

HCR™ RNA-ISH Setup Guide for the DISCOVERY ULTRA

This Setup Guide demonstrates the use of an HCR™ 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™ 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™ RNA-ISH Kit 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™ RNA-ISH Kit Information

Upon receiving an HCR™ RNA-ISH Kit, please check all reagents and their storage conditions listed below.

HCR™ RNA-ISH Starter Kit

HCR™ Reagents	Amount for an HCR™ RNA-ISH Starter Kit	Storage Temperature
HCR™ Probe A <i>PPIB/Ppib</i> ¹ – Positive Control ²	1 mL	2 to 8 °C
HCR™ Probe B <i>PPIB/Ppib</i> ¹ – Positive Control ²	1 mL	2 to 8 °C
HCR™ Probe A Diluent	3 mL	2 to 8 °C
HCR™ Probe B Diluent	3 mL	2 to 8 °C
HCR™ Probe A <i>dapB</i> – Negative Control ²	1 mL	2 to 8 °C
HCR™ Probe B <i>dapB</i> – Negative Control ²	1 mL	2 to 8 °C
HCR™ Probe A Diluent	3 mL	2 to 8 °C
HCR™ Probe B Diluent	3 mL	2 to 8 °C
HCR™ Membrane Stain ³	1.5 mL	2 to 8 °C
HCR™ Control Slides ⁴	3 Slides	2 to 8 °C
HCR™ Pretreat A	7 mL	2 to 8 °C
HCR™ Pretreat B	14 mL	2 to 8 °C
HCR™ Pretreat C	5 mL	2 to 8 °C
HCR™ Detect A	5 mL	2 to 8 °C
HCR™ Detect B	5 mL	2 to 8 °C
HCR™ Detect C	5 mL	2 to 8 °C
HCR™ Detect D	5 mL	2 to 8 °C
HCR™ Detect E	5 mL	2 to 8 °C
HCR™ Detect F AP/HRP ⁵	5 mL	2 to 8 °C

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

² These are the HCR™ Probes included in the HCR™ RNA-ISH Starter Kit, and they are provided in volumes sufficient to perform the assay on 10 slides each.

³ 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 15-16 for more information on how to perform an HCR™ RNA-ISH + IHC/IF co-detection assay.

⁴ The HCR™ Control Slides include 3 human or mouse liver FFPE tissue sections. Please allocate one slide for the positive control, one slide for the negative control, and one slide for the HCR™ RNA-ISH + IHC/IF co-detection assay using the HCR™ Membrane Stain.

⁵ HCR™ Detect F AP is included in the HCR™ RNA-ISH AP Starter Kit, and HCR™ Detect F HRP is included in the HCR™ RNA-ISH HRP Starter Kit.

HCR™ RNA-ISH Kit

HCR™ Reagents	Amount for a 20 Slide Kit	Amount for a 90 Slide Kit	Storage Temperature
HCR™ Probe A ¹	1.75 mL	7 mL	2 to 8 °C
HCR™ Probe B	1.75 mL	7 mL	2 to 8 °C
HCR™ Probe A Diluent	5.25 mL	21 mL	2 to 8 °C
HCR™ Probe B Diluent	5.25 mL	21 mL	2 to 8 °C
HCR™ Pretreat A	7 mL	28 mL	2 to 8 °C
HCR™ Pretreat B	14 mL	55 mL	2 to 8 °C
HCR™ Pretreat C	5 mL	15 mL	2 to 8 °C
HCR™ Detect A	5 mL	19 mL	2 to 8 °C
HCR™ Detect B	5 mL	19 mL	2 to 8 °C
HCR™ Detect C	5 mL	15 mL	2 to 8 °C
HCR™ Detect D	5 mL	19 mL	2 to 8 °C
HCR™ Detect E	5 mL	19 mL	2 to 8 °C
HCR™ Detect F AP/HRP ²	5 mL	19 mL	2 to 8 °C

¹ Every HCR™ Probe includes 4 components: HCR™ Probe A, HCR™ Probe B, HCR™ Probe A Diluent, and HCR™ Probe B Diluent. Please reference Page 7 for more information on how to prepare the HCR™ Probe solution.

² HCR™ Detect F AP is included in the HCR™ RNA-ISH AP Kit, and HCR™ Detect F HRP is included in the HCR™ RNA-ISH HRP Kit.

Required Materials for the DISCOVERY ULTRA

The HCR™ 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™ RNA-ISH experiment. For more information, please inquire with your Roche representative.

Materials from Roche		
	Catalog #	Quantity
PRETREATMENT Barcodes and Open Dispensers	Varies	4
PROBE Barcodes and Open Dispensers	Varies	Varies ¹
DETECTION Dispensers and Barcodes	Varies	6 ²

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

² You will need to obtain additional detection barcodes if you are using third-party tyramide dyes.

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™ 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

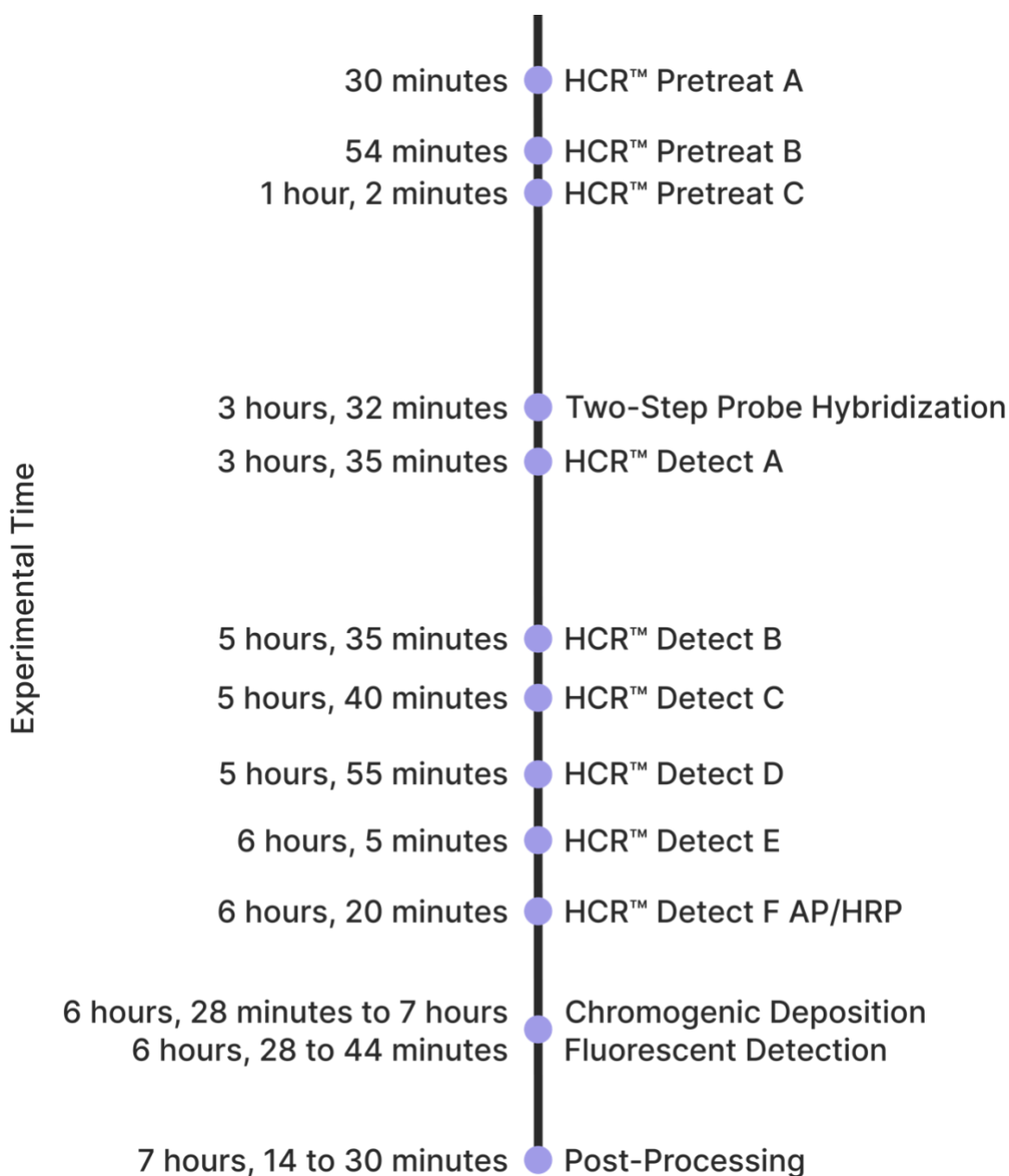
Materials from Roche		
	Catalog #	Quantity
ANTIBODY Dispensers and Barcodes	Varies	1
DISCOVERY Inhibitor RUO	07017944001	1
OmniMAP or UltraMAP HRP/AP ¹	Varies	1

¹ Our HCR™ 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™ 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.

Probe Solution Preparation

HCR™ Probe Hybridization is a two-step process that requires two separate probe solutions. HCR™ Probe A solution and HCR™ Probe B solution can be prepared separately by mixing HCR™ Probe A with HCR™ Probe A Diluent and HCR™ Probe B with HCR™ Probe B Diluent, respectively. We recommend transferring the entirety of HCR™ Probe A and HCR™ Probe B into the HCR™ Probe A Diluent and HCR™ Probe B Diluent bottles. After the solutions have been transferred, you can either vortex the bottles or invert the bottle 4-5 times to ensure proper mixture.

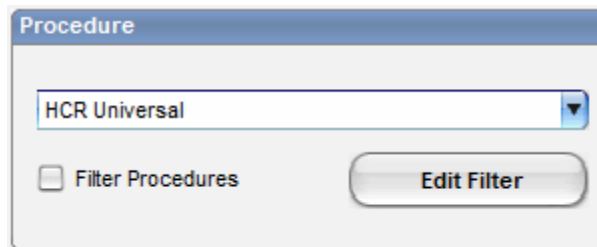
Filling and Registering Reagents

Refer to the Roche Manual (pages 296-309) for directions on how to fill and register user-fillable dispensers.

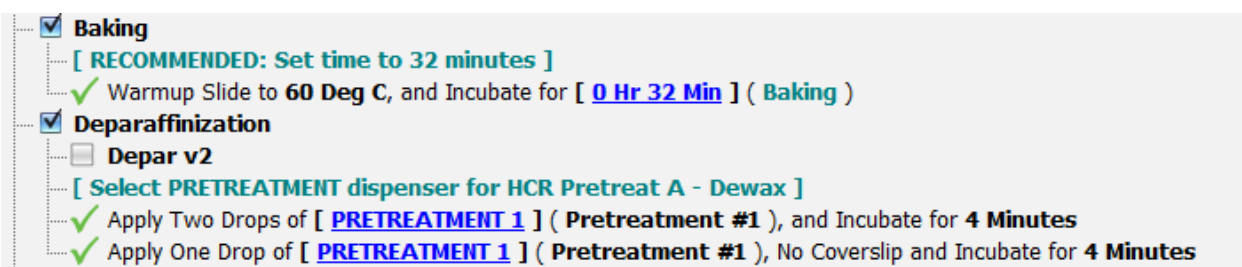
Reagent	Dispenser Barcode	Recommended Incubation Time
HCR™ Pretreat A	PRETREATMENT	Default
HCR™ Pretreat B	PRETREATMENT	16-24 minutes
HCR™ Pretreat C	PRETREATMENT	8 minutes
HCR™ Probe A Solution	PROBE	1 hour, 16 mins
HCR™ Probe B Solution	PROBE	1 hour, 16 mins
HCR™ Detect A	DETECTION	Default
HCR™ Detect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

Creating an HCR™ 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**. **HCR™ Pretreat A** must be placed into an open **PRETREATMENT dispenser**. Make sure that the **Pretreatment** selections are the same.

STEP 3: Select **Pretreatment**.



The **HCR™ Pretreat B** must be placed into an open **PRETREATMENT dispenser** that is a different value than the one assigned to HCR™ 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 HCR™ Pretreat B solution.

☒ **Pretreatment**
 [Recommended Temp and Time = 97 C and 16-24 minutes]

☒ **HCR Target Retrieval**
 [Select PRETREATMENT dispenser for HCR RNA-CISH Antigen Retrieval]

☒ Apply Three Drops of [PRETREATMENT 2] (Pretreatment #2) and Incubate for 4 Minutes

☒ Warmup Slide to [97 Deg C] from All Temperatures (Cycle 1)

☒ **16 Minutes**

☒ **24 minutes**

☐ **32 minutes**

Optional: You can forego the use of HCR™ 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.

☒ **Pretreatment**
 [Recommended Temp and Time = 97 C and 16-24 minutes]

☐ **HCR Target Retrieval**

☐ **HCR Target Retrieval v2**

☒ **CC1 Reservoir**

☐ **Low Temp CC1**

☒ Warmup Slide to [Very High Temperature], and Incubate for 4 Minutes (Cell Conditioner #1)

☐ **CC1 8 Min**

☐ **CC2 Reservoir**

☐ **CC Option**

☐ **Protease Options**

☐ **Blocker**

☐ **Inhibitor**

☒ **Pretreatment**
 [Recommended Temp and Time = 97 C and 16-24 minutes]

☐ **HCR Target Retrieval**

☐ **HCR Target Retrieval v2**

☐ **CC1 Reservoir**

☒ **CC2 Reservoir**

☐ **Low Temp CC2**

☐ [91°C is the standard temperature]

☒ Warmup Slide to [Very High Temperature], and Incubate for 4 Minutes (Cell Conditioner #2)

☐ **CC2 8 Min**

☐ **CC Option**

☐ **Protease Options**

☐ **Blocker**

☐ **Inhibitor**

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.

☒ **Pretreatment**
 [Recommended Temp and Time = 97 C and 16-24 minutes]

☐ HCR Target Retrieval

☐ HCR Target Retrieval v2

☐ CC1 Reservoir

☐ CC2 Reservoir

☐ CC Option

☒ **Protease Options**
 [RECOMMENDED: If using temperatures above 42°C, set incubation for less than 1 hour]

☒ Warmup Slide to [37 Deg C], and Incubate for 4 Minutes (Enzyme Temp RB)

☒ Apply One Drop of [PROTEASE 3] (Enzyme), and Incubate for [0 Hr 4 Min]

☐ **Blocker**

☐ **Inhibitor**

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

☒ **Pretreatment**
 [Recommended Temp and Time = 97 C and 16-24 minutes]

☐ HCR Target Retrieval

☒ **HCR Target Retrieval v2**
 [Select PRETREATMENT dispenser for HCR Pretreat B - Target Retrieval]
 [Tissue Pretreatment]

☒ Apply Three Drops of [PRETREATMENT 2] (Pretreatment #2) and Incubate for 4 Minutes

☒ Warmup Slide to [97 Deg C] from Very High Temperatures (Cycle 1)

☒ **16 Minutes**
☒ Apply One Drop of [PRETREATMENT 2] (Pretreatment #2), Apply Coverslip, and Incubate for 4 Minutes

☒ **24 minutes**
☒ Apply One Drop of [PRETREATMENT 2] (Pretreatment #2), Apply Coverslip, and Incubate for 4 Minutes

☐ 32 minutes

☐ CC1 Reservoir

☐ CC2 Reservoir

☐ CC Option

☐ **Protease Options**

☒ **Blocker**
 [Select PRETREATMENT dispenser for HCR Pretreat C - Endogenous Enzyme Block]

☒ Apply One Drop of [PRETREATMENT 3] (Pretreatment #3), and Incubate for [0 Hr 8 Min]

☐ **Inhibitor**

HCR™ 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 RNA ISH**

☐ **Pre-Hybridization**

[Select PROBE dispenser for HCR Probe & Incubate for 4 minutes]

[HCR Probe A incubate for 1hr 16min at 43C]

✓ Apply Three Drops of [[PROBE 1](#)] (**Probe #1**), Apply Coverslip, and Incubate for [[4 Minutes](#)]

✓ Warmup Slide to [[43 Deg C](#)], and Incubate for [[1 Hr 16 Min](#)] (**Hybridization #1**)

[HCR Probe B incubate for 1hr 16min at 43C]

✓ Apply Three Drops of [[PROBE 2](#)] (**Probe #2**), Apply Coverslip, and Incubate for [[4 Minutes](#)]

✓ Warmup Slide to [[43 Deg C](#)], and Incubate for [[1 Hr 16 Min](#)] (**Hybridization #2**)

HCR™ Probe A and HCR™ Probe B 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.*

✓ Apply Two Drops of [[DETECTION 1](#)] (**Detection #1**), and Incubate for **4 Minutes**

[Select DETECTION dispenser for HCR Detect B]

✓ Apply Two Drops of [[DETECTION 2](#)] (**Detection #2**), Apply Coverslip, and Incubate for **4 Minutes**

[Recommended temp = 42C; Target Time = 2 hours]

✓ Warmup Slide to [[42 Deg C](#)], and Incubate for [[2 Hours](#)] (**Hybridization #3**)

[Select DETECTION dispenser for HCR Detect C]

✓ Apply One Drop of [[DETECTION 3](#)] (**Detection #3**), No Coverslip and Incubate for **4 Minutes**

[Select DETECTION dispenser for HCR Detect D]

[Recommended incubation time is 32 minutes]

✓ Apply Two Drops of [[DETECTION 4](#)] (**Detection #4**), Apply Coverslip, and Incubate for [[0 Hr 32 Min](#)]

[Select DETECTION dispenser for HCR Detect E]

[Recommended incubation time is 8 minutes]

✓ Apply Two Drops of [[DETECTION 5](#)] (**Detection #5**), Apply Coverslip, and Incubate for [[0 Hr 8 Min](#)]

[Select DETECTION dispenser for HCR Detect F HRP/AP]

[Recommended incubation time is 32 minutes]

✓ Apply Two Drops of [[DETECTION 6](#)] (**Detection #6**), Apply Coverslip, and Incubate for [[0 Hr 32 Min](#)]

HCR™ Detect A to F must be placed into **Detection dispensers**. Select **DETECTION dispensers** for each of the HCR™ Detect reagents and follow the comments (displayed in green text) for recommendations on a standard starting protocol.

STEP 5a: To perform **chromogenic ISH**, select the appropriate **Chromogen** that corresponds to your HCR™ 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™ RNA-ISH with mRNA DAB detection.*

☐ [Disabling heat is recommended]

☒ **Disable heat for 1st Detection**

☐ [Default detection is mRNA DAB unless a chromogen or fluorescent detection is selected]

☐ mRNA Purple

☐ mRNA Green

☐ mRNA Teal

☒ **DISCOVERY Red**

☐ [To be used with HCR RNA-CISH AP detection]

☐ [Recommended incubation time is 12 minutes]

☒ Apply One Drop of **DISC Naphthol** and One Drop of **DISC Fast Red**, Apply Coverslip, Incubate for [**12 Minutes**]

☐ **Fluorescent 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™ RNA-ISH HRP Kit. See **Appendix B** for an example protocol summary for HCR™ RNA-FISH with Cy5 Detection.*

☒ **Disable heat for 1st Detection**

☐ [Default detection is mRNA DAB unless a chromogen or fluorescent detection is selected]

☐ mRNA Purple

☐ mRNA Green

☐ mRNA Teal

☐ DISCOVERY Red

☒ **Fluorescent Detection**

☒ **Cy5**

☒ Apply One Drop of **Cy5 H2O2**, and Incubate for [**0 Hr 12 Min**]

☐ Rhodamine 6G

☐ DCC

☐ FAM

☐ Red 610

☐ Open Detection Kit

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 use third-party TSA dyes by selecting **Open Detection Kit** (see **Appendix E** for recommendations).

NOTE: This selection requires another open Detection dispenser.

☒ **Disable heat for 1st Detection**
 [Default detection is mRNA DAB unless a chromogen or fluorescent detection is selected]

☐ mRNA Purple
☐ mRNA Green
☐ mRNA Teal
☐ DISCOVERY Red

☒ **Fluorescent Detection**
☐ Cy5
☐ Rhodamine 6G
☐ DCC
☐ FAM
☐ Red 610
☒ **Open Detection Kit**
 [User Provided Fluorescent Dye]
 ✓ Apply Two Drops of [[DETECTION 7](#)] (**Detection #7**), Apply Coverslip, and Incubate for [[0 Hr 12 Min](#)]

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

[Disabling heat is recommended]

☒ **Disable heat for 1st Detection**
☐ SxS Automated Open Detection
☒ **Chromogen Block**
☐ mRNA Purple
☒ **DISCOVERY Red**
 [To be used with HCR RNA-CISH AP detection]
 [Recommended incubation time is 12 minutes]
 ✓ Apply One Drop of **DISC Naphthol** and One Drop of **DISC Fast Red**, Apply Coverslip, Incubate for [[12 Minutes](#)]

☐ Research Fork #16
☐ DAB
☐ DISCOVERY Purple
☐ Silver
☐ DISCOVERY Green HRP
☐ DISCOVERY Yellow HRP
☐ DISCOVERY Blue HRP

☐ **IHC**
☒ **Counterstain**
 ✓ Apply One Drop of [[HEMATOXYLIN II](#)] (**Counterstain**), Apply Coverslip, and Incubate for [[4 Minutes](#)]
☒ **Post Counterstain**
 ✓ Apply One Drop of [[BLUING REAGENT](#)] (**Post Counterstain**), Apply Coverslip, and Incubate for [[4 Minutes](#)]

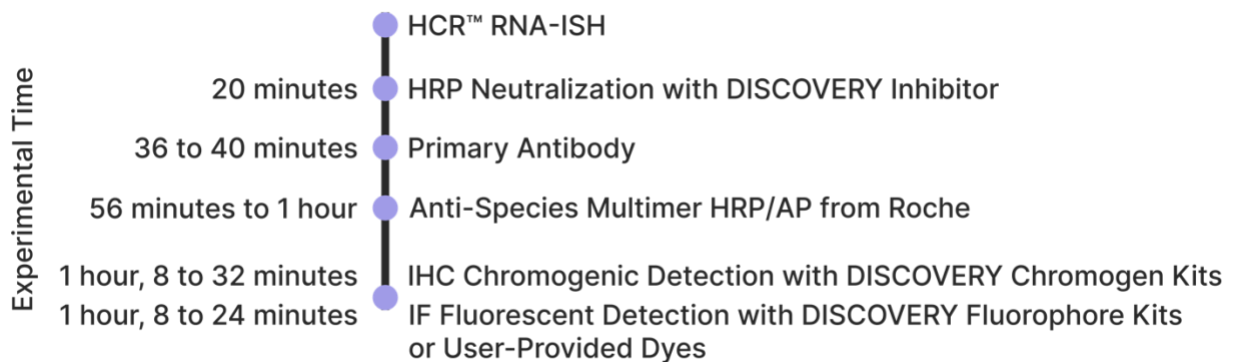
Post-Processing for AP-Based Detection

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 coverslipping.

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.

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



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

Chromogenic ISH + IHC Co-Detection

Program the HCR™ RNA-ISH Protocol as outlined previously (see Page 8). 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™ RNA-CISH + IHC Co-Detection with mRNA Purple for CISH and Discovery Green for IHC.

☒ **IHC/IF**

- ☐ DS Pretreatment
- ☐ DS Option
- ☒ **DS Inhibitor**
 - [Use of HRP conjugates requires DISC Inhib to inactivate endogenous peroxidase]
 - ☒ **Neutralize**
 - [Neutralize step of previously bound HRP conjugates]
- ☐ DS ISH
- ☒ **DS Antibody**
 - ☐ DS Antibody Manual Application
 - ☐ DS Antibody Blocking
 - ☐ Disable heat for DS Antibody
 - ☐ DS High Temp Ab incubation
 - ☒ Warmup Slide to [37 Deg C] from Very Low Temperatures (DS Primary Antibody)
 - ☐ DS Extended Ab incubation
 - ☒ Apply One Drop of [ANTIBODY 1] (DS Antibody), and Incubate for [16 Minutes]
 - ☐ Disable heat for 2nd Fixative
 - ☐ DS Post-Antibody Fixative
 - ☐ DS Linking Antibody
 - ☒ Disable heat for 2nd Detection
 - ☒ **DS Multimer HRP**
 - ☐ DS Multimer HRP Blocker
 - [Select Multimer species]
 - ☒ Apply One Drop of [OMap anti-Rb HRP] (DS Multimer HRP), and Incubate for [16 Minutes]
 - ☐ DS Multimer AP
 - ☐ DS Enzyme conjugate
 - ☐ DS DISCOVERY Amplification
 - ☐ DS DAB
 - ☐ DS Silver
 - ☒ **DS DISCOVERY Purple**
 - ☒ Apply One Drop of DISC H2O2 P, and Incubate for [0 Hr 32 Min]

Fluorescent ISH + IF Co-Detection

Program the HCR[™] RNA-ISH Protocol as outlined previously (see Page 8). 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[™] RNA-FISH + IF Co-Detection with Cy5 for FISH and Rhodamine 6G for IF.

☐ DS Cy5
☐ DS DCC
☐ DS FAM
☐ DS Red 610
☒ DS Rhodamine 6G
✓ Apply One Drop of Rhod 6G H2O2, and Incubate for [0 Hr 4 Min]
☐ DS Open Detection Kit
☐ Triple Stain

Appendix

Appendix A: HCRTM 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 1801	Protocol Name mRNA DAB Protocol Summary	Version 1	Creation Date 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: HCRTM RNA-FISH Protocol Summary [Fluorescent Detection - Cy5 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 778	Protocol Name FISH Cy5 Detection	Version 2	Creation Date 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: HCRTM 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™ 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
Opal 520	8-24 min	1:250	Akoya Biosciences	FP1487001KT
Opal 570	8-24 min	1:250	Akoya Biosciences	FP1488001KT
Opal 620	8-24 min	1:250	Akoya Biosciences	FP1495001KT
Opal 690	8-24 min	1:250	Akoya Biosciences	FP1497001KT