HCR™ Pro RNA-ISH Setup Guide for the DISCOVERY ULTRA

This Setup Guide demonstrates the use of an HCR™ Pro RNA-ISH kit on the DISCOVERY ULTRA platform from Roche Diagnostics. Reagent preparation steps, including registering individual ULTRA Dispensers for their respective reagents, will be described in further detail. Each DISCOVERY ULTRA run takes approximately 10.5-11.5 hours followed by a short post-processing of stained slides. This time range depends on the type of chromogen or fluorophore used in the assay. 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. Please note that this Setup Guide is for use with VSS 12.5.4 and above.

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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 listed below.

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™ Pro 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]	1.5 mL	2 to 8 °C
HCR™ Control Slides[3]	2 Slides	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 17-18 for more information on how to perform an HCR™ Pro RNA-ISH + IHC/IF co-detection assay.*

[3]*Please allocate one slide for the positive control and one slide for the negative control.*

Ready-to-Use HCR™ Pro Detect

All HCR™ Pro Detect reagents for the Starter Kit come pre-filled in amber dispensers along with their corresponding barcodes, as these reagents are sensitive to light.

HCR™ Reagents	Amount for an HCR™ Pro RNA-ISH Starter Kit	Storage Temperature	Dispenser Barcode
Pretreat A	7 mL	2 to 8 °C	PRETREATMENT 1
Pretreat B	14 mL	2 to 8 °C	PRETREATMENT 2
Pretreat C	5 mL	2 to 8 °C	PRETREATMENT 3
HCR™ Pro Detect A	5 mL	2 to 8 °C	DETECTION 1
HCR™ Pro Detect B	5 mL	2 to 8 °C	DETECTION 2
HCR™ Pro Detect C	5 mL	2 to 8 °C	DETECTION 3
HCR™ Pro Detect D	5 mL	2 to 8 °C	DETECTION 4
HCR™ Pro Detect E	5 mL	2 to 8 °C	DETECTION 5
HCR™ Pro Detect F HRP[1]	5 mL	2 to 8 °C	DETECTION 6
HCR™ Pro Detect F AP[1]	5 mL	2 to 8 °C	DETECTION 7

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

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 dispensers 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 F**.

HCR™ HiFi Probe

HCR™ Reagents	Amount for 20 Slides	Amount for 90 Slides	Storage Temperature
HCR™ HiFi Probe 1A	7 mL	28 mL	2 to 8 °C
HCR™ HiFi Probe 1B	7 mL	28 mL	2 to 8 °C

HCR™ Pro Detect Kit

All HCR™ Pro Detect reagents are either provided in amber bottles or pre-filled in amber dispensers, as these reagents are sensitive to light. Please note that HCR™ Pro Detect reagents at a 90-slide scale always come in a pre-filled format for ease of use.

HCR™ Reagents	Amount for 20 Slides	Amount for 90 Slides	Storage Temperature
Pretreat A	7 mL	28 mL	2 to 8 °C
Pretreat B	14 mL	55 mL[1]	2 to 8 °C
Pretreat C	5 mL	15 mL	2 to 8 °C
HCR™ Pro Detect A	5 mL	19 mL	2 to 8 °C
HCR™ Pro Detect B	5 mL	19 mL	2 to 8 °C
HCR™ Pro Detect C	5 mL	15 mL	2 to 8 °C
HCR™ Pro Detect D	5 mL	19 mL	2 to 8 °C
HCR™ Pro Detect E	5 mL	19 mL	2 to 8 °C
HCR™ Pro Detect F HRP/AP[2]	5 mL	19 mL	2 to 8 °C

[1]*Pretreat B at a 90-slide scale is provided in two pre-filled dispensers: one containing 30 mL and the other containing 25 mL.*

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

Ready-to-Use HCR™ Pro Detect

Ready-to-use HCR™ Pro Detect reagents are provided with pre-filled dispensers along with the appropriate barcodes for each reagent.

HCR™ Reagents	Amount for 20 Slides	Amount for 90 Slides	Dispenser Barcode
Pretreat A	7 mL	28 mL[1]	PRETREATMENT 1
Pretreat B	14 mL	55 mL[1]	PRETREATMENT 2
Pretreat C	5 mL	15 mL	PRETREATMENT 3
HCR™ Pro Detect A	5 mL	19 mL	DETECTION 1
HCR™ Pro Detect B	5 mL	19 mL	DETECTION 2
HCR™ Pro Detect C	5 mL	15 mL	DETECTION 3
HCR™ Pro Detect D	5 mL	19 mL	DETECTION 4
HCR™ Pro Detect E	5 mL	19 mL	DETECTION 5
HCR™ Pro Detect F HRP[2]	5 mL	19 mL	DETECTION 6
HCR™ Pro Detect F AP[2]	5 mL	19 mL	DETECTION 7

[1]*Pretreat A and B are used at faster rates than other reagents, causing their associated barcodes to reach the test limit sooner. To accommodate this, Pretreat A and Pretreat B at the 90-slide scale are provided with two PRETREATMENT 1 and two PRETREATMENT 2 barcodes.*

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

Note: When running HCR™ Pro RNA-ISH HRP and AP assays simultaneously, be sure to use the appropriate DETECTION 6 and DETECTION 7 barcodes as provided by MI. To avoid confusion, please do not use the DETECTION 6 barcode for HCR™ Pro Detect F AP or the DETECTION 7 barcode for HCR™ Pro Detect F HRP.

Required Materials for the DISCOVERY ULTRA

The HCR™ Pro RNA-ISH protocol requires specific materials available only from Roche. It is essential to check the availability of these materials prior to setting up an HCR™ Pro RNA-ISH experiment. Please note that ready-to-use HCR™ Pro Detect reagents are pre-filled into their appropriate dispensers and are provided with their corresponding barcodes, ensuring the reagents are ready for immediate use. For more information, please inquire with your Roche/MI representative.

Materials from Roche		
	Catalog #	**Quantity**
PRETREATMENT Barcodes and Open Dispensers	Varies	3
PROBE Barcodes and Open Dispensers	Varies	Varies[1]
DETECTION Dispensers and Barcodes	Varies	6[2]
Light Protective Prep Kit[3]	Varies	6

[1]*Each HCR™ HiFi Probe requires two probe barcodes. For example, running the dapB HCR™ HiFi Probe, the Ppib HCR™ HiFi Probe, and one target HCR™ HiFi Probe would require 6 probe barcodes.*

[2]*You will need to obtain additional detection barcodes if you are using third-party Tyramide dyes.*

[3]*The Light Protective Prep Kit contains one amber dispenser that needs to be used for HCR™ Pro Detect reagents A-F. The ordering code for this kit is 07475144001.*

Recommended Materials for the DISCOVERY ULTRA for Running a Chromogenic ISH Assay

Materials from Roche		
	Catalog #	**Storage Temperature**
DISCOVERY mRNA Teal Kit	08352941001	2 to 8 °C
DISCOVERY mRNA Green HRP Kit	08952612001	2 to 8 °C
DISCOVERY mRNA DAB Detection	06614353001	2 to 8 °C
DISCOVERY mRNA Purple	08352909001	2 to 8 °C
DISCOVERY Red Kit[1]	07425333001	2 to 8 °C
Hematoxylin II	05277965001	2 to 8 °C
Bluing Reagent	05266769001	2 to 8 °C
DISCOVERY Inhibitor RUO[2]	07017944001	2 to 8 °C

[1]*The HCR™ Pro RNA-ISH AP Kit requires the use of the DISCOVERY Red Detection Kit.*

[2]*DISCOVERY Inhibitor is necessary for multiplex ISH and IHC staining.*

Recommended Materials for the DISCOVERY ULTRA for Running a Fluorescent ISH Assay

Materials from Roche		
	Catalog #	**Storage Temperature**
DISCOVERY Cy5 Kit	07551215001	2 to 8 °C
DISCOVERY Rhodamine 6G Kit	07988168001	2 to 8 °C
DISCOVERY DCC Kit	07988192001	2 to 8 °C
DISCOVERY FAM Kit	07988150001	2 to 8 °C
DISCOVERY Red 610 Kit	07988176001	2 to 8 °C

Required Materials for the DISCOVERY ULTRA for Running an ISH + IHC/IF Co-Detection Assay

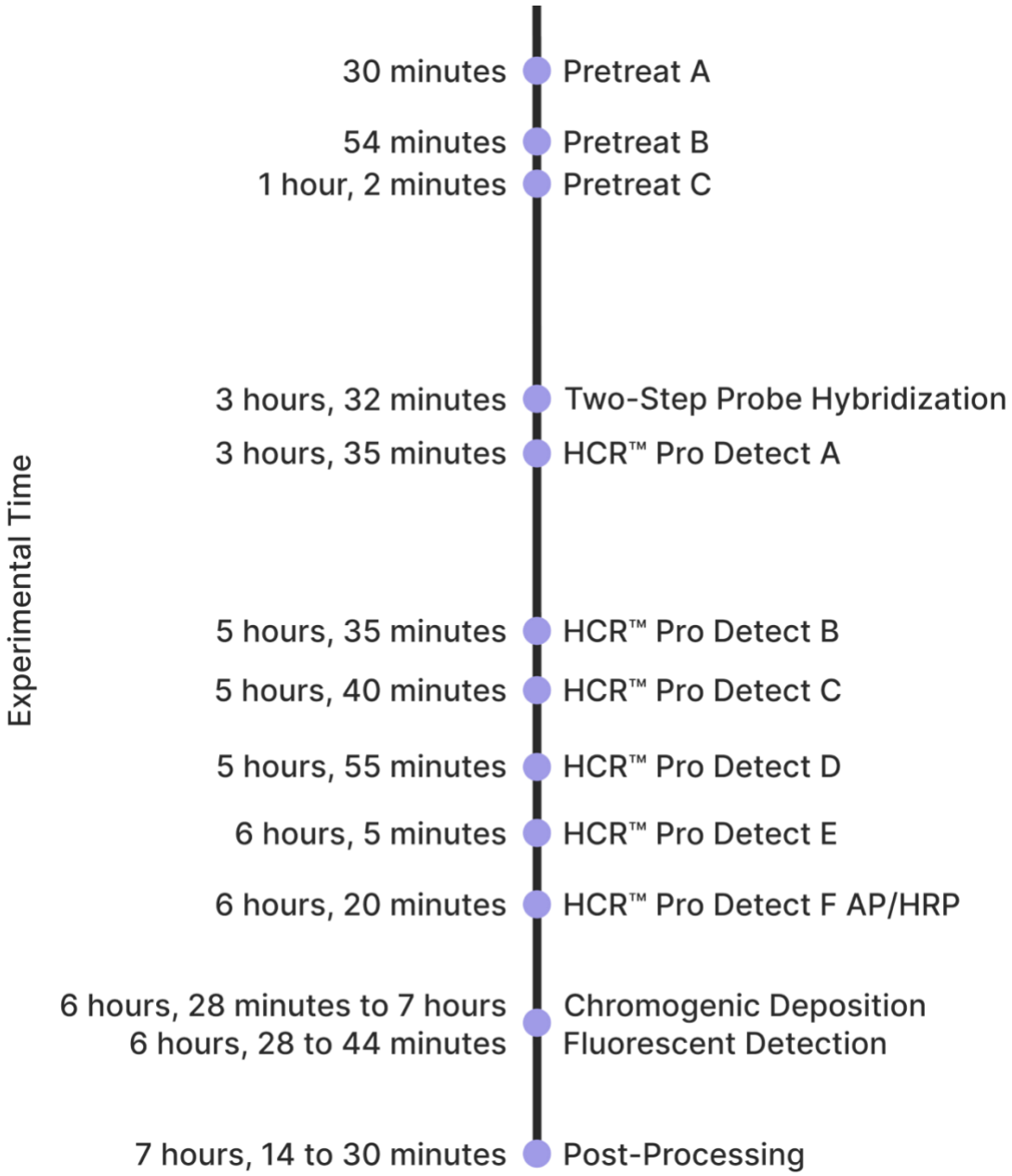
Materials from Roche		
	Catalog #	**Quantity**
ANTIBODY Dispensers and Barcodes	Varies	1
DISCOVERY Inhibitor RUO	07017944001	1
OmniMAP or UltraMAP HRP/AP[1]	Varies	1

[1]*The HCR™ Pro RNA-ISH Starter Kit requires an anti-Rb secondary antibody for IHC/IF detection given that the HCR™ Membrane Stain's host species is in rabbit. You should use anti-species multimer HRP/AP kits required by their primary antibodies.*

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 DISCOVERY 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 DISCOVERY ULTRA run takes approximately 10.5 to 11.5 hours. The timeline above only accounts for 7 hours and 14-30 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 DISCOVERY ULTRA washing steps.

Reagent Preparation

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

Refer to the Roche Manual (pages 296-309) for directions on how to fill and register user-fillable dispensers. For ready-to-use HCR™ Pro Detect reagents, refer to the same manual for instructions on how to register the barcodes provided with each dispenser. After registration, apply each barcode to its respective dispenser as shown in the table below.

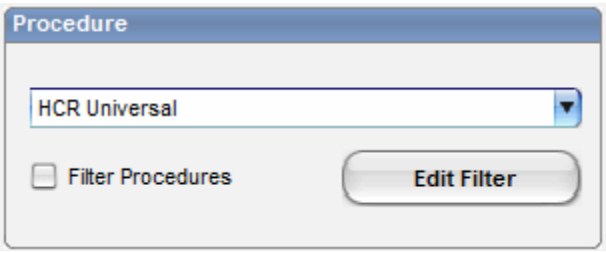
Reagent	Dispenser Barcode	Recommended Incubation Time
Pretreat A	PRETREATMENT 1	Default
Pretreat B	PRETREATMENT 2	16-24 minutes
Pretreat C	PRETREATMENT 3	8 minutes
HCR™ HiFi Probe 1A	PROBE	1 hour, 16 mins
HCR™ HiFi Probe 1B	PROBE	1 hour, 16 mins
HCR™ Pro Detect A	DETECTION 1	Default
HCR™ Pro Detect B	DETECTION 2	2 hours
HCR™ Pro Detect C	DETECTION 3	Default
HCR™ Pro Detect D	DETECTION 4	32 mins
HCR™ Pro Detect E	DETECTION 5	8 mins
HCR™ Pro Detect F HRP	DETECTION 6	32 mins
HCR™ Pro Detect F AP	DETECTION 7	32 mins
HCR™ Membrane Marker	ANTIBODY	16 mins

Other important notes:

1. Please avoid excessive bubbles when transferring the HCR™ HiFi Probe and HCR™ Pro Detect solutions to the DISCOVERY ULTRA Dispensers.
2. If the reagent-filled DISCOVERY ULTRA Dispensers have not been used for more than a week, it is highly recommended to gently invert the dispensers several times to ensure solution uniformity before next use.

Creating an HCR™ Pro RNA-ISH Protocol

STEP 1: Select **HCR™ Universal** from the **Procedure** drop-down menu.



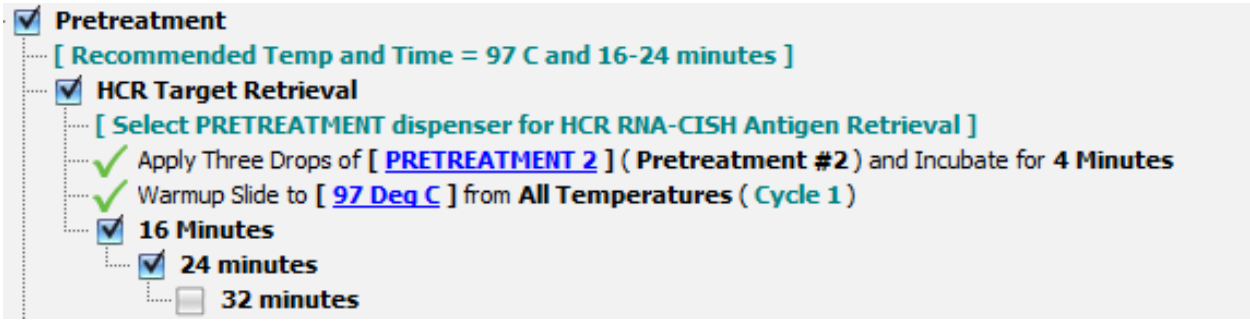
STEP 2: Begin building the HCR™ protocol. To start, select **Baking and Deparaffinization**.

If you prefer baking slides offline, de-select **Baking**. **Pretreat A** must be placed into an open **PRETREATMENT dispenser**. Make sure that the **Pretreatment** selections are the same.

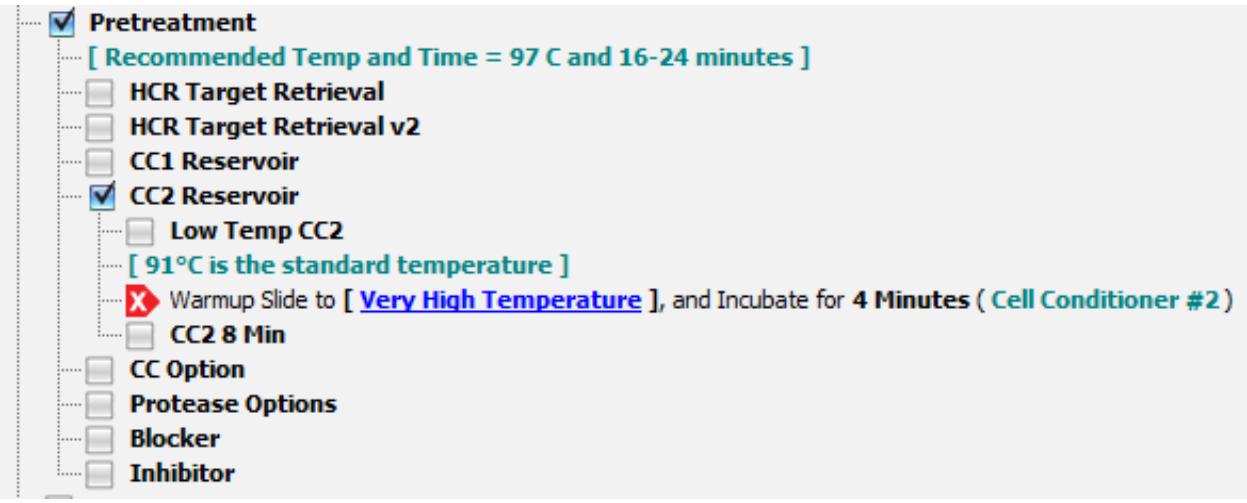
STEP 3: Select **Pretreatment**.

The **Pretreat B** must be placed into an open **PRETREATMENT dispenser** that is a different value than the one assigned to Pretreat A. The recommended target retrieval is HCR™ Target Retrieval v2 for 16 minutes and 24 minutes at 97 °C for FFPE cell pellets and tissues, respectively. For over-fixed tissues, you can increase the time to 32 minutes. To perform a less rigorous target retrieval, you can switch over to the following HCR™ Target Retrieval option shown below.

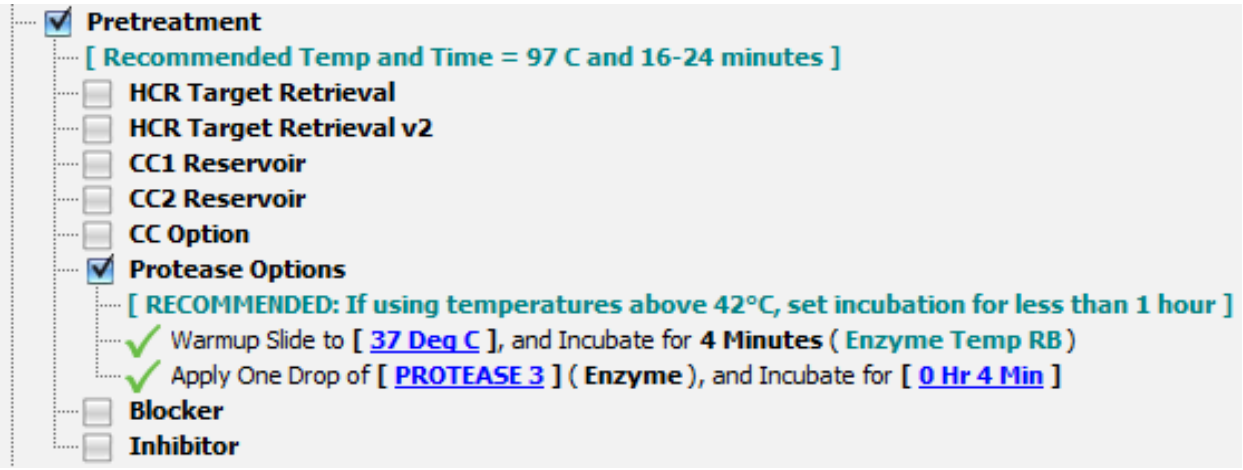
Optional: **HCR™ Target Retrieval** uses less Pretreat B solution.



Optional: You can forego the use of Pretreat B and rely on the DISCOVERY ULTRA onboard buffers for their antigen retrieval. You may also use CC1 or CC2 in conjunction with **HCR™ Target Retrieval** selections.



Optional: A mild protease pretreatment can be done in addition to any antigen retrieval. Please keep in mind that protease can have harmful effects on targeting proteins with any downstream IHC/IF assays.



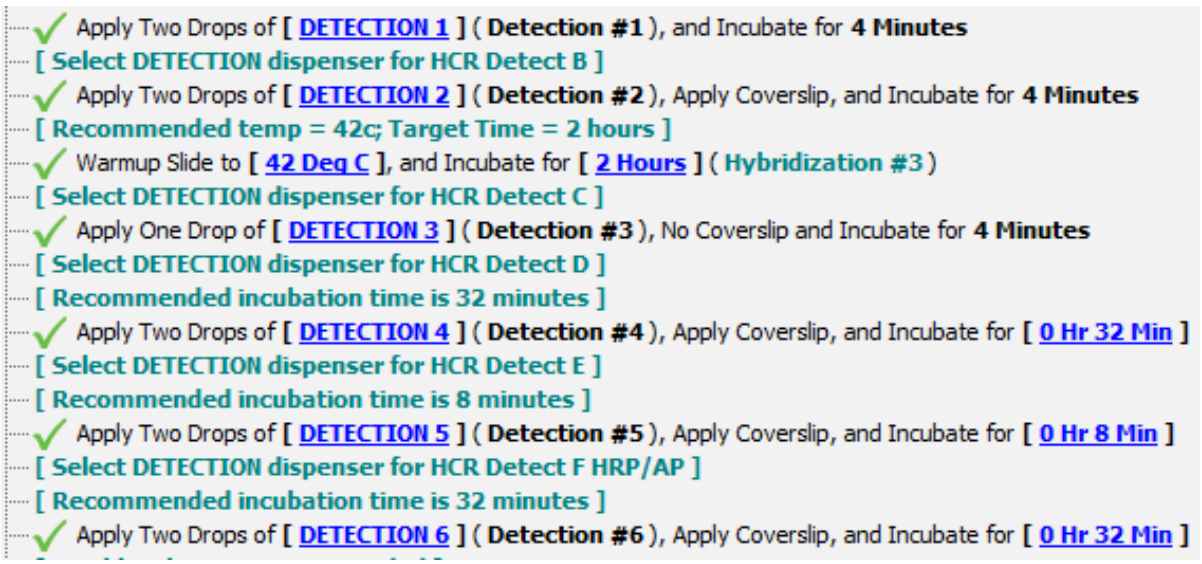
STEP 4: Select **Blocker** OR **DISCOVERY Inhibitor** to place an enzyme inhibitor solution onto the slide.

Pretreat C must be placed into an open **PRETREATMENT dispenser** different from the aforementioned dispensers. If you select **Blocker**, incubate for 8 minutes.

Optional: If **Inhibitor** is selected, then Roche's DISCOVERY Inhibitor RUO (Catalog #: 07017944001) needs to be present on the reagent rack. Incubate the solution for 12 minutes.

STEP 5: Select **HCR™ RNA-ISH**.

HCR™ HiFi Probe 1A and HCR™ HiFi Probe 1B must be placed into **PROBE dispensers**. Follow the comments (displayed in green text) for recommendations on a standard starting protocol.
*NOTE: Each target requires **two** probe dispensers.*
NOTE: While the protocol on the DISCOVERY ULTRA instrument refers to our probes as HCR Probes, these have been rebranded as HCR™ HiFi Probes. Both names refer to the same reagent.



HCR™ Pro Detect A to F must be placed into **Detection dispensers**. Select **DETECTION dispensers** for each of the HCR™ Pro Detect reagents and follow the comments (displayed in green text) for recommendations on a standard starting protocol. Please note that all HCR™ Pro Detect reagents must be placed into light-protective amber dispensers, as these reagents are sensitive to light.

STEP 5a: To perform **chromogenic ISH**, select the appropriate **Chromogen** that corresponds to your HCR™ Pro RNA-ISH kit.
*NOTE: The Chromogen defaults to mRNA DAB unless a Chromogen or Fluorescent Detection is selected. See **Appendix A** for an example protocol summary for HCR™ Pro RNA-ISH with mRNA DAB detection.*

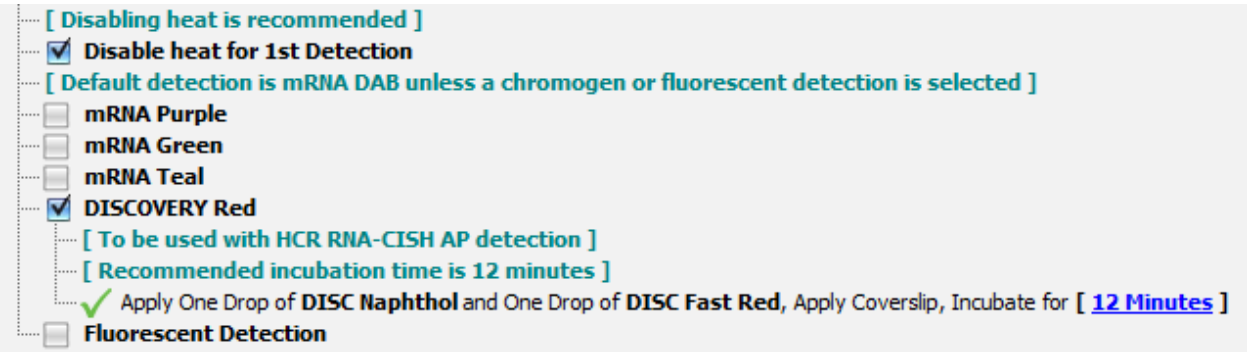


Table of Chromogens with Recommended Incubation Times

Chromogen	Enzyme	Incubation Time	Activator Time
mRNA DAB	HRP	Defaults to 8 minutes	N/A
DISCOVERY Red	AP	12-16 minutes	N/A
mRNA Purple	HRP	8 – 32 minutes	N/A
mRNA Teal	HRP	16 minutes	16 minutes
mRNA Green	HRP	24 minutes	16 minutes

STEP 5b: To perform **Fluorescent ISH**, select **Fluorescent Detection.**
*NOTE: This selection requires the use of an HCR™ Pro RNA-ISH HRP kit. See **Appendix B** for an example protocol summary for HCR™ Pro RNA-FISH with Cy5 Detection.*

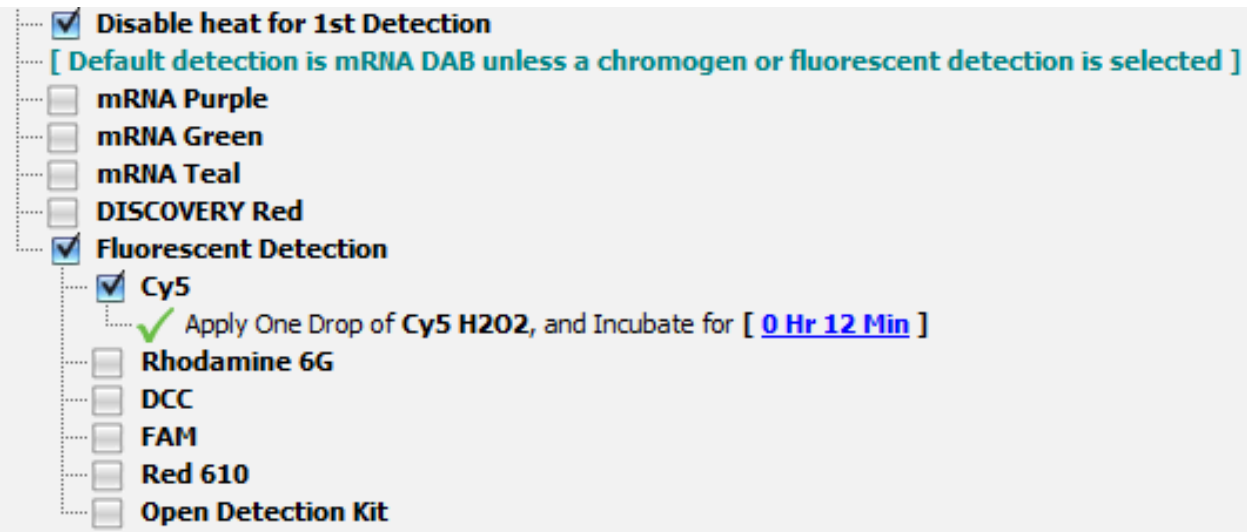
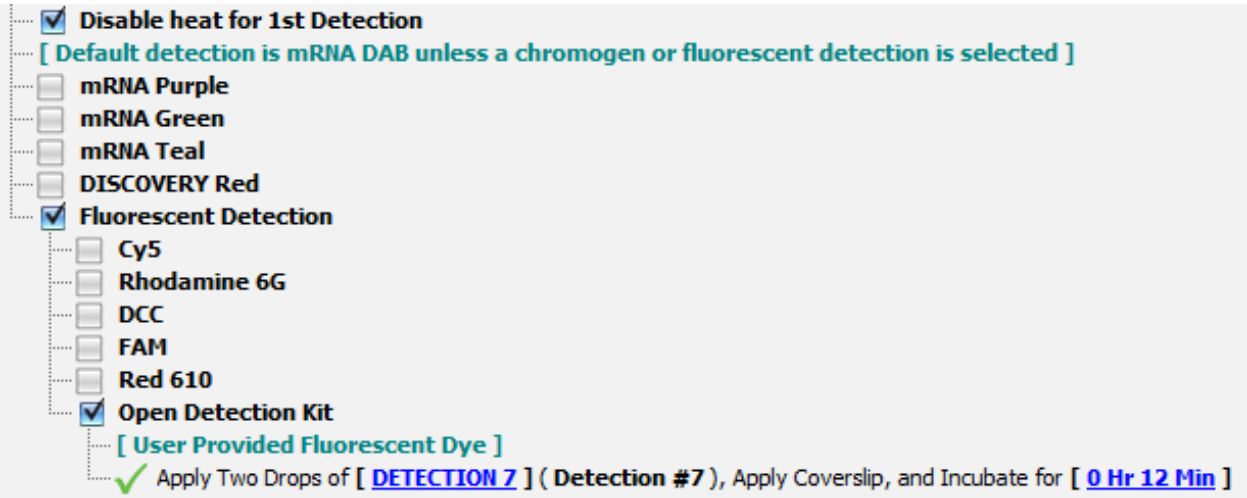


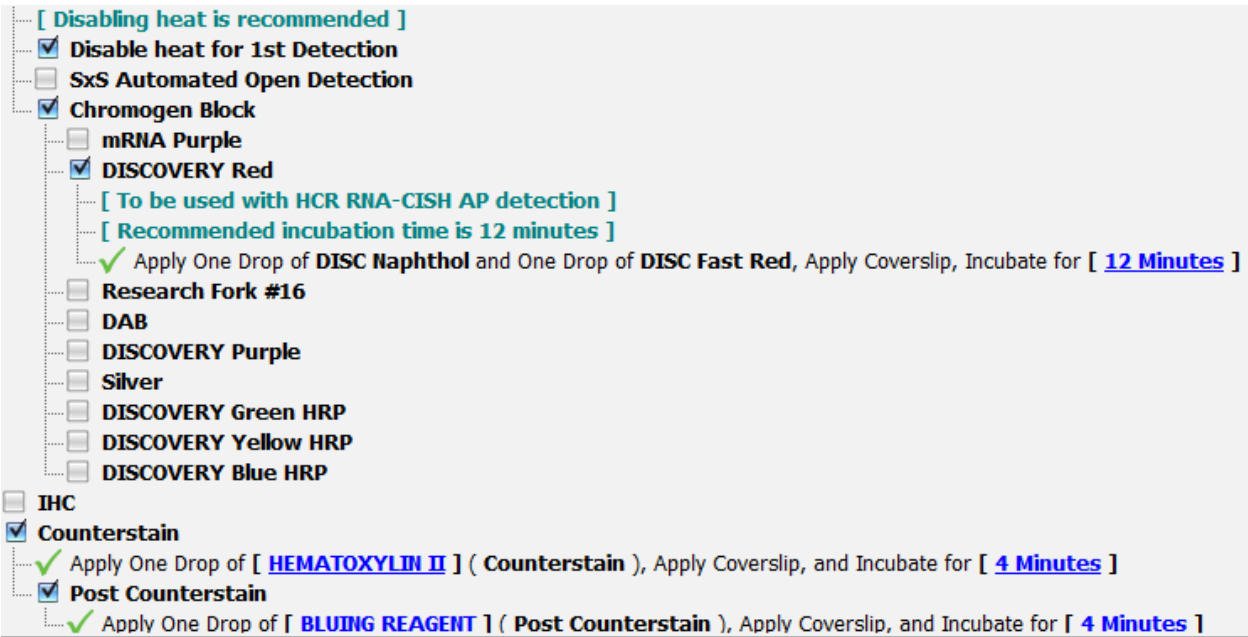
Table of Roche Fluorescent Dyes with Recommended Incubation Times

Tyramide Dyes	Recommended Incubation Time Range
Cy5	8-24 minutes
Rhodamine 6G	8-24 minutes
DCC	8-24 minutes
FAM	8-24 minutes
Red 610	8-24 minutes

Instead of using Roche's Fluorophore Kits, you can also use third-party TSA dyes by selecting **Open Detection Kit** (see **Appendix E** for recommendations).
NOTE: This selection requires another open Detection dispenser.



STEP 6 (Chromogenic ISH only): For counterstain and post-counterstain, select **Hematoxylin II** and **Bluing reagent,** respectively, and incubate for 4 mins each.



Post-Processing for AP-Based Detection

After slides are unloaded from the DISCOVERY ULTRA, we recommend washing the slides thoroughly with soapy water to remove any liquid coverslip. Bake the slides for 15 minutes (or until dry) at 60 °C. We recommend using EcoMount (Biocare) or VectaMount (Vectorlabs) mounting media for cover-slipping.

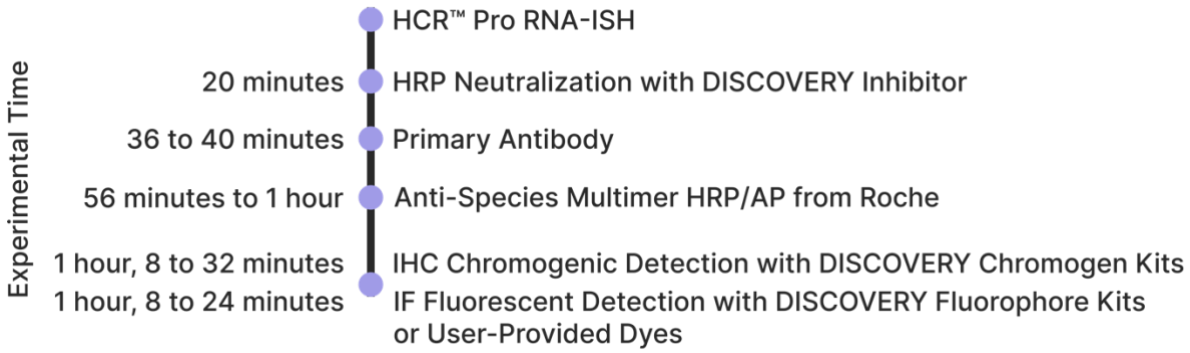
Post-Processing for HRP-Based Detection

After slides are unloaded from the DISCOVERY ULTRA, we recommend washing the slides thoroughly with soapy water to remove any liquid coverslip. After washing the slides, rinse them with water. Dehydrate by immersing the slides in 95% ethanol for 2 minutes twice followed by 100% ethanol for 2 minutes twice. Then, immerse the slides in a xylene (or xylene substitute) solution for 5 minutes and lay the slides flat inside a fume hood. Mount slides one 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 DISCOVERY ULTRA, we recommend washing the slides thoroughly with soapy water to remove any liquid coverslip. After washing the slides, rinse them with water. Immerse the washed slides in 1 X PBST and mount each slide one at a time with ProLong™ Gold Antifade mounting medium (or any other suitable mounting medium that you are familiar with).

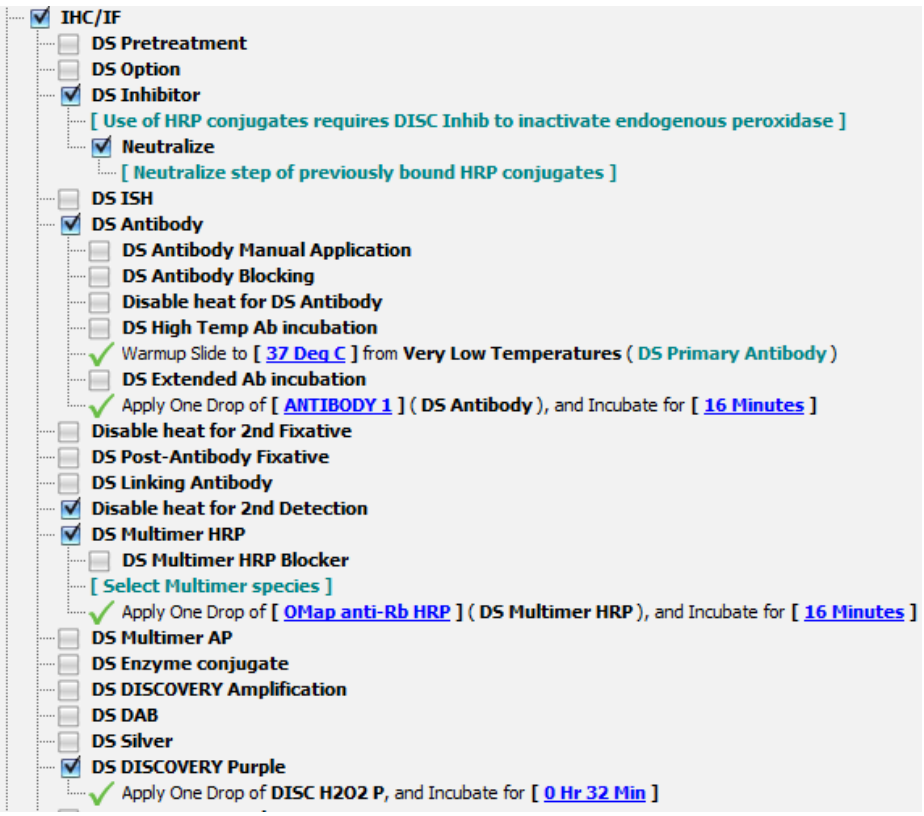
Overall Workflow of the HCR™ Pro RNA-ISH + IHC/IF Protocol



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

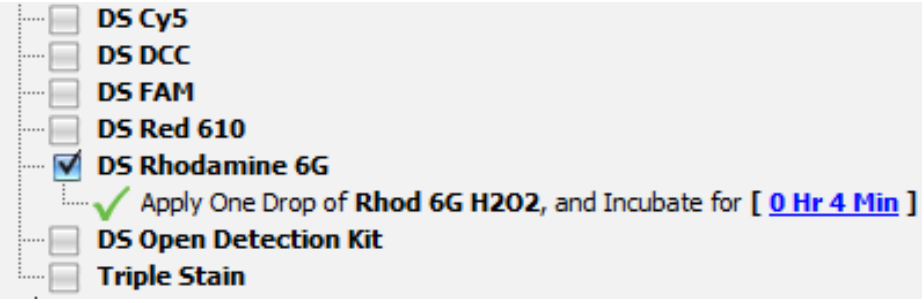
Chromogenic ISH + IHC Co-Detection

Program the HCR™ Pro RNA-ISH Protocol as outlined previously (see page 10). A dropdown list will appear once IHC is selected. An example for the selection of primary and HRP-conjugated secondary antibodies is shown below. You should determine the appropriate selection for which secondary antibodies to use (e.g., anti-species, HRP/AP-conjugated, and OmniMap/UltraMap). To deactivate HRP that was introduced from the ISH assay, select **DS Inhibitor** and **Neutralize**. Please note that this step is *NOT* required if you opt to use an AP-driven chromogen for the IHC staining. The last step is to select the appropriate chromogen needed for the IHC staining (example shown below). See **Appendix C** for an example protocol summary for HCR™ Pro RNA-CISH + IHC Co-Detection with mRNA Purple for CISH and Discovery Green for IHC.



Fluorescent ISH + IF Co-Detection

Program the HCR™ Pro RNA-ISH Protocol as outlined previously (see page 10). The setup for IF is identical to the setup for IHC, except that you will choose a Roche provided dye instead of a chromogen. See **Appendix D** for an example protocol summary for HCR™ Pro RNA-FISH + IF Co-Detection with Cy5 for FISH and Rhodamine 6G for IF.



Appendix

Appendix A: HCR™ Pro RNA-CISH Protocol Summary [Chromogenic Detection - mRNA DAB detection]

Protocol Summary

Procedure: HCR Universal (v3.01.0000)

DISCOVERY ULTRA

Ventana Medical Systems, Inc., 1910 Innovation Park Drive Tucson, Arizona USA

Validated: No **Active:** Yes

Protocol No	Protocol Name	Version	Creation Date
1801	mRNA DAB Protocol Summary	1	11/28/2023 3:58:05 PM

1	Baking [Selected]
2	Warmup Slide to 60 Deg C, and Incubate for [0 Hr 32 Min] (Baking)
3	Deparaffinization [Selected]
4	Apply Two Drops of [PRETREATMENT 1] (Pretreatment #1), and Incubate for 4 Minutes
5	Apply One Drop of [PRETREATMENT 1] (Pretreatment #1), No Coverslip and Incubate for 4 Minutes
6	Pretreatment [Selected]
7	HCR Target Retrieval v2 [Selected]
8	Apply Three Drops of [PRETREATMENT 2] (Pretreatment #2) and Incubate for 4 Minutes
9	Warmup Slide to [97 Deg C] from Very High Temperatures (Cycle 1)
10	16 Minutes [Selected]
11	Apply One Drop of [PRETREATMENT 2] (Pretreatment #2), Apply Coverslip, and Incubate for 4 Minutes
12	24 minutes [Selected]
13	Blocker [Selected]
14	Apply One Drop of [PRETREATMENT 3] (Pretreatment #3), and Incubate for [0 Hr 8 Min]
15	HCR RNA ISH [Selected]
16	Apply Three Drops of [PROBE 1] (Probe #1), Apply Coverslip, and Incubate for [4 Minutes]
17	Warmup Slide to [43 Deg C], and Incubate for [1 Hr 16 Min] (Hybridization #1)
18	Apply Three Drops of [PROBE 2] (Probe #2), Apply Coverslip, and Incubate for [4 Minutes]
19	Warmup Slide to [43 Deg C], and Incubate for [1 Hr 16 Min] (Hybridization #2)
20	Apply Two Drops of [DETECTION 1] (Detection #1), and Incubate for 4 Minutes
21	Apply Two Drops of [DETECTION 2] (Detection #2), Apply Coverslip, and Incubate for 4 Minutes
22	Warmup Slide to [42 Deg C], and Incubate for [2 Hours] (Hybridization #3)
23	Apply One Drop of [DETECTION 3] (Detection #3), No Coverslip and Incubate for 4 Minutes
24	Apply Two Drops of [DETECTION 4] (Detection #4), Apply Coverslip, and Incubate for [0 Hr 32 Min]
25	Apply Two Drops of [DETECTION 5] (Detection #5), Apply Coverslip, and Incubate for [0 Hr 8 Min]
26	Apply Two Drops of [DETECTION 6] (Detection #6), Apply Coverslip, and Incubate for [0 Hr 32 Min]
27	Disable heat for 1st Detection [Selected]
28	Counterstain [Selected]
29	Apply One Drop of [HEMATOXYLIN II] (Counterstain), Apply Coverslip, and Incubate for [4 Minutes]
30	Post Counterstain [Selected]
31	Apply One Drop of [BLUING REAGENT] (Post Counterstain), Apply Coverslip, and Incubate for [4 Minutes]

* one drop is one reagent dispense

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NOTE: Chromogen defaults to mRNA DAB when no chromogen is selected.

Appendix B: HCR™ Pro RNA-FISH Protocol Summary [Fluorescent Detection - Cy5 Detection]

Protocol Summary

Procedure: HCR Universal (v3.01.0000)

DISCOVERY ULTRA

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Validated: No **Active:** Yes

Protocol No	Protocol Name	Version	Creation Date
778	FISH Cy5 Detection	2	10/09/2023 1:31:09 PM

1 Baking [Selected]
2 Warmup Slide to 60 Deg C, and Incubate for [0 Hr 32 Min] (Baking)
3 Deparaffinization [Selected]
4 Apply Two Drops of [PRETREATMENT 1] (Pretreatment #1), and Incubate for 4 Minutes
5 Apply One Drop of [PRETREATMENT 1] (Pretreatment #1), No Coverslip and Incubate for 4 Minutes
6 Pretreatment [Selected]
7 HCR Target Retrieval v2 [Selected]
8 Apply Three Drops of [PRETREATMENT 2] (Pretreatment #2) and Incubate for 4 Minutes
9 Warmup Slide to [97 Deg C] from Very High Temperatures (Cycle 1)
10 16 Minutes [Selected]
11 Apply One Drop of [PRETREATMENT 2] (Pretreatment #2), Apply Coverslip, and Incubate for 4 Minutes
12 24 minutes [Selected]
13 Blocker [Selected]
14 Apply One Drop of [PRETREATMENT 3] (Pretreatment #3), and Incubate for [0 Hr 8 Min]
15 HCR RNA ISH [Selected]
16 Apply Three Drops of [PROBE 1] (Probe #1), Apply Coverslip, and Incubate for [4 Minutes]
17 Warmup Slide to [43 Deg C], and Incubate for [1 Hr 16 Min] (Hybridization #1)
18 Apply Three Drops of [PROBE 2] (Probe #2), Apply Coverslip, and Incubate for [4 Minutes]
19 Warmup Slide to [43 Deg C], and Incubate for [1 Hr 16 Min] (Hybridization #2)
20 Apply Two Drops of [DETECTION 1] (Detection #1), and Incubate for 4 Minutes
21 Apply Two Drops of [DETECTION 2] (Detection #2), Apply Coverslip, and Incubate for 4 Minutes
22 Warmup Slide to [42 Deg C], and Incubate for [2 Hours] (Hybridization #3)
23 Apply One Drop of [DETECTION 3] (Detection #3), No Coverslip and Incubate for 4 Minutes
24 Apply Two Drops of [DETECTION 4] (Detection #4), Apply Coverslip, and Incubate for [0 Hr 32 Min]
25 Apply Two Drops of [DETECTION 5] (Detection #5), Apply Coverslip, and Incubate for [0 Hr 8 Min]
26 Apply Two Drops of [DETECTION 6] (Detection #6), Apply Coverslip, and Incubate for [0 Hr 32 Min]
27 Disable heat for 1st Detection [Selected]
28 Fluorescent Detection [Selected]
29 Cy5 [Selected]
30 Apply One Drop of Cy5 H2O2, and Incubate for [0 Hr 16 Min]

* one drop is one reagent dispense
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Appendix C: HCR™ Pro RNA-CISH + IHC Co-Detection Protocol Summary [CISH - mRNA Purple and IHC - Discovery Green]

Protocol Summary

Procedure: HCR Universal (v3.01.0000)

DISCOVERY ULTRA

Ventana Medical Systems, Inc., 1910 Innovation Park Drive Tucson, Arizona USA

Validated: No **Active: Yes**

Protocol No	Protocol Name	Version	Creation Date
1802	CISH mRNA Purple IHC green duplex	1	11/28/2023 4:05:30 PM

1	Baking [Selected]
2	Warmup Slide to 60 Deg C, and Incubate for [0 Hr 32 Min] (Baking)
3	Deparaffinization [Selected]
4	Apply Two Drops of [PRETREATMENT 1] (Pretreatment #1), and Incubate for 4 Minutes
5	Apply One Drop of [PRETREATMENT 1] (Pretreatment #1), No Coverslip and Incubate for 4 Minutes
6	Pretreatment [Selected]
7	HCR Target Retrieval v2 [Selected]
8	Apply Three Drops of [PRETREATMENT 2] (Pretreatment #2) and Incubate for 4 Minutes
9	Warmup Slide to [97 Deg C] from Very High Temperatures (Cycle 1)
10	16 Minutes [Selected]
11	Apply One Drop of [PRETREATMENT 2] (Pretreatment #2), Apply Coverslip, and Incubate for 4 Minutes
12	24 minutes [Selected]
13	Blocker [Selected]
14	Apply One Drop of [PRETREATMENT 3] (Pretreatment #3), and Incubate for [0 Hr 8 Min]
15	HCR RNA ISH [Selected]
16	Apply Three Drops of [PROBE 1] (Probe #1), Apply Coverslip, and Incubate for [4 Minutes]
17	Warmup Slide to [43 Deg C], and Incubate for [1 Hr 16 Min] (Hybridization #1)
18	Apply Three Drops of [PROBE 2] (Probe #2), Apply Coverslip, and Incubate for [4 Minutes]
19	Warmup Slide to [43 Deg C], and Incubate for [1 Hr 16 Min] (Hybridization #2)
20	Apply Two Drops of [DETECTION 1] (Detection #1), and Incubate for 4 Minutes
21	Apply Two Drops of [DETECTION 2] (Detection #2), Apply Coverslip, and Incubate for 4 Minutes
22	Warmup Slide to [42 Deg C], and Incubate for [2 Hours] (Hybridization #3)
23	Apply One Drop of [DETECTION 3] (Detection #3), No Coverslip and Incubate for 4 Minutes
24	Apply Two Drops of [DETECTION 4] (Detection #4), Apply Coverslip, and Incubate for [0 Hr 32 Min]
25	Apply Two Drops of [DETECTION 5] (Detection #5), Apply Coverslip, and Incubate for [0 Hr 4 Min]
26	Apply Two Drops of [DETECTION 6] (Detection #6), Apply Coverslip, and Incubate for [0 Hr 32 Min]
27	Disable heat for 1st Detection [Selected]
28	mRNA Purple [Selected]
29	Apply One Drop of mRNA Purple H2O2, and Incubate for [0 Hr 20 Min]
30	IHC/IF [Selected]
31	DS Inhibitor [Selected]
32	Neutralize [Selected]
33	DS Antibody [Selected]
34	Warmup Slide to [37 Deg C] from Very Low Temperatures (DS Primary Antibody)
35	Apply One Drop of [ANTIBODY 1] (DS Antibody), and Incubate for [16 Minutes]
36	Disable heat for 2nd Detection [Selected]
37	DS Multimer HRP [Selected]
38	Apply One Drop of [OMap anti-Rb HRP] (DS Multimer HRP), and Incubate for [16 Minutes]
39	DS DISCOVERY Green HRP [Selected]
40	Apply One Drop of Green H2O2, and Incubate for [28 Minutes]
41	Apply One Drop of Green Activator, and Incubate for [16 Minutes]
42	Counterstain [Selected]

* one drop is one reagent dispense

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Protocol Summary

Procedure: HCR Universal (v3.01.0000)

DISCOVERY ULTRA

Ventana Medical Systems, Inc., 1910 Innovation Park Drive Tucson, Arizona USA

Validated: No **Active: Yes**

Protocol No	Protocol Name	Version	Creation Date
1802	CISH mRNA Purple IHC green duplex	1	11/28/2023 4:05:30 PM

43	Apply One Drop of [HEMATOXYLIN II] (Counterstain), Apply Coverslip, and Incubate for [4 Minutes]
44	Post Counterstain [Selected]
45	Apply One Drop of [BLUING REAGENT] (Post Counterstain), Apply Coverslip, and Incubate for [4 Minutes]

Appendix D: HCR™ Pro RNA-FISH + IF Co-Detection Protocol Summary [FISH - Cy5 and IF - Rhodamine 6G]

Protocol Summary

Procedure: HCR Universal (v3.01.0000)

DISCOVERY ULTRA

Ventana Medical Systems, Inc., 1910 Innovation Park Drive Tucson, Arizona USA

Validated: No **Active:** Yes

Protocol No	Protocol Name	Version	Creation Date
776	FISH Cy5 IF Rho 6G	2	10/09/2023 1:34:53 PM

1 Baking [Selected]
2 Warmup Slide to 60 Deg C, and Incubate for [0 Hr 32 Min] (Baking)
3 Deparaffinization [Selected]
4 Apply Two Drops of [PRETREATMENT 1] (Pretreatment #1), and Incubate for 4 Minutes
5 Apply One Drop of [PRETREATMENT 1] (Pretreatment #1), No Coverslip and Incubate for 4 Minutes
6 Pretreatment [Selected]
7 HCR Target Retrieval v2 [Selected]
8 Apply Three Drops of [PRETREATMENT 2] (Pretreatment #2) and Incubate for 4 Minutes
9 Warmup Slide to [97 Deg C] from Very High Temperatures (Cycle 1)
10 16 Minutes [Selected]
11 Apply One Drop of [PRETREATMENT 2] (Pretreatment #2), Apply Coverslip, and Incubate for 4 Minutes
12 24 minutes [Selected]
13 Blocker [Selected]
14 Apply One Drop of [PRETREATMENT 3] (Pretreatment #3), and Incubate for [0 Hr 8 Min]
15 HCR RNA ISH [Selected]
16 Apply Three Drops of [PROBE 1] (Probe #1), Apply Coverslip, and Incubate for [4 Minutes]
17 Warmup Slide to [43 Deg C], and Incubate for [1 Hr 16 Min] (Hybridization #1)
18 Apply Three Drops of [PROBE 2] (Probe #2), Apply Coverslip, and Incubate for [4 Minutes]
19 Warmup Slide to [43 Deg C], and Incubate for [1 Hr 16 Min] (Hybridization #2)
20 Apply Two Drops of [DETECTION 1] (Detection #1), and Incubate for 4 Minutes
21 Apply Two Drops of [DETECTION 2] (Detection #2), Apply Coverslip, and Incubate for 4 Minutes
22 Warmup Slide to [42 Deg C], and Incubate for [2 Hours] (Hybridization #3)
23 Apply One Drop of [DETECTION 3] (Detection #3), No Coverslip and Incubate for 4 Minutes
24 Apply Two Drops of [DETECTION 4] (Detection #4), Apply Coverslip, and Incubate for [0 Hr 32 Min]
25 Apply Two Drops of [DETECTION 5] (Detection #5), Apply Coverslip, and Incubate for [0 Hr 8 Min]
26 Apply Two Drops of [DETECTION 6] (Detection #6), Apply Coverslip, and Incubate for [0 Hr 32 Min]
27 Disable heat for 1st Detection [Selected]
28 Fluorescent Detection [Selected]
29 Cy5 [Selected]
30 Apply One Drop of Cy5 H2O2, and Incubate for [0 Hr 16 Min]
31 IHC/IF [Selected]
32 DS Inhibitor [Selected]
33 Neutralize [Selected]
34 DS Antibody [Selected]
35 Warmup Slide to [37 Deg C] from Very Low Temperatures (DS Primary Antibody)
36 Apply One Drop of [ANTIBODY 1] (DS Antibody), and Incubate for [16 Minutes]
37 Disable heat for 2nd Detection [Selected]
38 DS Multimer HRP [Selected]
39 Apply One Drop of [OMap anti-Rb HRP] (DS Multimer HRP), and Incubate for [16 Minutes]
40 DS Rhodamine 6G [Selected]
41 Apply One Drop of Rhod 6G H2O2, and Incubate for [0 Hr 4 Min]

* one drop is one reagent dispense
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Appendix E: Third-Party Recommended Tyramide Dyes

Validated Tyramide Dyes	Incubation Time	Recommended Starting Concentration	Vendor	Catalog #
CF488A	8-24 min	10 µM	Biotium	92171
CF550R	8-24 min	10 µM	Biotium	96077
CF555	8-24 min	10 µM	Biotium	96021
CF583R	8-24 min	10 µM	Biotium	96085
CF594	8-24 min	10 µM	Biotium	92174
CF640R	8-24 min	10 µM	Biotium	92175
CF754	8-24 min	10 µM	Biotium	96090
Alexa Fluor 488 - Tyramide	8-24 min	2x	ThermoFisher	B40953
Alexa Fluor 546 - Tyramide	8-24 min	2x	ThermoFisher	B40954
Alexa Fluor 647 - Tyramide	8-24 min	2x	ThermoFisher	B40958
Alexa Fluor 750 - Tyramide	8-24 min	2x	ThermoFisher	B56131

Appendix F: 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).

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:

   $$\mathbf{V_D = 300 * N_S + 600}$$

   Where 600 is the recommended dead volume (in µL) for the DISCOVERY ULTRA.

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 user-fillable dispensers. 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:

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 *** 4** + 600 V_D = 1800 µL	V_P = 1800 / 50 V_P = 36 µL	**HCR™ HiFi Probe A Solution** 36 µL HiFi Probe 1A 1800 µL HiFi Probe A Diluent **HCR™ HiFi Probe B Solution** 36 µL HiFi Probe 1B 1800 µL HiFi Probe B Diluent

The table below outlines the individual component volumes required to stain 3, 5, 10, and 20 slides:

# of Slides	HCR™ HiFi Probe A/B Diluent Volume	HCR™ HiFi Probe 1A/1B Volume
3	1.5 mL	30 µL
5	2.1 mL	42 µL
10	3.6 mL	72 µL
20	6.6 mL	132 µL

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

Appendix G: 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™ Pro Detect E** (Step #25 as written in Appendix A-D) from 8 minutes to **4 minutes**.
Fine-tuning[2]	To finely reduce signal intensity, you can either: 1. Reduce the incubation time of **HCR™ Pro Detect B** (Step #22 as written in Appendix A-D) from 120 minutes to **60-90 minutes.** 2. Reduce the **chromogenic deposition** time at an increment of **4 minutes** (except for DISCOVERY Red, which should be kept at 12 minutes).

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

Level of Tuning	Guidelines
Coarse-tuning[1]	To significantly increase the signal, you can either: 1. Increase the incubation time of **HCR™ Pro Detect E** (Step #25 as written in Appendix A-D) from 8 minutes to **16-24 minutes**. 2. De-select "**Disable heat for 1st Detection**" (Step #27 as written in Appendix A-D). Please note that the hematoxylin counterstain could be slightly more intense due to the heat.
Fine-tuning[2]	Increase the **chromogenic deposition** time at an increment of **4 minutes**. Please see the recommended incubation times for various chromogens on page 14 of this Setup Guide.

[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™ Pro Detect reagents to the target, increase the **Antigen Retrieval** time from 15 to up to **24-32 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 (Step #17 as written in Appendix A-D) 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 Roche's "protease 3" at **37 °C** for **4 to 8 minutes**.

Appendix H: 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 (or Roche's validated primaries), any of Roche's detection kits (e.g., Multimer detection HRP/AP Kits), and any of Roche's fluorophore kits (e.g., for IF). 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/DAB chromogen for RNA-ISH and any of Roche's translucent chromogens (e.g., green, purple, teal, and etc.) 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 Roche's OmniMap anti-Rb HRP (Ordering Code: 05269679001) followed by any of Roche's chromogens or fluorophores. 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. **Can you incorporate additional Antigen Retrieval Steps for an HCR™ Pro RNA-ISH + IHC/IF co-detection assay?**
    a. Yes! If the target retrieval for HCR™ Pro RNA-ISH is insufficient/sub-optimal for your protein target, you can incorporate a separate CC1/CC2 target retrieval step after ISH, but before the IHC/IF staining.
5. **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).
    b. In such cases, we recommend that you use a light protease digestion with Roche's "protease 3" at **37 °C** for **4 to 8 minutes**.