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

This Setup Guide demonstrates the use of a 2-Plex 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 2-Plex HCR™ Pro RNA-ISH run on the DISCOVERY ULTRA takes approximately 13 to 14 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 2-Plex HCR™ Pro RNA-ISH kit can be used to probe and visualize two RNA targets in FFPE tissue sections. Please read through the Setup Guide for additional information so that you can easily incorporate the 2-Plex 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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2-Plex HCR™ Pro RNA-ISH Kit Information

Upon receiving a 2-Plex HCR™ Pro RNA-ISH kit, please check all reagents and their storage conditions.

2-Plex HCR™ Pro RNA-ISH Kit

2-Plex 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™ HiFi Probe 2A	140 µL	580 µL	2 to 8 °C
HCR™ HiFi Probe 2B	140 µL	580 µL	2 to 8 °C

2-Plex HCR™ Pro Detect

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 2-Plex A	5 mL	19 mL	2 to 8 °C
HCR™ Pro Detect 2-Plex B	5 mL	19 mL	2 to 8 °C
HCR™ Pro Detect 2-Plex C	5 mL	19 mL	2 to 8 °C
HCR™ Pro Detect 2-Plex D	5 mL	19 mL	2 to 8 °C
HCR™ Pro Detect 2-Plex E	5 mL	19 mL	2 to 8 °C
HCR™ Pro Detect 2-Plex F AP/HRP[2]	5 mL	19 mL	2 to 8 °C
HCR™ Pro Detect 2-Plex G	5 mL	19 mL	2 to 8 °C
HCR™ Pro Detect 2-Plex H	5 mL	19 mL	2 to 8 °C
HCR™ Pro Detect 2-Plex J HRP	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 2-Plex A	5 mL	19 mL	DETECTION 8
HCR™ Pro Detect 2-Plex B	5 mL	19 mL	DETECTION 9
HCR™ Pro Detect 2-Plex C	5 mL	19 mL	DETECTION 10
HCR™ Pro Detect 2-Plex D	5 mL	19 mL	DETECTION 11
HCR™ Pro Detect 2-Plex E	5 mL	19 mL	DETECTION 12
HCR™ Pro Detect 2-Plex F HRP[2]	5 mL	19 mL	DETECTION 13
HCR™ Pro Detect 2-Plex F AP[2]	5 mL	19 mL	DETECTION 14
HCR™ Pro Detect 2-Plex G	5 mL	19 mL	DETECTION 15
HCR™ Pro Detect 2-Plex H	5 mL	19 mL	DETECTION 16
HCR™ Pro Detect 2-Plex J HRP	5 mL	19 mL	DETECTION 17

[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 2-plex HCR™ Pro RNA-ISH HRP and AP assays simultaneously, be sure to use the appropriate DETECTION 13 and DETECTION 14 barcodes as provided by MI. To avoid confusion, please do not use the DETECTION 13 barcode for HCR™ Pro Detect F AP or the DETECTION 14 barcode for HCR™ Pro Detect F HRP.

Required Materials for the DISCOVERY ULTRA

The 2-Plex HCR™ Pro RNA-ISH protocol requires specific materials available only from Roche. Ensure these materials are available before setting up a 2-Plex 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	9[2]
Light Protective Prep Kit[3]	Varies	9

[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 2-Plex reagents A-J. The ordering code for this kit is 07475144001.*

Recommended Materials for the DISCOVERY ULTRA for Running a 2-Plex 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 2-Plex HCR™ Pro RNA-ISH Kit requires the use of the DISCOVERY Red Detection Kit in the first channel. The DISCOVERY mRNA DAB Detection Kit should not be used in the first channel.*

[2]*The DISCOVERY Inhibitor is necessary for an ISH + IHC co-detection assay.*

Recommended Materials for the DISCOVERY ULTRA for Running a 2-Plex 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 a 2-Plex HCR™ Pro RNA-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]*Please 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
SlowFade Gold Antifade with DAPI (or without DAPI)	ThermoFisher	Suitable mounting medium for fluorescent detection
Cover Glass	Any	Dimension depends on the size of the tissue
100% Ethanol	Any	None

Reagent Preparation

The two main steps to prepare for an HCR™ Pro RNA-ISH run include:

i. Preparation of HiFi probe solutions
ii. Transfer of all reagents to the registered BOND Containers

Preparation of Probe Solutions

HCR™ HiFi Probe Hybridization is a two-step process that requires two separate probe solutions: 2-plex HiFi Probe A Solution and 2-plex HiFi Probe B Solution.

How you prepare these solutions depends on the concentrations of your probes. Refer to the following sections to prepare your 2-plex HCR™ HiFi Probe A/B Solutions according to whether your probes are provided at 1× and 50× or both at 50×.

Important: For short target applications, HiFi Probe solutions must be freshly prepared (within 48 hours of each experiment).

Scenario 1:
HCR™ HiFi Probe 1 = 1×
HCR™ HiFi Probe 2 = 50×

In standard configurations, HCR™ HiFi Probe 1 is supplied at 1× (ready-to-use), while HCR™ HiFi Probe 2 is supplied at 50× (concentrated). To prepare your working 2-plex HCR™ HiFi Probe A/B Solutions, dilute Probe 2 into Probe 1 based on the volumes specified below.

Prepare 2-Plex HCR™ HiFi Probe A Solution

1. **Measure out HCR™ HiFi Probe 1A**. Refer to the table below for your desired number of slides. Measure out the volume of HCR™ HiFi Probe 1A into a clean dispenser.
2. **Add HCR™ HiFi Probe 2A.** Measure out the volume listed in the third column of HCR™ HiFi Probe 2A and add to the same dispenser. Mix gently by pipetting up and down.

Prepare 2-Plex HCR™ HiFi Probe B Solution

1. **Measure out HCR™ HiFi Probe 1B**. Refer to the table below for your desired number of slides. Measure out the volume of HCR™ HiFi Probe 1A into a clean dispenser.
2. **Add HCR™ HiFi Probe 2B.** Measure out the volume listed in the third column of HCR™ HiFi Probe 2A and add to the same dispenser. Mix gently by pipetting up and down.

# of Slides	HCR™ HiFi Probe 1A/1B Volume	HCR™ HiFi Probe 2A/2B 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

For slide numbers not listed, see **Appendix E** for instructions on calculating the required volumes of HCR™ HiFi Probe 1A/1B and HCR™ HiFi Probe 2A/2B to prepare your 2-plex probe solutions.

Scenario 2:
HCR™ HiFi Probe 1 & 2 = 50×

In some experiments (e.g., when both probes target short transcripts), Probe 1 and Probe 2 are supplied at 50× concentration. In this case, your kit will include additional Probe Diluent reagents to bring both probes to working concentration.

Prepare 2-Plex HCR™ HiFi Probe A Solution

1. **Measure out HCR™ HiFi Probe A Diluent.** Refer to the table below for your desired number of slides. Measure out the volume of HCR™ HiFi Probe A Diluent into a clean dispenser.
2. **Add HCR™ HiFi Probe 1A**. Measure out the volume listed in the third column of HCR™ HiFi Probe 1A and add to the same dispenser. Mix gently by pipetting up and down.
3. **Add HCR™ HiFi Probe 2A.** Measure out the volume listed in the last column of HCR™ HiFi Probe 2A and add to the same dispenser. Mix gently by pipetting up and down.

Prepare 2-Plex HCR™ HiFi Probe B Solution

1. **Measure out HCR™ HiFi Probe B Diluent.** Refer to the table below for your desired number of slides. Measure out the volume of HCR™ HiFi Probe B Diluent into a clean dispenser.
2. **Add HCR™ HiFi Probe 1B**. Measure out the volume listed in the third column of HCR™ HiFi Probe 1B and add to the same dispenser. Mix gently by pipetting up and down.
3. **Add HCR™ HiFi Probe 2B.** Measure out the volume listed in the last column of HCR™ HiFi Probe 2B and add to the same dispenser. Mix gently by pipetting up and down.

# of Slides	HCR™ HiFi Probe A/B Diluent Volume	HCR™ HiFi Probe 1A/1B Volume	HCR™ HiFi Probe 2A/2B Volume
3	1.5 mL	30 µL	30 µL
5	2.1 mL	42 µL	42 µL
10	3.6 mL	72 µL	72 µL
20	6.6 mL	132 µL	132 µL

For slide numbers not listed, see **Appendix E** for instructions on calculating the required volumes of HCR™ HiFi Probe 1A/1B and HCR™ HiFi Probe 2A/2B to prepare your 2-plex probe solutions.

Registration of User-Filled Dispensers

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.

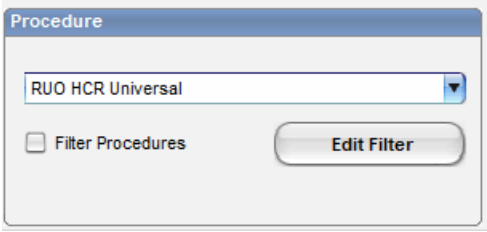
Reagent	Dispenser Barcode	Recommended Incubation Time
Pretreat A	PRETREATMENT 1	Default
Pretreat B	PRETREATMENT 2	16-24 minutes
Pretreat C	PRETREATMENT 3	8 minutes
HCR™ HiFi Probe 2-Plex A	PROBE	1 hour, 16 mins
HCR™ HiFi Probe 2-Plex B	PROBE	1 hour, 16 mins
HCR™ Pro Detect 2-Plex A	DETECTION 8	Default
HCR™ Pro Detect 2-Plex B	DETECTION 9	2 hours
HCR™ Pro Detect 2-Plex C	DETECTION 10	Default
HCR™ Pro Detect 2-Plex D	DETECTION 11	32 mins
HCR™ Pro Detect 2-Plex E	DETECTION 12	8 mins (16 mins for Discovery Red and mRNA Green detection)
HCR™ Pro Detect 2-Plex F HRP	DETECTION 13	
HCR™ Pro Detect 2-Plex F AP	DETECTION 14	32 mins
HCR™ Pro Detect 2-Plex G	DETECTION 15	32 mins
HCR™ Pro Detect 2-Plex H	DETECTION 16	8 mins (16 mins for mRNA Green Detection)
HCR™ Pro Detect 2-Plex J HRP	DETECTION 17	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 ULTRA Dispensers.
2. If the reagent-filled ULTRA Dispensers have not been used for more than a week, it is highly recommended to gently invert the dispensers several times with the lid closed to ensure solution uniformity before next use.

Creating a 2-Plex HCR™ Pro RNA-ISH Protocol

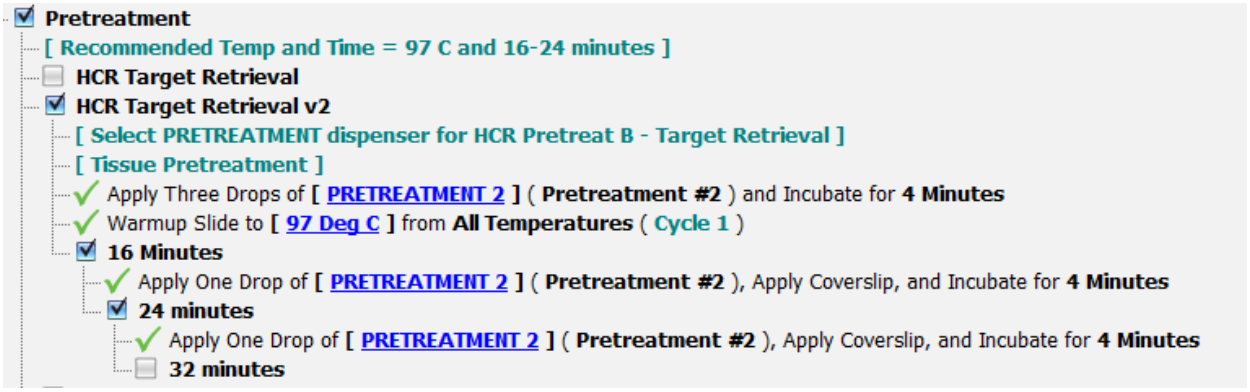
STEP 1: Select **HCR™ Universal** from the **Procedure** drop-down menu.



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

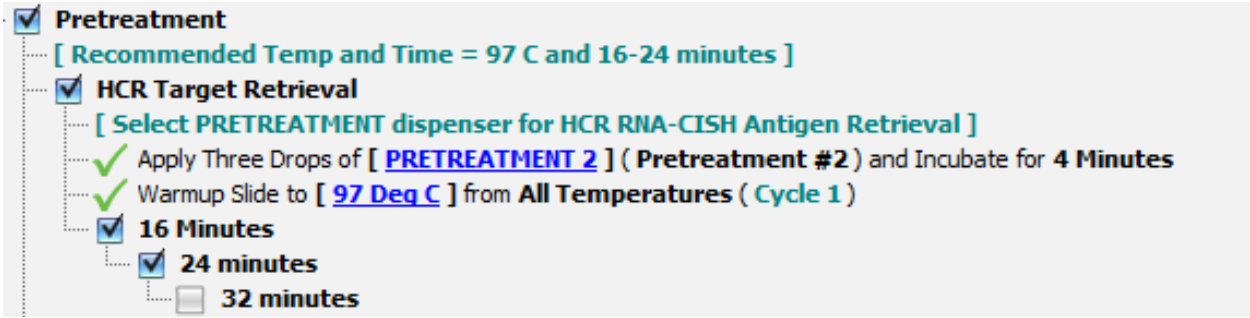
If you prefer baking slides offline, de-select **Baking**. **Pretreat A** must be placed into an open **PRETREATMENT dispenser**. Make sure that the **Pretreatment** selections are the same.

STEP 3: Select **Pretreatment**.

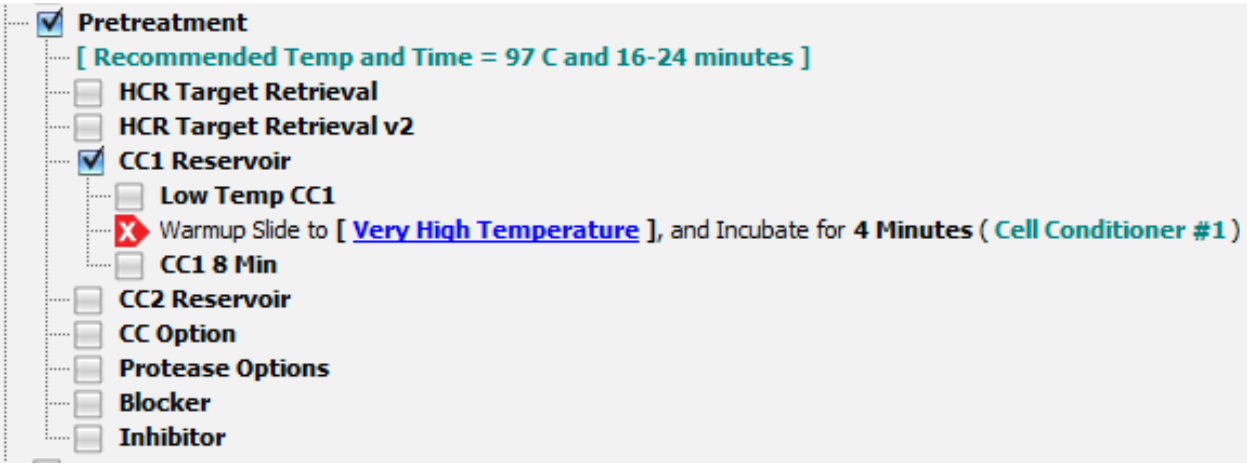


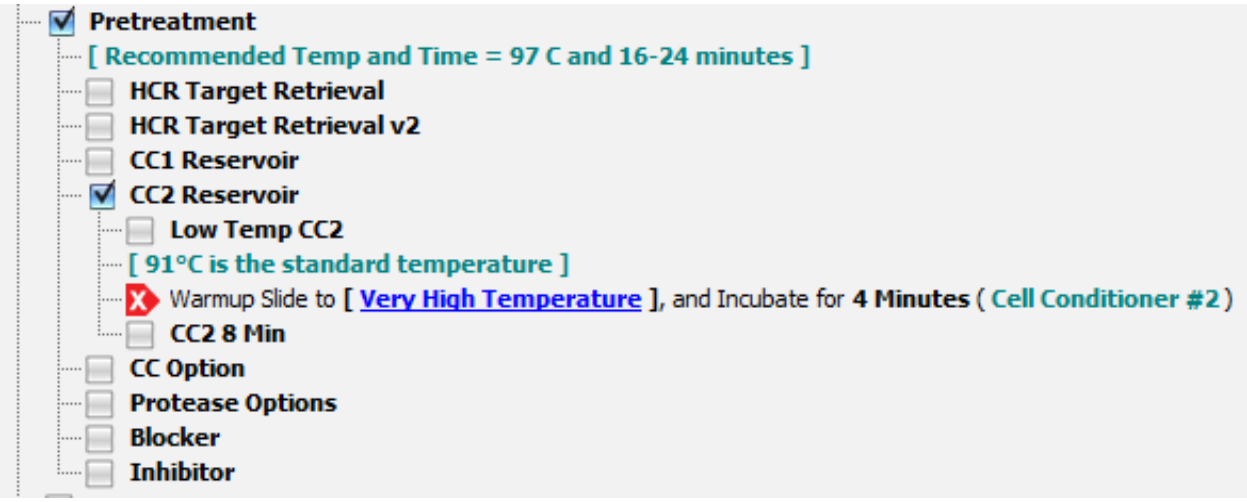
The **Pretreat B** must be placed into an open **PRETREATMENT dispenser** that is a different value than the one assigned to Pretreat A. The recommended target retrieval is HCR™ Target Retrieval v2 for 16 minutes and 24 minutes at 97 °C for FFPE cell pellets and tissues, respectively. For over-fixed 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.

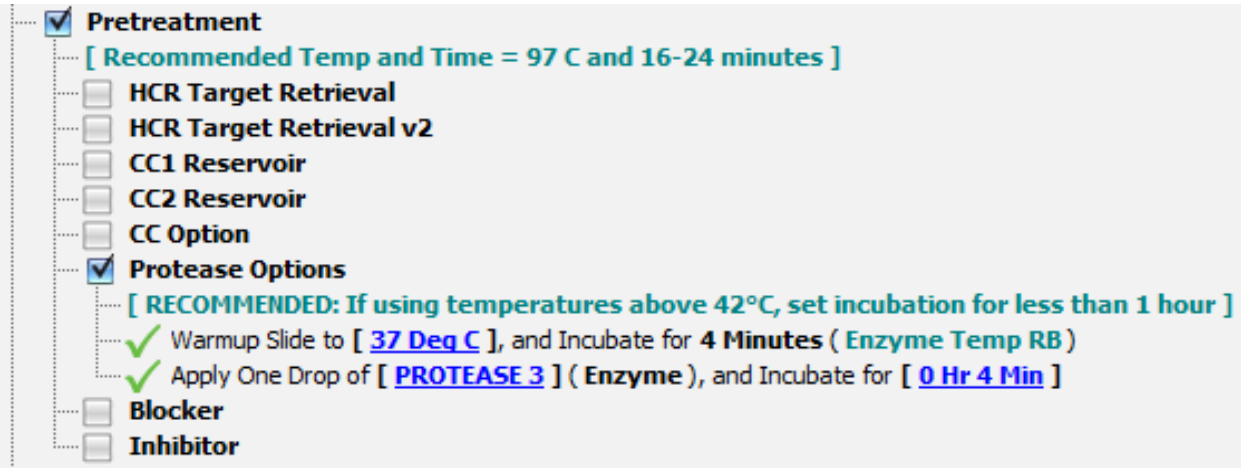


Optional: You can forego the use of Pretreat B and rely on the DISCOVERY ULTRA onboard buffers for their antigen retrieval. You may also use CC1 or CC2 in conjunction with **HCR™ Target Retrieval** selections.

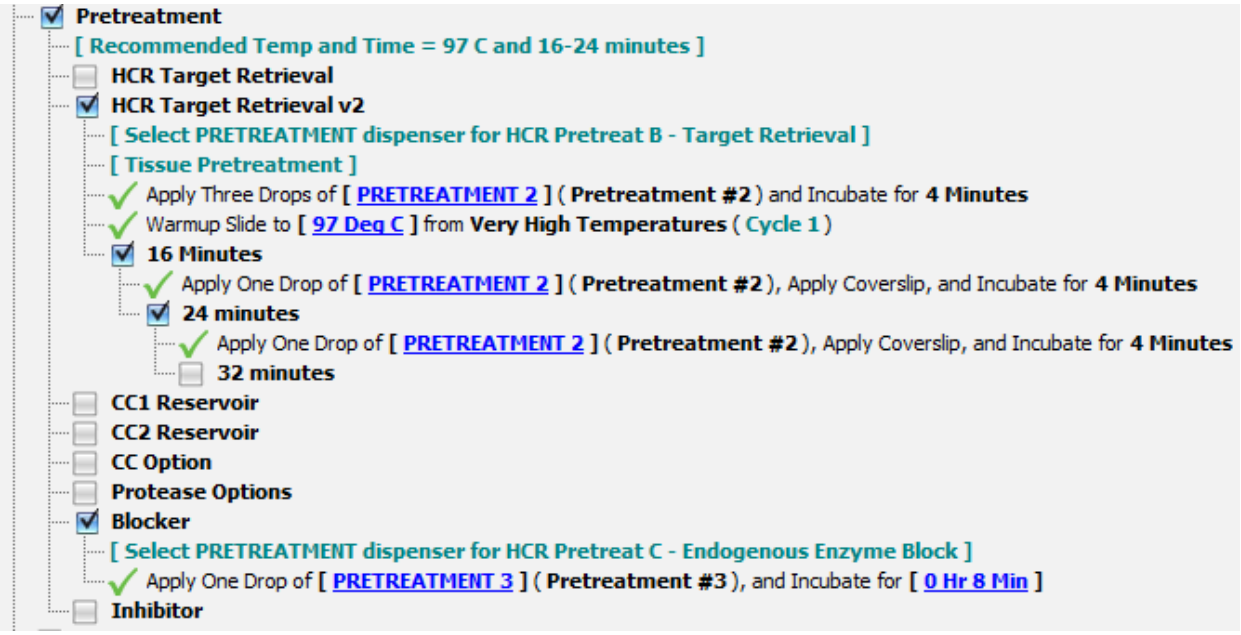




Optional: A mild protease pretreatment can be done in addition to any antigen retrieval. Please keep in mind that protease can have harmful effects on targeting proteins with any downstream IHC/IF assays.



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

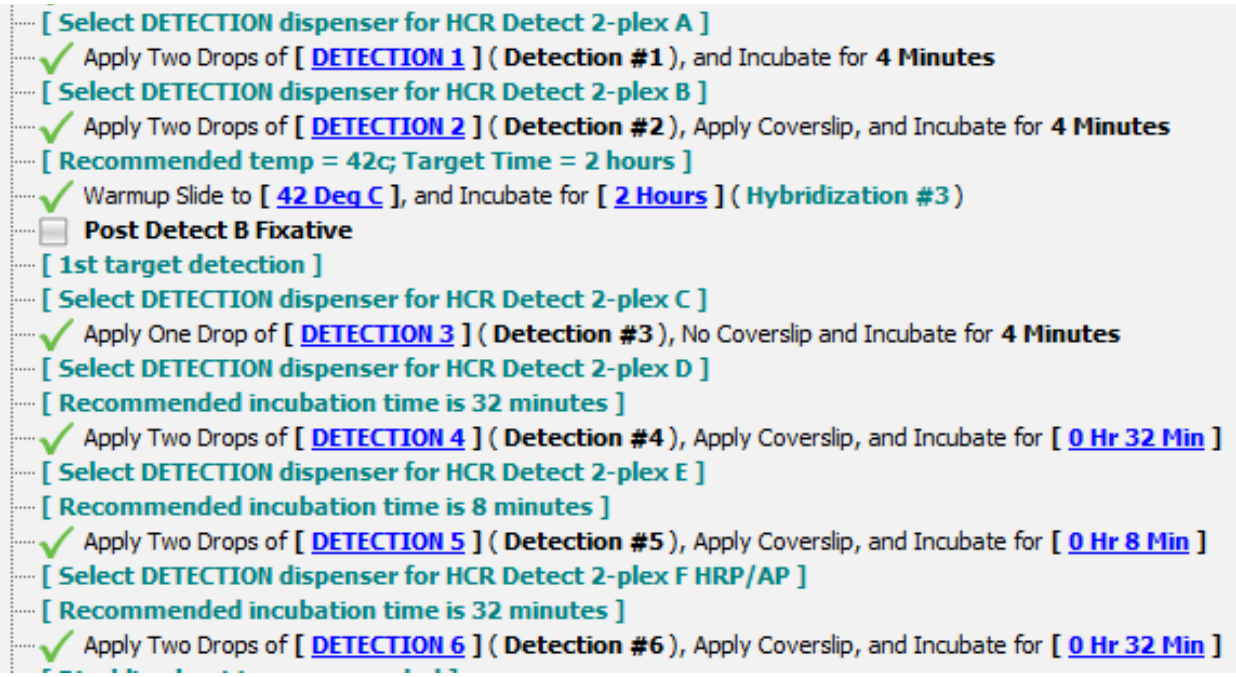


Pretreat C must be placed into an open **PRETREATMENT dispenser** different from the aforementioned dispensers. If you select **Blocker**, incubate for 8 minutes.

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 **2-Plex HCR™ RNA-ISH**.

HCR™ HiFi Probe 2-Plex A and HCR™ HiFi Probe 2-Plex 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.*
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.



STEP 5a: To perform **Chromogenic ISH** in the first channel, select the appropriate **Chromogen** that corresponds to your 2-Plex 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 a 2-Plex HCR™ Pro RNA-ISH assay with Discovery Red and mRNA DAB detection.*

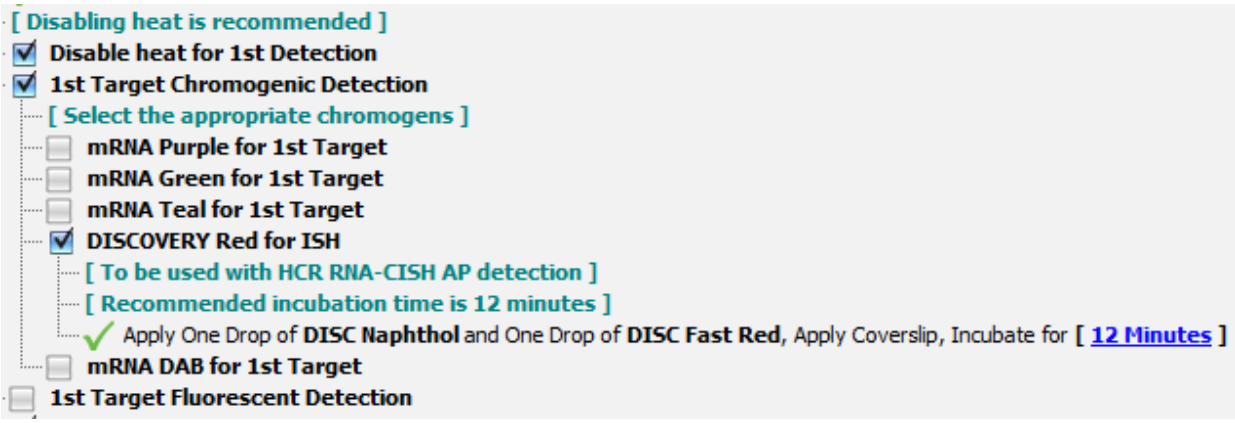


Table of Chromogens with Recommended Incubation Times

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	20 minutes	16 minutes
mRNA Green	HRP	24 minutes	20 minutes

STEP 5b: To perform **Fluorescent ISH** in the first channel, select **Fluorescent Detection**.
*NOTE: This selection requires the use of a 2-Plex HCR™ Pro RNA-ISH HRP/HRP Kit. See **Appendix B** for an example protocol summary for a 2-Plex HCR™ Pro RNA-FISH assay with Rhodamine 6G and Cy5 Detection.*

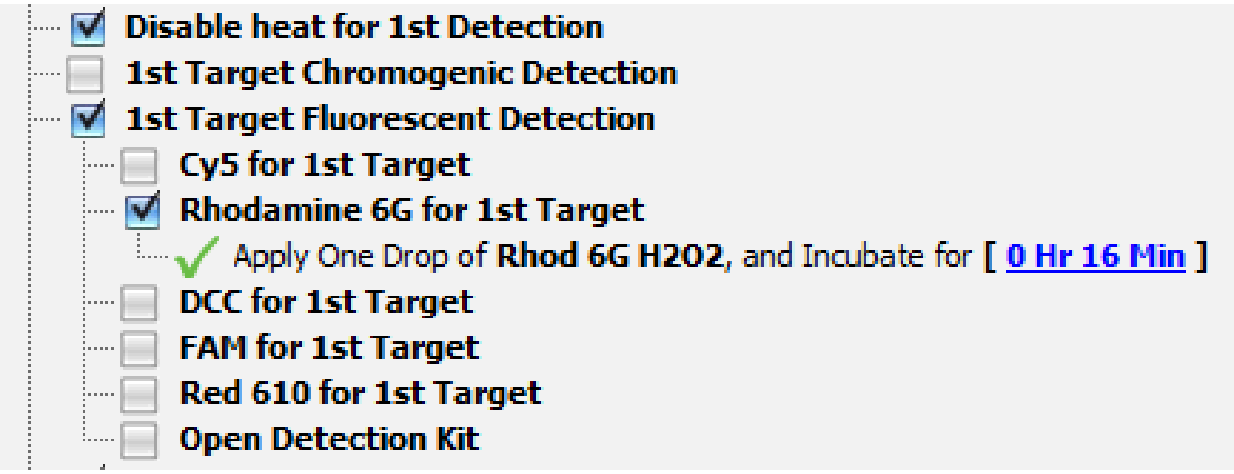
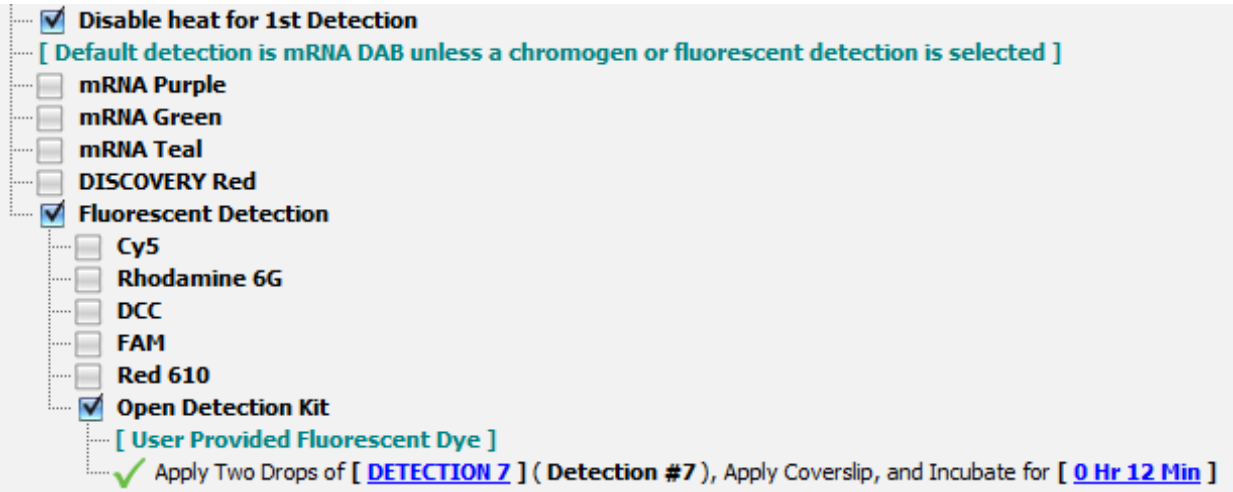


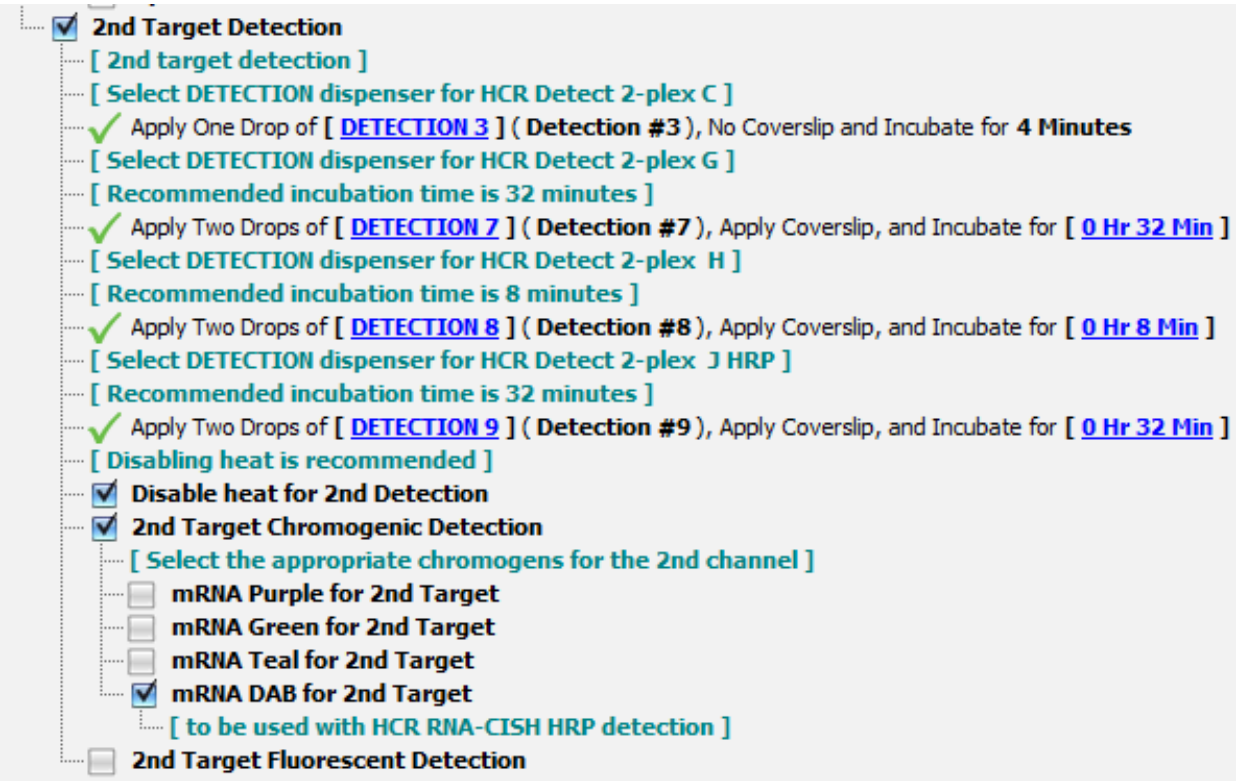
Table of Roche Fluorescent Dyes with Recommended Incubation Times

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

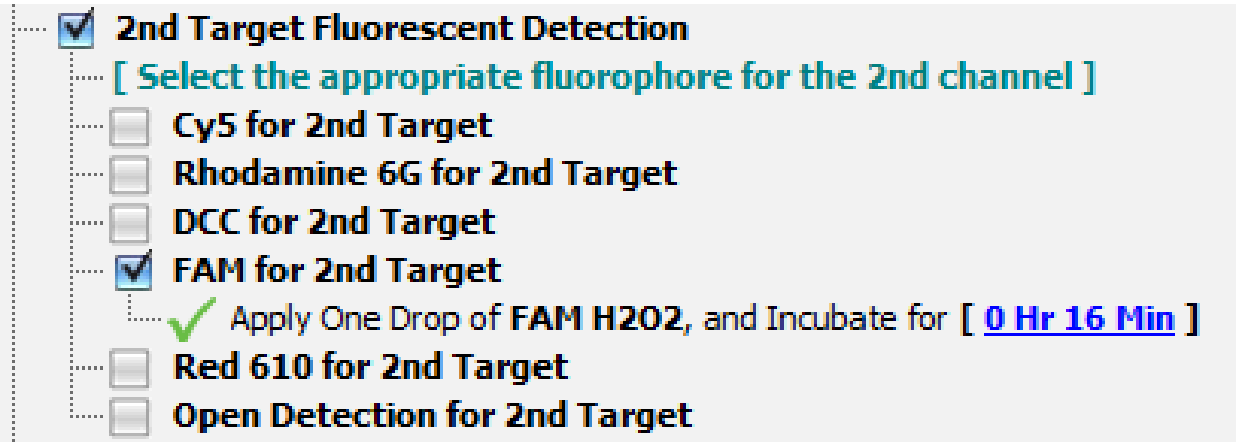
Instead of using Roche's Fluorophore Kits, you can also use third-party TSA dyes by selecting **Open Detection Kit** (see **Appendix E** for recommendations).
NOTE: This selection requires another open Detection dispenser.



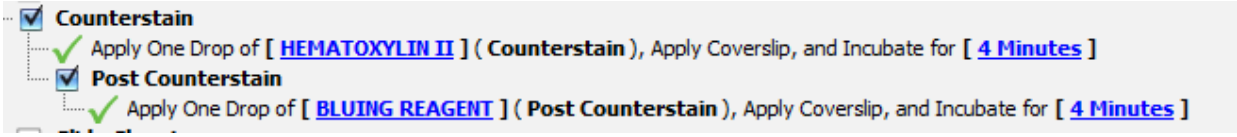
STEP 6a: To perform **Chromogenic ISH** in the second channel, make the following selections as shown below. **HCR™ Pro Detect 2-Plex A-J** must be placed into **DETECTION dispensers**. Select **DETECTION dispensers** for each of the HCR™ Pro Detect 2-Plex reagents and follow the comments (displayed in green text) for recommendations on a standard starting protocol.
NOTE: HCR™ Pro Detect 2-Plex A-J reagents must be placed into light-protective amber dispensers, as these reagents are sensitive to light.



STEP 6b: To perform **Fluorescent ISH** in the second channel, make the following selections as shown below.



STEP 7 (Chromogenic ISH only): For counterstain and post-counterstain, select **Hematoxylin II** and **Bluing reagent,** respectively, and incubate for 4 minutes each.



Post-Processing for AP to HRP 2-Plex Chromogenic ISH 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 at least 15 minutes (or until dry) at 60 °C. We recommend using EcoMount (Biocare) or VectaMount (Vectorlabs) mounting media for cover-slipping.

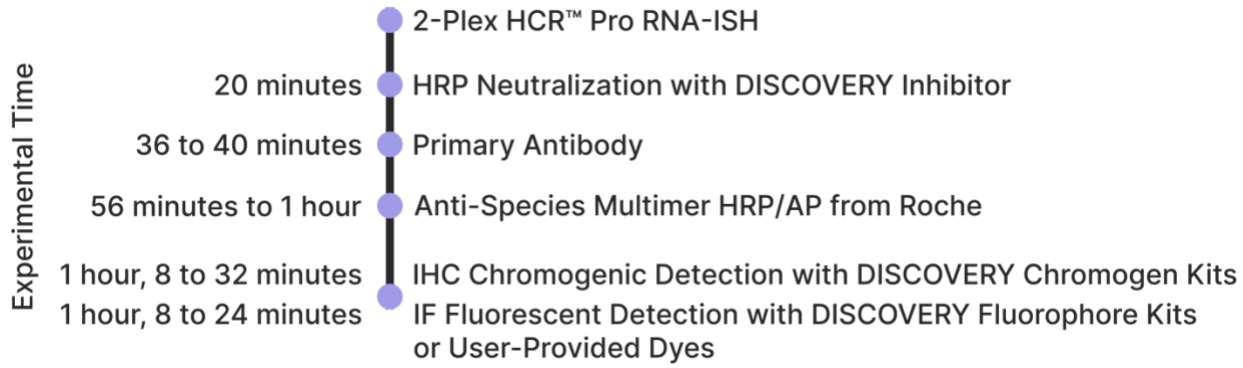
Post-Processing for HRP to HRP 2-Plex Chromogenic ISH Detection

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 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 lay the slides flat inside a fume hood. Mount slides one at a time with EcoMount (Biocare) or Cytoseal (or any other xylene-based mounting medium). Allow slides to air dry for 5 minutes before imaging. Dehydration with slide baking is also suitable.

Post-Processing for 2-Plex Fluorescent ISH Detection

After slides are unloaded from the DISCOVERY ULTRA, we recommend washing the slides thoroughly with soapy water to remove any liquid coverslip. Immerse the slides in 1 x PBST/TBST. Mount slides one at a time with SlowFade Gold Antifade with DAPI (or without DAPI).

Overall Workflow of the 2-Plex HCR™ Pro RNA-ISH + IHC/IF Protocol

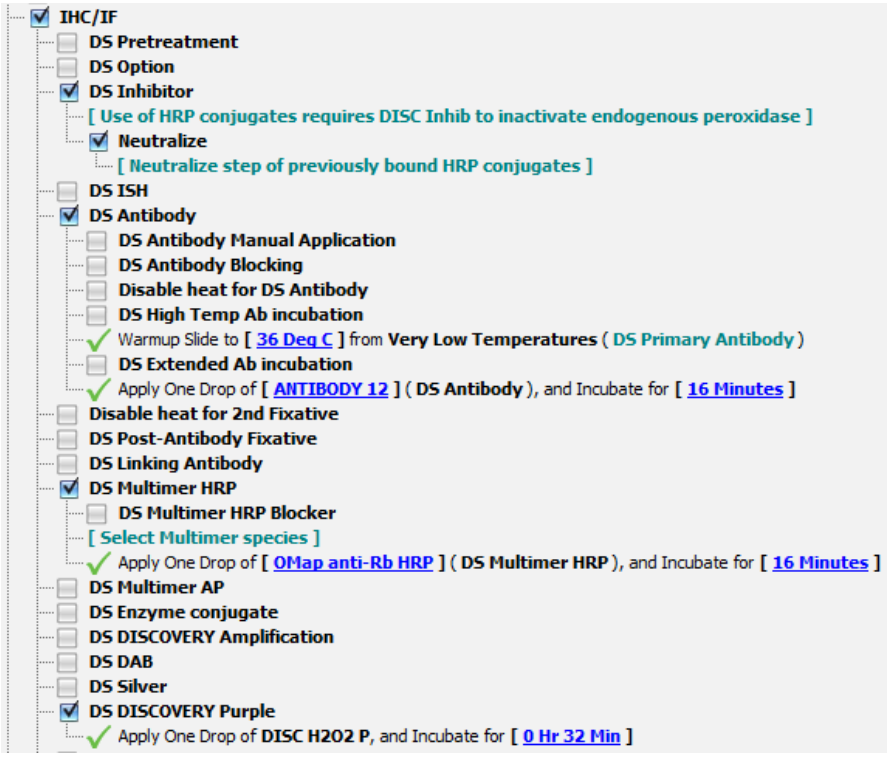


Creating a 2-Plex HCR™ Pro RNA-ISH + IHC/IF Co-Detection Protocol

2-Plex Chromogenic ISH + IHC Co-Detection

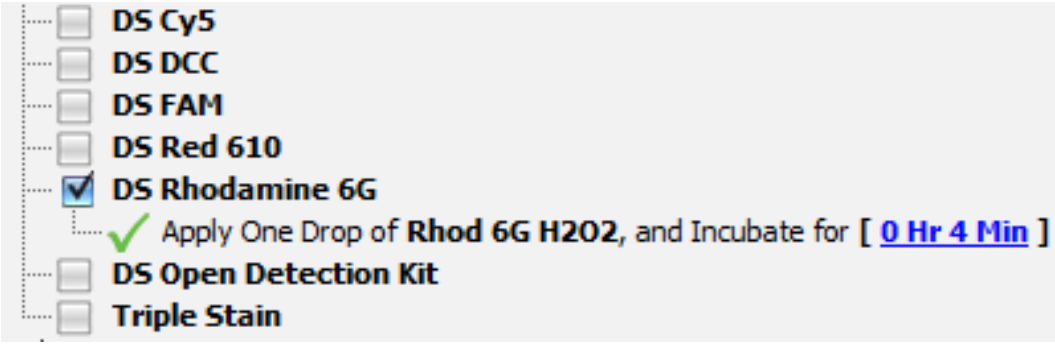
Program the 2-Plex HCR™ Pro RNA-ISH Protocol as outlined previously (see page 11). A dropdown list will appear once IHC is selected. An example for the selection of primary and HRP-conjugated secondary antibodies is shown below. You should determine the appropriate selection for which secondary antibodies 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 a 2-Plex HCR™ Pro RNA-CISH + IHC Co-Detection assay with Discovery Red and mRNA DAB for CISH and Discovery Purple for IHC.

NOTE: Only use HRP-driven chromogens for IHC if you are using a 2-Plex HCR™ Pro RNA-ISH AP/HRP kit.



Fluorescent ISH + IF Co-Detection

Program the 2-Plex HCR™ Pro RNA-ISH Protocol as outlined previously (see page 11). 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 a 2-Plex HCR™ Pro RNA-FISH + IF Co-Detection assay with Rhodamine 6G and FAM for FISH and Cy5 for IF.



Appendix

Appendix A: 2-Plex HCR™ Pro RNA-CISH Protocol Summary [2-Plex Chromogenic Detection – Discovery Red and mRNA DAB detection]

Protocol Summary

Procedure: RUO HCR Universal (v4.00.0000)

DISCOVERY ULTRA

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

Validated: No **Active: Yes**

Protocol No	Protocol Name	Version	Creation Date
1815	2-plex CISH Protocol	2	07/30/2024 2:23:40 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	2-plex 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 16 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	1st Target Chromogenic Detection [Selected]
29	DISCOVERY Red for ISH [Selected]
30	Apply One Drop of DISC Naphthol and One Drop of DISC Fast Red, Apply Coverslip, Incubate for [12 Minutes]
31	2nd Target Detection [Selected]
32	Apply Two Drops of [DETECTION 7] (Detection #7), Apply Coverslip, and Incubate for [0 Hr 32 Min]
33	Apply Two Drops of [DETECTION 8] (Detection #8), Apply Coverslip, and Incubate for [0 Hr 8 Min]
34	Apply Two Drops of [DETECTION 9] (Detection #9), Apply Coverslip, and Incubate for [0 Hr 32 Min]
35	Disable heat for 2nd Detection [Selected]
36	2nd Target Chromogenic Detection [Selected]
37	mRNA DAB for 2nd Target [Selected]
38	Counterstain [Selected]
39	Apply One Drop of [HEMATOXYLIN II] (Counterstain), Apply Coverslip, and Incubate for [4 Minutes]
40	Post Counterstain [Selected]
41	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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Appendix B: 2-Plex HCR™ Pro RNA-FISH Protocol Summary [2-Plex Fluorescent Detection – Rhodamine 6G and Cy5 detection]

Protocol Summary

Procedure: RUO HCR Universal (v4.00.0000)

DISCOVERY ULTRA

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

Validated: No **Active:** Yes

Protocol No	Protocol Name	Version	Creation Date
1816	2-plex FISH Protocol	2	07/22/2024 1:48:25 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	2-plex 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	1st Target Fluorescent Detection [Selected]
29	Rhodamine 6G for 1st Target [Selected]
30	Apply One Drop of Rhod 6G H2O2, and Incubate for [0 Hr 16 Min]
31	2nd Target Detection [Selected]
32	Apply Two Drops of [DETECTION 7] (Detection #7), Apply Coverslip, and Incubate for [0 Hr 32 Min]
33	Apply Two Drops of [DETECTION 8] (Detection #8), Apply Coverslip, and Incubate for [0 Hr 8 Min]
34	Apply Two Drops of [DETECTION 9] (Detection #9), Apply Coverslip, and Incubate for [0 Hr 32 Min]
35	Disable heat for 2nd Detection [Selected]
36	2nd Target Fluorescent Detection [Selected]
37	Cy5 for 2nd Target [Selected]
38	Apply One Drop of Cy5 H2O2, and Incubate for [0 Hr 12 Min]

* one drop is one reagent dispense

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Appendix C: 2-Plex HCR™ Pro RNA-CISH + IHC Co-Detection Protocol Summary [2-Plex CISH – Discovery Red and mRNA DAB and IHC - Discovery Purple]

Protocol Summary

Procedure: RUO HCR Universal (v4.00.0000)

DISCOVERY ULTRA

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

Validated: No **Active: Yes**

Protocol No	Protocol Name	Version	Creation Date
1814	2-plex CISH + IHC	2	07/30/2024 2:29:34 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 2-plex 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 16 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 1st Target Chromogenic Detection [Selected]
29 DISCOVERY Red for ISH [Selected]
30 Apply One Drop of DISC Naphthol and One Drop of DISC Fast Red, Apply Coverslip, Incubate for [12 Minutes]
31 2nd Target Detection [Selected]
32 Apply Two Drops of [DETECTION 7] (Detection #7), Apply Coverslip, and Incubate for [0 Hr 32 Min]
33 Apply Two Drops of [DETECTION 8] (Detection #8), Apply Coverslip, and Incubate for [0 Hr 8 Min]
34 Apply Two Drops of [DETECTION 9] (Detection #9), Apply Coverslip, and Incubate for [0 Hr 32 Min]
35 Disable heat for 2nd Detection [Selected]
36 2nd Target Chromogenic Detection [Selected]
37 mRNA DAB for 2nd Target [Selected]
38 IHC/IF [Selected]
39 DS Inhibitor [Selected]
40 Neutralize [Selected]
41 DS Antibody [Selected]
42 Warmup Slide to [36 Deg C] from Very Low Temperatures (DS Primary Antibody)

* one drop is one reagent dispense

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VSS v12.5.4 Build 23128.1

Validated: No **Active: Yes**

Protocol No	Protocol Name	Version	Creation Date
1814	2-plex CISH + IHC	2	07/30/2024 2:29:34 PM

43 Apply One Drop of [ANTIBODY 12] (DS Antibody), and Incubate for [16 Minutes]
44 DS Multimer HRP [Selected]
45 Apply One Drop of [OMap anti-Rb HRP] (DS Multimer HRP), and Incubate for [16 Minutes]
46 DS DISCOVERY Purple [Selected]
47 Apply One Drop of DISC H2O2 P, and Incubate for [0 Hr 32 Min]
48 Counterstain [Selected]
49 Apply One Drop of [HEMATOXYLIN II] (Counterstain), Apply Coverslip, and Incubate for [4 Minutes]
50 Post Counterstain [Selected]
51 Apply One Drop of [BLUING REAGENT] (Post Counterstain), Apply Coverslip, and Incubate for [4 Minutes]

Appendix D: 2-Plex HCR™ Pro RNA-FISH + IF Co-Detection Protocol Summary [2-Plex FISH – Rhodamine 6G and FAM and IF- Cy5]

Protocol Summary

Procedure: RUO HCR Universal (v4.00.0000)

DISCOVERY ULTRA

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

Validated: No **Active:** Yes

Protocol No	Protocol Name	Version	Creation Date
1813	2-plex FISH + IF	4	07/30/2024 3:32:59 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 2-plex 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 1st Target Fluorescent Detection [Selected]
29 Rhodamine 6G for 1st Target [Selected]
30 Apply One Drop of Rhod 6G H_2O_2, and Incubate for [0 Hr 16 Min]
31 2nd Target Detection [Selected]
32 Apply Two Drops of [DETECTION 7] (Detection #7), Apply Coverslip, and Incubate for [0 Hr 32 Min]
33 Apply Two Drops of [DETECTION 8] (Detection #8), Apply Coverslip, and Incubate for [0 Hr 8 Min]
34 Apply Two Drops of [DETECTION 9] (Detection #9), Apply Coverslip, and Incubate for [0 Hr 32 Min]
35 Disable heat for 2nd Detection [Selected]
36 2nd Target Fluorescent Detection [Selected]
37 FAM for 2nd Target [Selected]
38 Apply One Drop of FAM H_2O_2, and Incubate for [0 Hr 16 Min]
39 IHC/IF [Selected]
40 DS Inhibitor [Selected]
41 Neutralize [Selected]
42 DS Antibody [Selected]

* one drop is one reagent dispense
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VSS v12.5.4 Build 23128.1

Validated: No **Active:** Yes

Protocol No	Protocol Name	Version	Creation Date
1813	2-plex FISH + IF	4	07/30/2024 3:32:59 PM

43 Warmup Slide to [36 Deg C] from Very Low Temperatures (DS Primary Antibody)
44 Apply One Drop of [ANTIBODY 12] (DS Antibody), and Incubate for [16 Minutes]
45 DS Multimer HRP [Selected]
46 Apply One Drop of [OMap anti-Rb HRP] (DS Multimer HRP), and Incubate for [16 Minutes]
47 DS Cy5 [Selected]
48 Apply One Drop of Cy5 H_2O_2, and Incubate for [0 Hr 12 Min]

Appendix E: Detailed 2-Plex HCR™ HiFi Probe Preparation Guide

This section provides a detailed breakdown of how to calculate the required volumes of each component for preparing 2-plex HCR™ HiFi Probe A and B Solutions.

Important: For short target applications, HiFi Probes must be freshly prepared (within 48 hours of each experiment).

Scenario 1:
HCR™ HiFi Probe 1 = 1×
HCR™ HiFi Probe 2 = 50×

1. **Calculate the Volume of 1× HCR™ HiFi Probe 1A/1B**: To determine the total volume (V_1) of HCR™ HiFi Probe 1A/1B required for your selected number of slides (N_s), use the following equation:

   $$\mathbf{V_1 = 300 * N_S + 600}$$

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

2. **Calculate the Volume of 50× Concentrated HCR™ HiFi Probe 2A/2B:** To determine the volume of concentrated HCR™ HiFi Probe 2A/2B (V_2) needed, divide the previously calculated Probe 1 volume (V_1) by 50:

   $$\mathbf{V_2 = V_1 / 50}$$

After thoroughly mixing the 1× HCR™ HiFi Probe 1 and 50× HCR™ HiFi Probe 2, transfer the 2-plex 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 for a 2-plex assay:

Container Type	HCR™ HiFi Probe 1A & 1B Volume	50× HCR™ HiFi Probe 2A & 2B Volume	Component Volumes of 2-Plex HCR™ HiFi Probe A and B Solution
Dispenser	V_1 = 300 * **4** + 600 V_1 = 1800 µL	V_2 = 1800 / 50 V_2 = 36 µL	**2-plex HCR™ HiFi Probe A Solution** 1800 µL HiFi Probe 1A 36 µL HiFi Probe 2A **2-plex HCR™ HiFi Probe B Solution** 1800 µL HiFi Probe 1B 36 µL HiFi Probe 2B

Scenario 2:
HCR™ HiFi Probe 1 & 2 = 50×

When both probes are at 50x concentration, HCR™ HiFi Probe A/B Diluents are required to prepare your 2-plex 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 2-plex 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 for a 2-plex assay:

Container Type	**HCR™ HiFi Probe A & B Diluent Volume**	**50× HCR™ HiFi Probe A & B Volume**	**Component Volumes of 2-Plex HCR™ HiFi Probe A and B Solution**
Dispenser	$V_D = 300 * \mathbf{4} + 600$ $V_D = 1800\ \mu L$	$V_P = 1800 / 50$ $V_P = 36\ \mu L$	**2-plex HCR™ HiFi Probe A Solution** 36 µL HiFi Probe 1A 36 µL HiFi Probe 2A 1800 µL HiFi Probe A Diluent **2-plex HCR™ HiFi Probe B Solution** 36 µL HiFi Probe 1B 36 µL HiFi Probe 2B 1800 µL HiFi Probe B Diluent