid up: 1 sec (injection) Lid down: 3 min (A4)	1	240
30*	Detection	Warp Red*	220	00:07:00	RT	00:09:00	Lid up: 1 sec (injection) Lid down: 90 sec (A4)	1	240

Appendix B: Detailed Staining Protocol for HCR™ Pro RNA-FISH [Fluorescent Detection]

Step No.	Reagent Type	Reagent name	Reagent Vol. (µL)	Total Inc. Time	Temp (°C)	Heat Inc. Time	Agitation	#	Wash Buffer Vol. (µL)
1	Dewax	DS1 (No extraction)	240	00:05:00	65.00	00:04:30	Lid up: 1 sec (injection) Lid down: 59 sec (A4)	0	85
2	Dewax	DS2-50	200	00:04:30	58.00	00:02:30	Lid up: 1 sec (injection) Lid down: 59 sec (A4)	1	220
3	Retrieval	AR1, high pH	370	00:32:00	95.00	00:27:00	Lid up: 15 min (A3) Lid down: 17 min (A4)	1	200
4	Buffer	System Fluid	220	00:04:20	33.00	00:59:00	Lid up: 1 sec (injection) Lid down: 59 sec (A4)	1	85
5	Buffer	System Fluid	220	00:00:10	RT	00:00:00	Lid up: 1 sec (injection) Lid down: 1 sec (A4)	0	220
6	Special	HCR™ Pretreat	220	00:05:00	RT	00:05:00	Lid up: 1 sec (injection) Lid down: 1 min (A4)	1	240
7	Buffer	System Fluid	220	00:00:10	RT	00:00:10	Lid up: 1 sec (injection) Lid down: 1 sec (A4)	0	200
8	CISH RNA*	HiFi Probe A	220	01:30:00	45.00	01:30:00	Lid up: 1 sec (injection) Lid down: 30 min (A4)	1	240
9	Buffer	System Fluid	240	00:01:00	45.00	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85
10	Buffer	System Fluid	240	00:01:00	45.00	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85
11	Buffer	System Fluid	240	00:05:00	45.00	00:05:00	Lid up: 1 sec (injection) Lid down: 85 sec (A4)	0	85
12	CISH RNA*	HiFi Probe B	220	01:30:00	45.00	01:30:00	Lid up: 1 sec (injection) Lid down: 30 min (A4)	1	240
13	Buffer	System Fluid	240	00:01:00	45.00	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85
14	Buffer	System Fluid	240	00:01:00	45.00	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85

15	Buffer	System Fluid	240	00:05:00	45.00	00:05:00	Lid up: 1 sec (injection) Lid down: 85 sec (A4)	0	85
16	Special	HCR™ Detect A	220	00:03:00	42.00	00:03:00	Lid up: 1 sec (injection) Lid down: 1 min (A4)	0	240
17	Special	HCR™ Detect B	220	01:30:00	42.00	01:30:00	Lid up: 1 sec (injection) Lid down: 30 min (A4)	1	240
18	Buffer	System Fluid	240	00:01:00	42.00	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85
19	Buffer	System Fluid	240	00:05:00	42.00	00:05:00	Lid up: 1 sec (injection) Lid down: 90 sec (A4)	1	240
20	Special	HCR™ Detect C	220	00:05:00	RT	00:05:00	Lid up: 1 sec (injection) Lid down: 90 sec (A4)	0	85
21	Special	HCR™ Detect D	220	00:20:00	RT	00:20:00	Lid up: 1 sec (injection) Lid down: 3 min (A4)	1	240
22	Buffer	System Fluid	240	00:01:00	RT	00:01:00	Lid up: 1 sec (injection) Lid down: 15 sec (A4)	0	85
23	Buffer	System Fluid	240	00:03:00	RT	00:03:00	Lid up: 1 sec (injection) Lid down: 1 min (A4)	0	85
24	Special	HCR™ Detect E	220	00:12:00	RT	00:12:00	Lid up: 1 sec (injection) Lid down: 3 min (A4)	1	240
25	Buffer	System Fluid	240	00:01:00	RT	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85
26	Buffer	System Fluid	240	00:00:10	RT	00:00:10	Lid up: 1 sec (injection) Lid down: 1 sec (A4)	0	85
27	Special	HCR™ Detect F HRP	220	00:20:00	RT	00:20:00	Lid up: 1 sec (injection) Lid down: 3 min (A4)	1	240
28	Buffer	System Fluid	240	00:01:00	RT	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85
29	Buffer	System Fluid	240	00:01:00	RT	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85

30*	Special	Tyramide Dye 1**	220	00:15:00#	RT	00:17:00	Lid up: 1 sec (injection) Lid down: 2 min 30 sec (A4)	2	240
31	Buffer	System Fluid	240	00:01:00	RT	00:02:00	Lid up: 1 sec (injection) Lid down: 15 sec (A4)	0	240
32	Buffer	System Fluid	130	00:01:00	RT	00:05:00	Lid up: 1 sec (injection) Lid down: 15 sec (Down)	0	85

**Please keep in mind that while CISH RNA is used here as the reagent type, the HCR™ HiFi Probes will still work as expected for fluorescent detection.*

***The incubation time of the Tyramide dye listed here is only used for reference, and you will need to optimize the actual incubation time.*

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

Step No.	Reagent Type	Reagent name	Reagent Vol. (µL)	Total Inc. Time	Temp (°C)	Heat Inc. Time	Agitation	#	Wash Buffer Vol. (µL)
1	Dewax	DS1 (No extraction)	240	00:05:00	65.00	00:04:30	Lid up: 1 sec (injection) Lid down: 59 sec (A4)	0	85
2	Dewax	DS2-50	200	00:04:30	58.00	00:02:30	Lid up: 1 sec (injection) Lid down: 59 sec (A4)	1	220
3	Retrieval	AR1, high pH	370	00:32:00	95.00	00:27:00	Lid up: 15 min (A3) Lid down: 17 min (A4)	1	200
4	Buffer	System Fluid	220	00:04:20	33.00	00:59:00	Lid up: 1 sec (injection) Lid down: 59 sec (A4)	1	85
5	Buffer	System Fluid	220	00:00:10	RT	00:00:00	Lid up: 1 sec (injection) Lid down: 1 sec (A4)	0	220
6	Special	HCR™ Pretreat	220	00:05:00	RT	00:05:00	Lid up: 1 sec (injection) Lid down: 1 min (A4)	1	240
7	Buffer	System Fluid	220	00:00:10	RT	00:00:10	Lid up: 1 sec (injection) Lid down: 1 sec (A4)	0	200
8	CISH RNA	HiFi Probe A	220	01:30:00	45.00	01:30:00	Lid up: 1 sec (injection) Lid down: 30 min (A4)	1	240
9	Buffer	System Fluid	240	00:01:00	45.00	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85
10	Buffer	System Fluid	240	00:01:00	45.00	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85
11	Buffer	System Fluid	240	00:05:00	45.00	00:05:00	Lid up: 1 sec (injection) Lid down: 85 sec (A4)	0	85
12	CISH RNA	HiFi Probe B	220	01:30:00	45.00	01:30:00	Lid up: 1 sec (injection) Lid down: 30 min (A4)	1	240
13	Buffer	System Fluid	240	00:01:00	45.00	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85
14	Buffer	System Fluid	240	00:01:00	45.00	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85

15	Buffer	System Fluid	240	00:05:00	45.00	00:05:00	Lid up: 1 sec (injection) Lid down: 85 sec (A4)	0	85
16	Special	HCR™ Detect A	220	00:03:00	42.00	00:03:00	Lid up: 1 sec (injection) Lid down: 1 min (A4)	0	240
17	Special	HCR™ Detect B	220	01:30:00	42.00	01:30:00	Lid up: 1 sec (injection) Lid down: 30 min (A4)	1	240
18	Buffer	System Fluid	240	00:01:00	42.00	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85
19	Buffer	System Fluid	240	00:05:00	42.00	00:05:00	Lid up: 1 sec (injection) Lid down: 90 sec (A4)	1	240
20	Special	HCR™ Detect C	220	00:05:00	RT	00:05:00	Lid up: 1 sec (injection) Lid down: 90 sec (A4)	0	85
21	Special	HCR™ Detect D	220	00:20:00	RT	00:20:00	Lid up: 1 sec (injection) Lid down: 3 min (A4)	1	240
22	Buffer	System Fluid	240	00:01:00	RT	00:01:00	Lid up: 1 sec (injection) Lid down: 15 sec (A4)	0	85
23	Buffer	System Fluid	240	00:03:00	RT	00:03:00	Lid up: 1 sec (injection) Lid down: 1 min (A4)	0	85
24	Special	HCR™ Detect E	220	00:12:00	RT	00:12:00	Lid up: 1 sec (injection) Lid down: 3 min (A4)	1	240
25	Buffer	System Fluid	240	00:01:00	RT	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85
26	Buffer	System Fluid	240	00:00:10	RT	00:00:10	Lid up: 1 sec (injection) Lid down: 1 sec (A4)	0	85
27	Special	HCR™ Detect F AP	220	00:20:00	RT	00:20:00	Lid up: 1 sec (injection) Lid down: 3 min (A4)	1	240
28	Buffer	System Fluid	240	00:01:00	RT	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85
29	Buffer	System Fluid	240	00:01:00	RT	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85

30	Detection	Warp Red	220	00:07:00	RT	00:17:00	Lid up: 1 sec (injection) Lid down: 90 sec (A4)	2	240
31	Buffer	System Fluid	130	00:00:10	RT	00:00:10	Lid up: 1 sec (injection) Lid down: 1 sec (A7)	0	85
32	Buffer	System Fluid	240	00:00:10	RT	00:00:10	Lid up: 1 sec (injection) Lid down: 1 sec (A4)	0	85
33	Special	HCR™ Post-Process B	220	00:15:00	42.00	00:15:00	Lid up: 1 sec (injection) Lid down: 90 sec (A4)	1	240
34	Buffer	System Fluid	240	00:01:00	RT	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85
35	Special	Primary Antibody	130	00:30:00	RT	00:30:00	Lid up: 1 sec (injection) Lid down: 2 min 30 sec (A7)	1	240
36	Buffer	System Fluid	240	00:00:10	RT	00:00:10	Lid up: 1 sec (injection) Lid down: 1 sec (A4)	0	85
37	Detection	Rabbit HRP*	130	00:20:00	RT	00:20:00	Lid up: 1 sec (injection) Lid down: 90 sec (A7)	1	240
38	Buffer	System Fluid	240	00:04:20	35	00:04:00	Lid up: 1 sec (injection) Lid down: 2 min (A4)	0	85
39	Buffer	System Fluid	240	00:04:20	35	00:04:00	Lid up: 1 sec (injection) Lid down: 2 min (A4)	1	240
40	Detection	DAB**	220	00:08:40	37.00	00:08:00	Lid up: 1 sec (injection) Lid down: 90 sec (A4)	1	240
41	Buffer	System Fluid	240	00:01:00	RT	00:01:00	Lid up: 1 sec (injection) Lid down: 15 sec (A4)	0	85
42	Others	Hematoxylin	220	00:01:00	RT	00:01:00	Lid up: 1 sec (injection) Lid down: 30 sec (A4)	2	240
43	Buffer	System Fluid	220	00:01:00	RT	00:01:00	Lid up: 1 sec (injection) Lid down: 15 sec (A4)	0	85
44	Special	HCR™ Post-Process A	220	00:03:00	RT	00:03:00	Lid up: 1 sec (injection) Lid down: 1 min (A4)	1	240

45	Buffer	System Fluid	130	00:01:00	RT	00:01:00	Lid up: 1 sec (injection) Lid down: 15 sec (Down)	0	85

Choose the appropriate **ONCORE Polymer Detection based on the species (Rabbit or Mouse) of the primary antibody and the type of chromogen (HRP- or AP-driven) used.*

***DAB is used here as an example. You may use other chromogens instead.*

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

Step No.	Reagent Type	Reagent name	Reagent Vol. (µL)	Total Inc. Time	Temp (°C)	Heat Inc. Time	Agitation	#	Wash Buffer Vol. (µL)
1	Dewax	DS1 (No extraction)	240	00:05:00	65.00	00:04:30	Lid up: 1 sec (injection) Lid down: 59 sec (A4)	0	85
2	Dewax	DS2-50	200	00:04:30	58.00	00:02:30	Lid up: 1 sec (injection) Lid down: 59 sec (A4)	1	220
3	Retrieval	AR1, high pH	370	00:32:00	95.00	00:27:00	Lid up: 15 min (A3) Lid down: 17 min (A4)	1	200
4	Buffer	System Fluid	220	00:04:20	33.00	00:59:00	Lid up: 1 sec (injection) Lid down: 59 sec (A4)	1	85
5	Buffer	System Fluid	220	00:00:10	RT	00:00:00	Lid up: 1 sec (injection) Lid down: 1 sec (A4)	0	220
6	Special	HCR Pretreat	220	00:05:00	RT	00:05:00	Lid up: 1 sec (injection) Lid down: 1 min (A4)	1	240
7	Buffer	System Fluid	220	00:00:10	RT	00:00:10	Lid up: 1 sec (injection) Lid down: 1 sec (A4)	0	200
8	CISH RNA*	HiFi Probe A	220	01:30:00	45.00	01:30:00	Lid up: 1 sec (injection) Lid down: 30 min (A4)	1	240
9	Buffer	System Fluid	240	00:01:00	45.00	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85
10	Buffer	System Fluid	240	00:01:00	45.00	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85
11	Buffer	System Fluid	240	00:05:00	45.00	00:05:00	Lid up: 1 sec (injection) Lid down: 85 sec (A4)	0	85
12	CISH RNA*	HiFi Probe B	220	01:30:00	45.00	01:30:00	Lid up: 1 sec (injection) Lid down: 30 min (A4)	1	240
13	Buffer	System Fluid	240	00:01:00	45.00	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85
14	Buffer	System Fluid	240	00:01:00	45.00	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85

15	Buffer	System Fluid	240	00:05:00	45.00	00:05:00	Lid up: 1 sec (injection) Lid down: 85 sec (A4)	0	85
16	Special	HCR™ Detect A	220	00:03:00	42.00	00:03:00	Lid up: 1 sec (injection) Lid down: 1 min (A4)	0	240
17	Special	HCR™ Detect B	220	01:30:00	42.00	01:30:00	Lid up: 1 sec (injection) Lid down: 30 min (A4)	1	240
18	Buffer	System Fluid	240	00:01:00	42.00	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85
19	Buffer	System Fluid	240	00:05:00	42.00	00:05:00	Lid up: 1 sec (injection) Lid down: 90 sec (A4)	1	240
20	Special	HCR™ Detect C	220	00:05:00	RT	00:05:00	Lid up: 1 sec (injection) Lid down: 90 sec (A4)	0	85
21	Special	HCR™ Detect D	220	00:20:00	RT	00:20:00	Lid up: 1 sec (injection) Lid down: 3 min (A4)	1	240
22	Buffer	System Fluid	240	00:01:00	RT	00:01:00	Lid up: 1 sec (injection) Lid down: 15 sec (A4)	0	85
23	Buffer	System Fluid	240	00:03:00	RT	00:03:00	Lid up: 1 sec (injection) Lid down: 1 min (A4)	0	85
24	Special	HCR™ Detect E	220	00:12:00	RT	00:12:00	Lid up: 1 sec (injection) Lid down: 3 min (A4)	1	240
25	Buffer	System Fluid	240	00:01:00	RT	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85
26	Buffer	System Fluid	240	00:00:10	RT	00:00:10	Lid up: 1 sec (injection) Lid down: 1 sec (A4)	0	85
27	Special	HCR™ Detect F HRP	220	00:20:00	RT	00:20:00	Lid up: 1 sec (injection) Lid down: 3 min (A4)	1	240
28	Buffer	System Fluid	240	00:01:00	RT	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85
29	Buffer	System Fluid	240	00:01:00	RT	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85

30	Special	Tyramide Dye 1	220	00:15:00	RT	00:17:00	Lid up: 1 sec (injection) Lid down: 2 min 30 sec (A4)	2	240
31	Buffer	System Fluid	130	00:00:10	RT	00:00:10	Lid up: 1 sec (injection) Lid down: 1 sec (A7)	0	85
32	Buffer	System Fluid	240	00:00:10	RT	00:00:10	Lid up: 1 sec (injection) Lid down: 1 sec (A4)	0	85
33	Special	HCR™ Post-Process B	220	00:15:00	42.00	00:15:00	Lid up: 1 sec (injection) Lid down: 90 sec (A4)	1	240
34	Buffer	System Fluid	240	00:01:00	RT	00:01:00	Lid up: 1 sec (injection) Lid down: 25 sec (A4)	0	85
35	Special	Primary Antibody	130	00:30:00	RT	00:30:00	Lid up: 1 sec (injection) Lid down: 2 min 30 sec (A7)	1	240
36	Buffer	System Fluid	240	00:00:10	RT	00:00:10	Lid up: 1 sec (injection) Lid down: 1 sec (A4)	0	85
37	Detection	Rabbit HRP	130	00:20:00	RT	00:20:00	Lid up: 1 sec (injection) Lid down: 90 sec (A7)	1	240
38	Buffer	System Fluid	240	00:04:20	35	00:04:00	Lid up: 1 sec (injection) Lid down: 2 min (A4)	0	85
39	Buffer	System Fluid	240	00:04:20	35	00:04:00	Lid up: 1 sec (injection) Lid down: 2 min (A4)	1	240
40	Special	Tyramide Dye 2	220	00:15:00	RT	00:17:00	Lid up: 1 sec (injection) Lid down: 2 min 30 sec (A4)	2	240
41	Buffer	System Fluid	240	00:00:10	RT	00:00:10	Lid up: 1 sec (injection) Lid down: 1 sec (A4)	0	85
42	Buffer	System Fluid	130	00:00:10	RT	00:00:10	Lid up: 1 sec (injection) Lid down: 1 sec (Down)	0	85

**Please keep in mind that while CISH RNA is used here as the reagent type, the HCR™ HiFi Probes will still work as expected for fluorescent detection.*

Appendix E: 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 F: 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 HCR™ 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 probe hybridization time, this may not be optimal across all tissue types. You can increase the **Probe Hybridization** time (Steps #2 and #15 as written in Appendix A and B) 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 Leica's protease with the following parameters: **0.5 µg/mL, 1:34000 at 37 °C for 15 minutes.**

Appendix G: 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. 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 the Matisse® Red chromogen for RNA-ISH and Matisse® Green chromogen for IHC. 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'd like to incorporate protease in my sample. What are your suggestions?**
    a. The 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). In such cases, we recommend that you use a light protease digestion.