VS Universal RNAscope™ Assay combined with Immunohistochemistry: RNA-Protein Co-Detection on DISCOVERY ULTRA

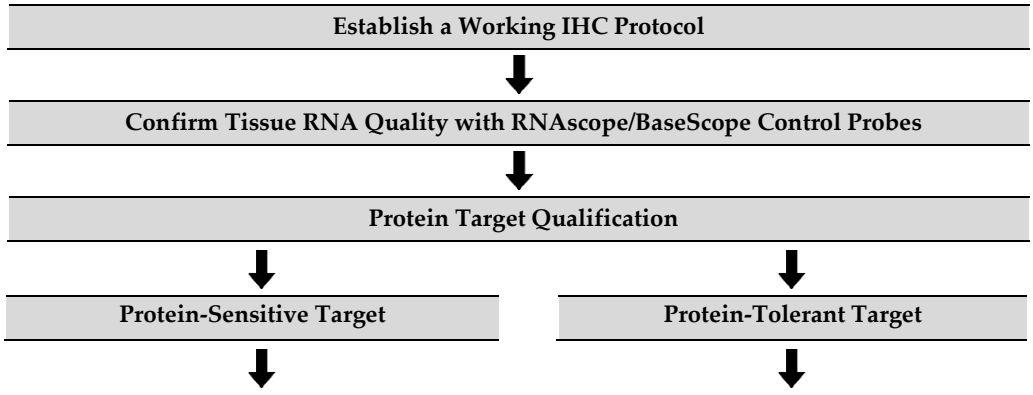
Introduction

This Technical Note provides guidelines for performing automated chromogenic co-detection of RNA and protein on the Ventana™ DISCOVERY ULTRA System. The Integrated Co-Detection Workflow (ICW) combines ACD's VS Universal RNAscope AP, VS Universal RNAscope HRP, VS RNAscope Duplex, or BaseScope™ VS detection assays with fully automated immunohistochemistry (IHC). RNA-Protein Co-Detection on the DISCOVERY ULTRA requires primary antibody, an RNAscope or BaseScope reagent kit, RNAscope or BaseScope target and control probes, the VS RNA-Protein Co-Detection Ancillary Kit, and additional materials from Roche Tissue Diagnostics. This workflow is compatible with NexES software versions 12.5.3 and greater from Roche Tissue Diagnostics, using the mRNA Universal procedure v6.03 or above. For every chemical, read the Safety Data Sheet (SDS) and follow handling instructions. Wear appropriate protective eyewear, clothing, and gloves. For the latest service and support information, go to **www.acdbio.com/support.**

Getting Started

Before you begin RNA-Protein Co-Detection, we recommend establishing a working IHC protocol with your primary antibody, tissue, and chromogen of interest on the DISCOVERY ULTRA, and confirming the RNA quality of your tissue using your RNAscope/BaseScope assay of interest with corresponding control probes. You will also need to create staining protocols for RNA-Protein Co-Detection under the mRNA Universal procedure within the NexES software with the help of your ACD FAS. Ensure you have all necessary materials.

Protease treatment is required for RNA detection but can be disruptive to some protein targets. To determine the optimal workflow for RNA-Protein Co-Detection, we recommend performing a qualification run to determine whether the protein target is sensitive to protease, using Chapter 1: Protein Target Qualification. For protease-tolerant targets, proceed to Chapter 2: Sequential ISH-IHC. For protease-sensitive targets, proceed to Chapter 3: Integrated Co-Detection Workflow.



Integrated Co-Detection Workflow	Sequential ISH-IHC Workflow

General Workflow for RNA-Protein Co-Detection on DISCOVERY ULTRA:

This w**orkflow** varies as indicated based on whether your protein target is protease-sensitive or protease-tolerant

Prepare and Load Slides onto DISCOVERY ULTRA

↓

Baking and Deparaffinization

↓

Cell Conditioning (Target Retrieval)

↓

Integrated Co-Detection Workflow Module (*Option for Protease-Sensitive Protein Targets*)

Primary Antibody Incubation

↓

Post-Primary Fixation

↓

ISH Assay Selection (VS Universal AP, VS Universal HRP, VS Duplex, BaseScope VS)

RNAscope VS Protease

OR

VS Co-Detection Protease (*Recommended following Post-Primary Fixation*)

↓

RNAscope or BaseScope Probe

↓

RNAscope or BaseScope Amplification

↓

mRNA Chromogen

↓

Dual Sequence Staining

VS Co-Detection Inhibitor

↓

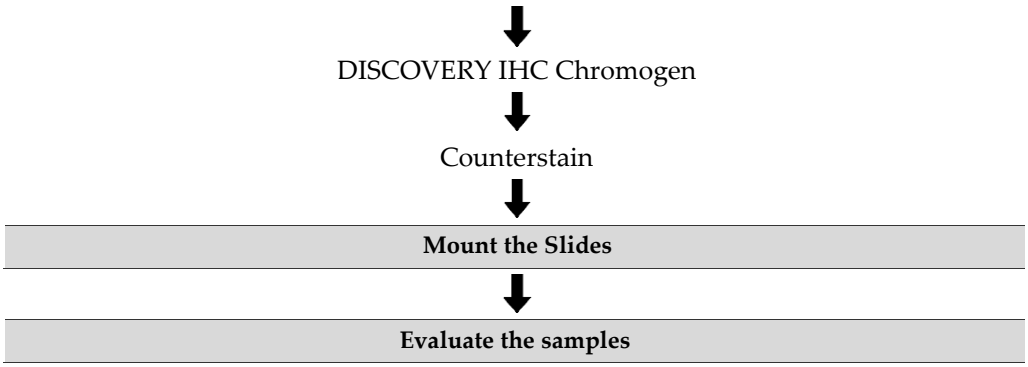
Primary Antibody (*Option for Protease-Tolerant Protein Targets*)

↓

Secondary Antibody Blocking (*Optional*)

↓

DISCOVERY Secondary Antibody


DISCOVERY IHC Chromogen
Counterstain
Mount the Slides
Evaluate the samples

Chromogen Combinations for RNA-Protein Co-Detection on DISCOVERY ULTRA

For available options for ISH – IHC chromogen combinations, see the following table:

ACD ISH Assay	Reagents for ISH Detection	IHC Detection System/Reagents
VS Universal RNAscope AP	RNAscope VS Universal AP Reagent Kit and **mRNA Red Detection Kit**	DISCOVERY AP-conjugated secondary with **DISCOVERY Yellow (AP)** IHC chromogen or DISCOVERY HRP-conjugated secondary with **DISCOVERY Teal HRP** or DISCOVERY HRP-conjugated secondary with **DISCOVERY Green HRP**
VS Universal RNAscope HRP (chromogenic)	RNAscope VS Universal HRP Reagent Kit and **mRNA Green Detection Kit** with DISCOVERY Inhibitor or **mRNA Teal Detection Kit** with DISCOVERY Inhibitor or **mRNA Purple Detection Kit** with DISCOVERY Inhibitor or **mRNA DAB Detection Kit**	DISCOVERY AP-conjugated secondary with **DISCOVERY Yellow (AP)** IHC Chromogen or DISCOVERY AP-conjugated secondary with **ChromoMap Red (AP)** or DISCOVERY HRP-conjugated secondary with **DISCOVERY Teal HRP** or DISCOVERY HRP-conjugated secondary with **DISCOVERY Green HRP** or DISCOVERY HRP-conjugated secondary with **DISCOVERY Purple HRP**
VS Universal RNAscope HRP (fluorescent)	RNAscope VS Universal HRP Reagent Kit and DISCOVERY Fluorescent Detection Kit with DISCOVERY Inhibitor	DISCOVERY HRP-conjugated secondary with DISCOVERY Fluorescent Detection Kit
VS Universal RNAscope Duplex	RNAscope VS Duplex Reagent Kit and **mRNA Red Detection Kit** and **mRNA Green Detection Kit** or **mRNA Teal Detection Kit** or **mRNA DAB Detection Kit**	DISCOVERY AP-conjugated secondary with **DISCOVERY Yellow (AP)** IHC Chromogen or DISCOVERY HRP-conjugated secondary with **DISCOVERY Teal HRP** or DISCOVERY HRP-conjugated secondary with **DISCOVERY Green HRP**
VS BaseScope (AP)	BaseScope VS Universal AP Reagent Kit and **mRNA Red Detection Kit**	DISCOVERY AP-conjugated secondary with **DISCOVERY Yellow (AP)** IHC chromogen or DISCOVERY HRP-conjugated secondary with **DISCOVERY Teal HRP** or DISCOVERY HRP-conjugated secondary with **DISCOVERY Green HRP**

Materials for RNA-Protein Co-Detection on DISCOVERY ULTRA

ACD VS Chromogenic ISH Detection Kits

RNAscope VS Reagent Kits

RNAscope 2.5 VS Probes and BaseScope VS Probes are available separately. Roche RNA-Protein Co-Detection can be performed with the RNAscope VS Universal AP Reagent Kit (Cat. No. 323250), RNAscope VS Universal HRP Reagent Kit (Cat. No. 323200), RNAscope VS Duplex Reagent Kit (Cat. No. 323300), and BaseScope VS Reagent Kit (Cat. No. 323700). Each RNAscope VS Reagent Kit includes the following Ready-To-Use (RTU) reagents to stain ~60 standard slides.

- RNAscope VS Universal Sample Prep Reagent Kit v2 (Cat. No. 323740)
- RNAscope VS Accessory Kit (Cat. No. 320630)
- Assay-Specific Detection Reagents

RNAscope VS Universal Sample Prep Reagent Kit v2 (Cat. No. 323740) and RNAscope VS Accessory Kit (Cat. No. 320630) are stored as indicated in the following tables:

RNAscope VS Sample Prep Reagent Kit v2 (Cat. No. 323740)			
☑	**Reagent**	**Quantity**	**Storage**
	RNAscope VS Universal Target Retrieval v2	10 mL x 2 bottle	Room Temp (15–30°C)
	RNAscope VS Universal Dewax	14 mL x 1 bottle	Room Temp (15–30°C)
RNAscope VS Accessory Kit (Cat. No. 320630)			
☑	**Reagent**	**Quantity**	**Storage**
	RNAscope VS Hematoxylin - RTU	7 mL x 1 bottle	2–8°C
	RNAscope VS Bluing Reagent - RTU	7 mL x 1 bottle	2–8°C

RTU Assay-specific Detection Reagents within each Reagent Kit are stored as indicated in the following tables:

RNAscope VS Universal AP Detection Reagents (Cat. No. 323260)			
☑	**Reagent**	**Quantity**	**Storage**
	RNAscope VS Universal AP AMP 1	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal AP AMP 2	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal AP AMP 3	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal AP AMP 4	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal AP AMP 5	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal AP AMP 6	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal AP AMP 7	14 mL x 1 bottle	2–8°C
	RNAscope VS Protease	14 mL x 1 bottle	2–8°C

RNAscope VS Universal HRP Detection Reagents (Cat. No. 323210)			
☑	**Reagent**	**Quantity**	**Storage**
	RNAscope VS Universal HRP AMP 1	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal HRP AMP 2	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal HRP AMP 3	14 mL x 1 bottle	2–8°C

RNAscope VS Universal HRP Detection Reagents (Cat. No. 323210)			
☑	**Reagent**	**Quantity**	**Storage**
	RNAscope VS Universal HRP AMP 4	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal HRP AMP 5	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal HRP AMP 6	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal HRP AMP 7	14 mL x 1 bottle	2–8°C
	RNAscope VS Protease	14 mL x 1 bottle	2–8°C

RNAscope VS Duplex Detection Reagents (Cat. No. 323310)			
☑	**Reagent**	**Quantity**	**Storage**
	RNAscope VS Duplex AMP 1	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP 2	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP 3	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP 4	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP 5	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP 6	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP 7	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP 8	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP 9	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP Wash	14 mL x 2 bottles	2–8°C
	RNAscope VS Protease	14 mL x 1 bottle	2–8°C

BaseScope VS Detection Reagents (Cat. No. 323710)			
☑	**Reagent**	**Quantity**	**Storage**
	BaseScope VS AMP 1	14 mL x 1 bottle	2–8°C
	BaseScope VS AMP 2	14 mL x 1 bottle	2–8°C
	BaseScope VS AMP 3	14 mL x 1 bottle	2–8°C
	BaseScope VS AMP 4	14 mL x 1 bottle	2–8°C
	BaseScope VS AMP 5	14 mL x 1 bottle	2–8°C
	BaseScope VS AMP 6	14 mL x 1 bottle	2–8°C
	BaseScope VS AMP 7	14 mL x 1 bottle	2–8°C
	BaseScope VS AMP 8	14 mL x 1 bottle	2–8°C
	RNAscope 2.5 VS Pretreat 3 - Protease	14 mL x 1 bottle	2–8°C

ACD Reagents for RNA-Protein Co-Detection on DISCOVERY ULTRA

The VS RNA-Protein Co-Detection Ancillary Kit includes materials for combined RNA and protein detection. Materials needed from this kit for ISH-IHC depend on the protease sensitivity of the protein target and if the staining is performed in an integrated or sequential manner.

Co-Detection Antibody Diluent helps preserve RNA prior to RNAscope detection and is recommended for use with the integrated co-detection workflow (ICW).

VS Co-Detection Protease helps counteract the impact of post-primary fixation on RNA accessibility and is recommended for use with the integrated co-detection workflow (ICW) on tissue samples.

VS Co-Detection Inhibitor is a chemical enzyme inhibitor recommended for use following the RNAscope/BaseScope assay prior to IHC detection. Use this reagent with both sequential and integrated co-detection workflows.

VS RNA-Protein Co-Detection Ancillary Kit - Cat No. 323760				
☑	**Reagent**	**Source / Ordering Info**	**Quantity**	**Storage**
	Co-Detection Antibody Diluent	ACD/Cat No. 323160	120 mL x 1 bottle	2–8°C
	VS RNA-Protein Co-Detection Protease and Inhibitor	ACD/Cat No. 323190	See the following table	

VS RNA-Protein Co-Detection Protease and Inhibitor - Cat No. 323190			
☑	**Reagent**	**Quantity**	**Storage**
	VS Co-Detection Protease	14 mL x 1 bottle	2–8°C
	VS Co-Detection Inhibitor	14 mL x 1 bottle	2–8°C

Required Materials from Roche Tissue Diagnostics

The Integrated Co-Detection Workflow (ICW) requires specific materials and equipment available *only* from Roche Tissue Diagnostics (Ventana Medical Systems, Inc.). Catalog numbers are valid in the United States only. For other regions, please check catalog or ordering numbers with your local lab supplier.

Roche Materials Required for RNA-Protein Co-Detection					
☑	**Component**	**Cat. No.**	**Ordering Code**	**Fill with:**	**Application:**
	Probe Dispensers	960-761 to 960-780	Contact local Roche representative	RNAscope VS Probes	Integrated Co-Detection Workflow Sequential Co-Detection Workflow
	mRNA Sample Prep Kit	760-248	08127166001	RNAscope VS Sample Prep Reagent Kit v2 and VS Protease reagent from Detection Kit	Protein Target Qualification Integrated Co-Detection Workflow Sequential Co-Detection Workflow
	Antibody Dispensers	770-001 to 770-099	Contact local Roche representative	Primary Antibody Concentrate diluted in Co-Detection Antibody Diluent	Protein Target Qualification Integrated Co-Detection Workflow Sequential Co-Detection Workflow
	User-fillable Enzyme 1 Dispenser	771-721	05271614001	VS Co-Detection Protease	Integrated Co-Detection Workflow

Roche Materials Required for RNA-Protein Co-Detection					
☑	Component	Cat. No.	Ordering Code	Fill with:	Application:
	User-fillable Fixative 1 Dispenser	771-731	05271614001	10% Neutral Buffered Formalin	Integrated Co-Detection Workflow
	User-fillable Pretreatment 1 Dispenser	960-901	5280095001	VS Co-Detection Inhibitor	Integrated Co-Detection Workflow Sequential Co-Detection Workflow
	Counterstain 1 dispenser	771-741	05271720001	VS Hematoxylin	Protein Target Qualification Integrated Co-Detection Workflow Sequential Co-Detection Workflow
	Counterstain 2 dispenser	771-742	05271738001	VS Bluing Reagent	Protein Target Qualification Integrated Co-Detection Workflow Sequential Co-Detection Workflow
	DISCOVERY Antibody Block	760-4204	05268869001	Pre-filled	Protein Target Qualification Integrated Co-Detection Workflow Sequential Co-Detection Workflow
	Additional ISH Assay-Specific Materials	(See the following table)			Integrated Co-Detection Workflow Sequential Co-Detection Workflow
	Additional IHC Detection Materials	(See the following table)			Protein Target Qualification Integrated Co-Detection Workflow Sequential Co-Detection Workflow

Additional Roche Materials Required for VS Universal AP ISH				
☑	Component	Cat. No.	Ordering Code	Fill with:
	mRNA RED Probe Amplification Kit	760-236	07095341001	RNAscope VS Universal AP Detection Reagents AMP 1-7
	mRNA RED Detection Kit	760-234	07099037001	Pre-filled

Additional Roche Materials Required for VS Universal HRP ISH				
☑	Component	Cat. No.	Ordering Code	Fill with:
	mRNA Probe Amplification Kit	760-222	06614337001	RNAscope VS Universal HRP Detection Reagents AMP 1–7
	DISCOVERY Inhibitor*	760-4840	07017944001	Pre-filled
	mRNA Purple HRP Detection Kit†	760-255	08127166001	Pre-filled
	mRNA Green HRP Detection Kit†	760-278	08952612001	Pre-filled
	mRNA Teal HRP Detection Kit†	760-256	08352941001	Pre-filled
	mRNA DAB Detection Kit†	760-224	06614353001	Pre-filled
	DISCOVERY DCC Kit†	760-240	07988192001	Pre-filled
	DISCOVERY FAM Kit†	760-243	07988150001	Pre-filled
	DISCOVERY FITC Kit†	760-232	07259212001	Pre-filled
	DISCOVERY Rhodamine Kit†	760-233	07259883001	Pre-filled
	DISCOVERY Rhodamine 6G Kit†	760-244	07988168001	Pre-filled

Additional Roche Materials Required for VS Universal HRP ISH				
	DISCOVERY Red 610 Kit†	760-245	07988176001	Pre-filled
	DISCOVERY Cy5 Kit†	760-238	07551215001	Pre-filled

* DISCOVERY Inhibitor is not required if using mRNA DAB Detection Kit

† Choose one mRNA Detection Kit for RNAscope HRP detection

Additional Roche Materials Required for VS Duplex ISH				
☑	**Component**	**Cat. No.**	**Ordering Code**	**Fill with:**
	mRNA Duplex Amp Kit	760-249	08127174001	RNAscope VS Duplex Detection Reagents AMP 1–9, AMP Wash
	mRNA Link (pre-filled)	760-6014	08127115001	Pre-filled
	mRNA RED Detection Kit	760-234	07099037001	Pre-filled
	mRNA Green HRP Detection Kit*	760-278	08952612001	Pre-filled
	mRNA Teal HRP Detection Kit*	760-256	08352941001	Pre-filled
	mRNA DAB Detection Kit*	760-224	06614353001	Pre-filled

* Choose one mRNA Detection Kit for VS Duplex Channel 1 detection

Additional Roche Materials Required for VS BaseScope ISH				
☑	**Component**	**Cat. No.**	**Ordering Code**	**Fill with:**
	mRNA RED Probe Amplification Kit	760-236	07095341001	BaseScope VS Detection Reagents AMP 1–7
	Option 8 dispenser	771-758	05271916001	BaseScope VS AMP 8 reagent
	mRNA RED Detection Kit	760-234	07099037001	Pre-filled

Reagent Options for Ventana IHC AP Detection				
☑	**Component**	**Cat. No.**	**Ordering Code**	**Storage**
	DISCOVERY UltraMap anti-Ms Alk Phos*	760-4312	05269687001	2–8°C
	DISCOVERY UltraMap anti-Rb Alk Phos*	760-4314	05269709001	2–8°C
	DISCOVERY Yellow Kit †	760-239	07698445001	2–8°C
	DISCOVERY ChromoMap Red Kit*	760-160	05266653001	2–8°C

Reagent Options for Ventana IHC HRP Detection				
☑	Product	Cat. No.	Ordering Code	Storage
	DISCOVERY UltraMap anti-Ms HRP*	760-4313	05269695001	2–8°C
	DISCOVERY UltraMap anti-Rb HRP*	760-4315	05269717001	2–8°C
	DISCOVERY UltraMap anti-Rat HRP*	760-4456	05891884001	2–8°C
	DISCOVERY UltraMap anti-Gt HRP*	760-4648	06607241001	2–8°C
	DISCOVERY Purple Kit †	760-229	07053983001	2–8°C
	DISCOVERY Green HRP Kit †	760-278	07053983001	2–8°C
	DISCOVERY Teal HRP Kit †	760-247	08254338001	2–8°C
	DISCOVERY ChromoMap DAB Kit †	760-159	05266645001	2–8°C
	DISCOVERY DCC Kit †	760-240	07988192001	2–8°C
	DISCOVERY FAM Kit †	760-243	07988150001	2–8°C
	DISCOVERY FITC Kit†	760-232	07259212001	2–8°C
	DISCOVERY Rhodamine Kit †	760-233	07259883001	2–8°C
	DISCOVERY Rhodamine 6G Kit †	760-244	07988168001	2–8°C
	DISCOVERY Red 610 Kit†	760-245	07988176001	2–8°C
	DISCOVERY Cy5 Kit †	760-238	07551215001	2–8°C

* Choose one secondary detection antibody depending on the primary antibody species and desired IHC chromogen
† Choose one IHC chromogen

Instrument buffers

☑	Component	Cat. No.	Ordering Code	Storage
	10X DISCOVERY Wash (RUO)	950-510	7311079001	Room Temp (15–30°C)
	ULTRA LCS (predilute)	650-210	5424534001	Room Temp (15–30°C)
	SSC Buffer (10X)	950-110	5353947001	Room Temp (15–30°C)
	Reaction Buffer (10X)	760-107	5266262001	Room Temp (15–30°C)
	DISCOVERY CC1	950-500	6414575001	Room Temp (15–30°C)

Additional Required Materials

Materials for Co-Detection

☑	Reagent	Source / Ordering Info	Quantity	Usage
	Primary Antibody (RTU)	User	As needed	• Protein Target Qualification (*optional*) • Sequential ISH-IHC Workflow (*optional*) • Integrated Co-Detection Workflow (*optional*)
	Primary Antibody Concentrate	User	As needed	• Protein Target Qualification (*optional*) • Sequential ISH-IHC Workflow (*optional*)

☑	Reagent	Source / Ordering Info	Quantity	Usage
				• Integrated Co-Detection Workflow (*optional*)
	10% Neutral Buffered Formalin	User	10 mL	• Integrated Co-Detection Workflow

Other materials

☑	Description	Supplier	Cat. No.
	SuperFrost® Plus Slides (required)	Fisher Scientific	12-550-15
	ProLong® Gold Antifade Reagent	Life Technologies	P36930
	EcoMount (if using Red detection)	Biocare	EM897L
	Tissue-Tek® Vertical 24 Slide Rack	American Master Tech Scientific/MLS	LWSRA24
	Tissue-Tek Staining Dishes	American Master Tech Scientific/MLS	LWT4457EA
	Cover Glass 24 x 50 mm	Fisher Scientific/MLS	12--545-F
	Distilled water	MLS	—
	Mild liquid dishwashing detergent (Dawn detergent or similar)	MLS	—
	Drying oven, capable of holding temperature at 60 +/– 1°C	MLS	—
	Fume hood	MLS	—
	100% ethanol (EtOH)	MLS	—
	Xylene	MLS	—
	Tissue-Tek Clearing Agent Dishes, xylene-resistant	American Master Tech Scientific/MLS	LWT4456EA
	Optional: Glass beaker (1 or 2 L)	MLS	—
	Optional: Hot plate	Fisher Scientific/MLS	11-300-49SHP

Chapter 1: Protein Target Qualification

Materials

The following tables provide general information on materials needed for protein target qualification. The full material list varies depending on your choice of primary antibody, IHC protein detection reagents, and IHC chromogen. Please refer to ***Materials for RNA-Protein Co-Detection on DISCOVERY ULTRA***.

ACD reagents for protein target qualification

Protein target qualification requires the following Ready-To-Use (RTU) reagents from ACD:

- RNAscope VS Protease (found within RNAscope or BaseScope Reagent Kit)
- RNAscope VS Universal Sample Prep Reagent Kit v2 (Cat. No. 323740)
- RNAscope VS Accessory Kit (Cat. No. 320630)

RNAscope VS Universal Sample Prep Reagent Kit v2 (Cat. No. 323740) and RNAscope VS Accessory Kit (Cat. No. 320630) are stored as indicated in the following tables:

RNAscope VS Sample Prep Reagent Kit v2 (Cat. No. 323740)			
☑	**Reagent**	**Quantity**	**Storage**
	RNAscope VS Universal Target Retrieval v2	10 mL x 2 bottle	Room Temp (15–30°C)
	RNAscope VS Universal Dewax	14 mL x 1 bottle	Room Temp (15–30°C)

RNAscope VS Protease reagent is a component of each ACD Detection Kit and is stored as indicated in the following tables:

RNAscope Protease Component					
☑	**ACD Detection Kit**	**Part No.**	**Reagent**	**Quantity**	**Storage**
	RNAscope VS Universal AP Detection Reagents	323260	RNAscope VS Protease	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal HRP Detection Reagents	323610	RNAscope VS Protease	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal Duplex Detection Reagents	323310	RNAscope VS Protease	14 mL x 1 bottle	2–8°C
	BaseScope VS Detection Reagents	323710	RNAscope 2.5 VS Pretreat 3 - Protease	14 mL x 1 bottle	2–8°C

Optional ACD reagents for protein target qualification

The following additional reagents from ACD can be used for protein target qualification on Roche DISCOVERY ULTRA:

- Co-Detection Antibody Diluent from VS RNA-Protein Co-Detection Ancillary Kit (Cat No. 323760)
- RNAscope VS Accessory Kit (Cat. No. 320630

VS RNA-Protein Co-Detection Ancillary Kit - Cat No. 323760				
☑	**Reagent**	**Source / Ordering Info**	**Quantity**	**Storage**
	Co-Detection Antibody Diluent	ACD/Cat No. 323160	120 mL x 1 bottle	2–8°C

RNAscope VS Accessory Kit (Cat. No. 320630)			
☑	**Reagent**	**Quantity**	**Storage**
	RNAscope VS Hematoxylin	7 mL x 1 bottle	2–8°C
	RNAscope VS Bluing Reagent	7 mL x 1 bottle	2–8°C

Roche materials for protein target qualification

Protein target qualification can be performed with either Roche RTU primary antibody or your choice of primary antibody concentrate diluted in the ACD Co-Detection Antibody Diluent. For a list of available Roche RTU primary antibodies and ordering information, please contact your local Roche representative.

The following additional materials from Roche are used for protein target qualification on DISCOVERY ULTRA. Catalog numbers are valid in the United States only. For other regions, please check catalog or ordering numbers with your local lab supplier.

Reagent dispensers

User-filled Dispensers Required for Protein Target Qualification					
☑	**Component**	**Source**	**Cat. No.**	**Ordering Code**	**Fill with:**
	mRNA Sample Prep Kit	Roche	760-248	08127166001	RNAscope VS Sample Prep Reagent Kit v2 and VS Protease reagent from Detection Kit
	User-fillable Antibody Dispensers (*Optional**)	770-001 to 770-099	770-001 to 770-099	Contact local Roche representative	Primary antibody concentrate diluted in Co-Detection Antibody Diluent
	Counterstain 1 dispenser	771-741	771-741	05271720001	VS Hematoxylin
	Counterstain 2 dispenser	771-742	771-742	05271738001	VS Bluing Reagent

*Protein target qualification can be performed with either Roche RTU primary antibody or your choice of primary antibody.

Reagent Options for Ventana IHC AP Detection				
☑	Component	Cat. No.	Ordering Code	Storage
	DISCOVERY UltraMap anti-Ms Alk Phos*	760-4312	05269687001	2–8°C
	DISCOVERY UltraMap anti-Rb Alk Phos*	760-4314	05269709001	2–8°C
	DISCOVERY Yellow Kit*	760-239	07698445001	2–8°C
	DISCOVERY Red Kit	760-228	07425333001	2–8°C
	DISCOVERY ChromoMap Red Kit*	760-160	05266653001	2–8°C

Reagent Options for Ventana IHC HRP Detection				
☑	Product	Cat. No.	Ordering Code	Storage
	DISCOVERY UltraMap anti-Ms HRP*	760-4313	05269695001	2–8°C
	DISCOVERY UltraMap anti-Rb HRP*	760-4315	05269717001	2–8°C
	DISCOVERY UltraMap anti-Rat HRP*	760-4456	05891884001	2–8°C
	DISCOVERY UltraMap anti-Gt HRP*	760-4648	06607241001	2–8°C
	DISCOVERY Purple Kit †	760-229	07053983001	2–8°C
	DISCOVERY Green HRP Kit †	760-278	07053983001	2–8°C
	DISCOVERY Teal HRP Kit †	760-247	08254338001	2–8°C
	DISCOVERY ChromoMap DAB Kit †	760-159	05266645001	2–8°C
	DISCOVERY DCC Kit †	760-240	07988192001	2–8°C
	DISCOVERY FAM Kit †	760-243	07988150001	2–8°C
	DISCOVERY FITC Kit†	760-232	07259212001	2–8°C
	DISCOVERY Rhodamine Kit †	760-233	07259883001	2–8°C
	DISCOVERY Rhodamine 6G Kit †	760-244	07988168001	2–8°C
	DISCOVERY Red 610 Kit†	760-245	07988176001	2–8°C
	DISCOVERY Cy5 Kit †	760-238	07551215001	2–8°C

* Choose one secondary detection antibody depending on the primary antibody species and desired IHC chromogen
† Choose one IHC chromogen

Instrument buffers

☑	Component	Cat. No.	Ordering Code	Storage
	10X DISCOVERY Wash (RUO)	950-510	7311079001	Room Temp (15–30°C)
	ULTRA LCS (Predilute)	650-210	5424534001	Room Temp (15–30°C)
	SSC Buffer (10X)	950-110	5353947001	Room Temp (15–30°C)
	Reaction Buffer (10X)	760-107	5266262001	Room Temp (15–30°C)
	DISCOVERY CC1	950-500	6414575001	Room Temp (15–30°C)

User-Sourced Materials

☑	Description	Supplier	Cat. No.
	Primary Antibody (RTU)	User	Various
	Primary Antibody Concentrate	User	Various
	SuperFrost Plus Slides (required)	Fisher Scientific	12-550-15
	ProLong Gold Antifade Reagent	Life Technologies	P36930
	EcoMount (if using Red detection)	Biocare	EM897L
	Tissue-Tek Vertical 24 Slide Rack	American Master Tech Scientific/MLS	LWSRA24
	Tissue-Tek Staining Dishes	American Master Tech Scientific/MLS	LWT4457EA
	Cover Glass 24 x 50 mm	Fisher Scientific/MLS	12--545-F
	Distilled water	MLS	—
	Mild liquid dishwashing detergent (Dawn detergent or similar)	MLS	—
	Drying oven, capable of holding temperature at 60 +/– 1°C	MLS	—
	Fume hood	MLS	—
	100% ethanol (EtOH)	MLS	—
	Xylene	MLS	—
	Tissue-Tek Clearing Agent Dishes, xylene-resistant	American Master Tech Scientific/MLS	LWT4456EA
	Optional: Glass beaker (1 or 2 L)	MLS	—
	Optional: Hot plate	Fisher Scientific/MLS	11-300-49SHP

Assay Procedure

Prepare the DISCOVERY ULTRA

Prepare the instrument

If the instrument has not been used for > 1 week, follow guidelines for instrument maintenance from Roche Tissue Diagnostics. Before use, empty the waste carboys if needed.

Dilute instrument bulk reagents

1. Prepare the instrument bulk fluids according to the manufacturer's instructions.
2. Fill bulk solution containers for 1X DISCOVERY Wash, ULTRA LCS (predilute), and CC1 (predilute) to be at least half full. Fully fill bulk solution containers for 2X SSC and 1X Reaction Buffer.

IMPORTANT! Do not use expired reagents.

Register new reagents

Reagent dispensers come with appropriate barcode labels and registration buttons for dispensing RNAscope VS Universal Reagents. Refer to the *Ventana DISCOVERY ULTRA System User Manual* for details. To register reagents:

- Log all ACD reagents and probes into the software as **log user-fillable reagents** and **log user-fillable probes**, respectively.
- Use the reagent registration wand that comes with the instrument to register new reagent kits from Roche Tissue Diagnostics

Prepare user-fillable reagents for protein target qualification

Refer to the table on page 13 to determine the proper dispenser for each user-fillable reagent.

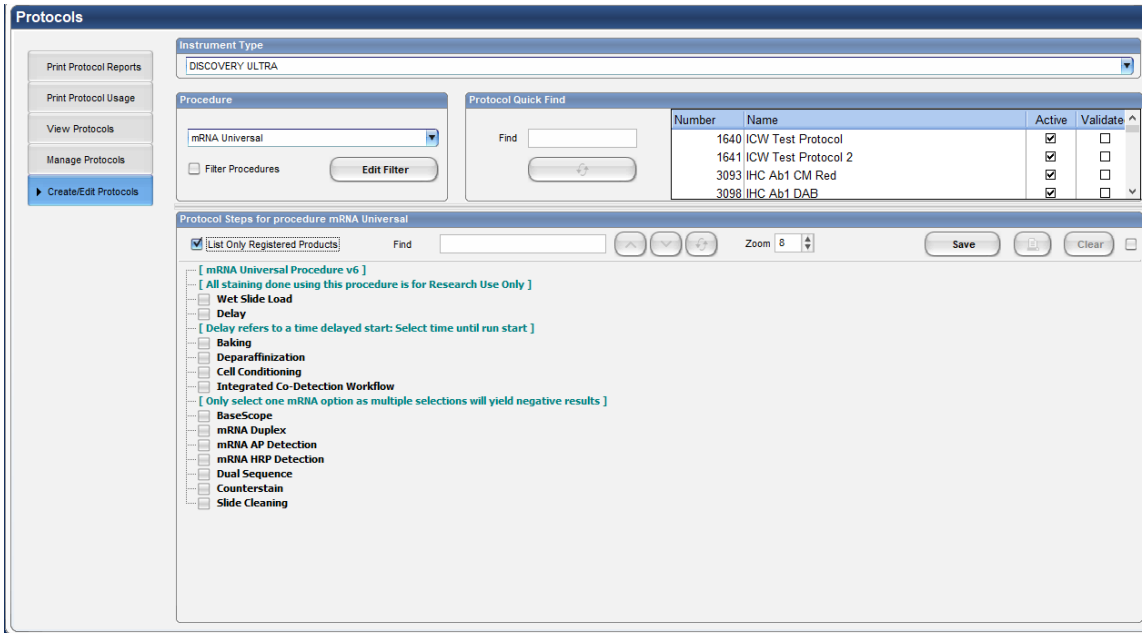
IMPORTANT! Avoid cross contamination between reagents. Dewax must be warmed to room temperature and be completely in solution before use.

1. If working with primary antibody concentrate, prepare the primary antibody:
   a. Dilute the primary antibody in Co-Detection Antibody Diluent using previously established conditions for IHC staining on the DISCOVERY ULTRA or antibody manufacturer's recommendations.
   b. Transfer the diluted primary antibody to an Antibody dispenser.
2. Fill the mRNA Sample Prep Kit:
   a. Transfer the contents of both bottles of VS Target Retrieval v2 from the RNAscope VS Sample Prep Reagent Kit v2 to the mRNA Target Retrieval Dispenser.
   b. Transfer the VS Dewax reagent from the RNAscope VS Sample Prep Reagent Kit v2 to the mRNA Dewax Dispenser.

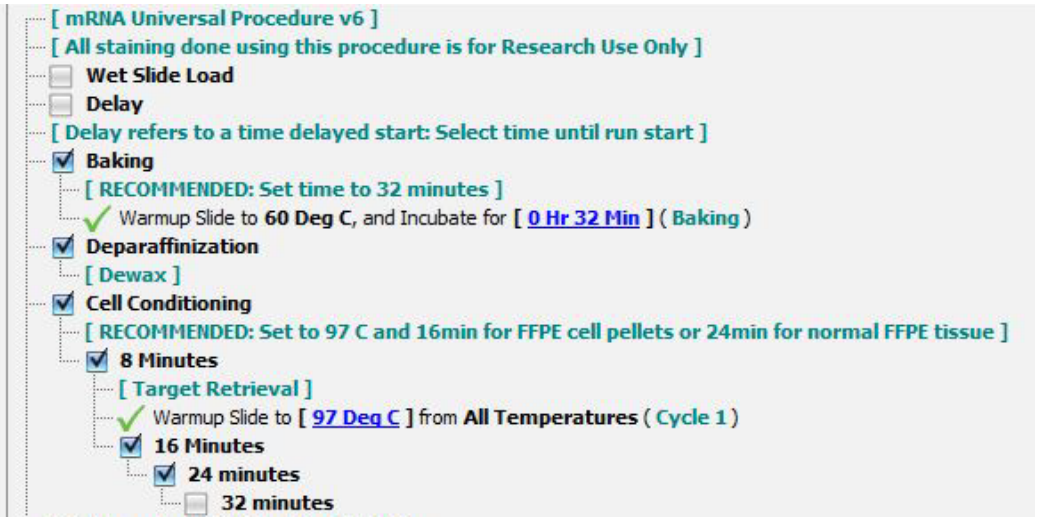
   Note: Leave the mRNA Protease dispenser empty. This dispenser is not used but must be on-instrument for the run to start successfully.
3. Fill the user-fillable dispensers:
   a. Transfer the VS Protease from the Detection Kit to the Enzyme 2 dispenser
   b. If using VS Accessory reagents for counterstaining, transfer the VS Hematoxylin and VS Bluing to the Counterstain 1 and Counterstain 2 dispensers.
4. Follow the dispenser product insert instructions to properly prime and handle the dispensers.
5. Store tightly capped dispensers (except the mRNA Dewax dispenser) at **4°C** when not in use.
6. Store tightly capped mRNA Dewax dispenser at room temperature when not in use.

Create instrument protocols for protein target qualification

1. Open the NexES software and click the **Protocol** button.
2. Click **Create/Edit Protocols**, go to the Procedure drop-down menu and select **mRNA Universal.** Main protocol selections appear as shown:

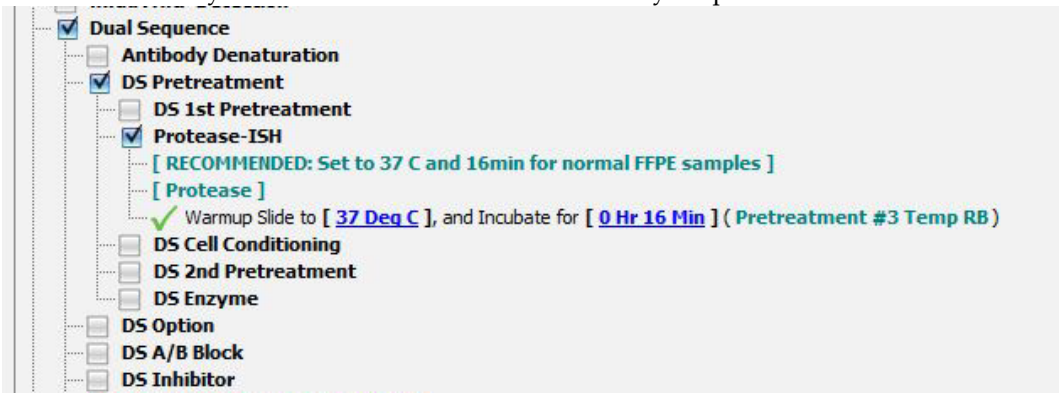


3. To create a protocol to test the sensitivity of your protein target to RNAscope VS Protease, select the appropriate pretreatment conditions as shown in the following screenshot. After the main step selections, drop-down menus become available for further selection. For Cell Conditioning, we recommend the same conditions previously used on your tissue for ISH-only staining.



4. Select Dual Sequence to view and select IHC options.

5. Select **DS Pretreatment** followed by **Protease-ISH** to enable use of the mRNA Protease dispenser outside of the mRNA assay selections. Select **37°C** and **16 MIN** as your protease conditions.



6. For HRP detection, select **DS Inhibitor** followed by **Neutralize**. You do not need to select these options for AP detection.
7. Select **DS Antibody**, followed by the desired primary antibody incubation time, temperature, and barcode according to your established IHC protocol.
8. Choose a secondary antibody selection corresponding to your desired secondary detection method.
   *For example, if using an anti-rabbit HRP multimer, select **DS Multimer HRP** and choose **UMap anti-Rb HRP**. Select the secondary incubation time according to your established IHC protocol.*
9. Select the appropriate detection kit and available incubation conditions according to your established IHC protocol. For a list of compatible IHC chromogen detection kits, see the table on page 10.
10. Select your preferred counterstain/post-counterstain reagents and assay time.
11. Click **Save As**, then select a unique protocol number from the drop-down menu and choose a protocol name. Click **Active**, add relevant comments in the available field, and click **Save**.
12. To create a control protocol without the RNAscope VS Protease application, de-select **DS Pretreatment**. Click **Save As**, then select a unique protocol number from the drop-down menu and choose a protocol name. Click **Active**, add relevant comments in the available field, and click **Save**.

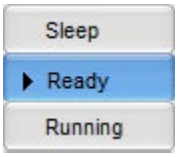
Print the labels

1. Select the **Print Label** icon from the upper right corner of the home screen.
2. Select your preferred template or create a new template. To create a new template, refer to the *Ventana DISCOVERY ULTRA System User Manual* for details.
3. Click **Protocol**.
4. Select the protocol with the RNAscope VS Protease application and the control protocol without the RNAscope VS Protease application created in the previous section. Click the **Add** button after selecting each protocol.
5. When the protocols for all slides have been assigned, click **Close/Print**.
6. Fill in the template for each slide. Click **Print** when completed

Load the reagents

1. Remove the nozzle caps from the filled dispensers and place each cap on the post located on the back of the dispenser.
2. Prime the user-fillable dispensers. For guidance, refer to the instructions provided by Roche Tissue Diagnostics.

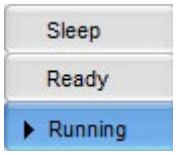
3. If needed, remove any air bubbles at the nozzle tip by pushing down on the nozzle until the liquid reaches the tip of the nozzle or forms a small meniscus at the tip of the nozzle.
4. Remove the yellow locking ring from the dispensers in all the prefilled dispensers. Refer to the instructions provided by Roche Tissue Diagnostics.
5. Load the dispensers onto the reagent racks.
6. Load the reagent racks onto the reagent carousel.
7. Select the **Ready** button.



8. Open the slide drawers.
9. Load each slide onto a heater pad with the label facing upward and inward. Ensure that the slides sit securely on the pads.

IMPORTANT! Prior to loading the slides, ensure heater pads are completely dry. Wipe off any liquid using laboratory tissue paper.

10. Close the slide drawers.
11. Select the **Running** button.



12. The assay duration varies from **15 – 20 HRS** based on assay selections.

IMPORTANT! Before leaving the instrument unattended, ensure all reagents and slides are successfully registered and the instrument is running.

Complete the run

1. After the run is complete, remove the Dewax reagent, place nozzle cap on the dispenser, and store at room temperature.
2. For the remaining reagents, place nozzle caps back on the dispensers and place racks onto the magnet locking tray.

IMPORTANT! Store reagent racks at **4°C** until next use. Store the Dewax dispenser at room temperature.

Wash and dry the slides

1. Prepare 200 mL of diluted detergent by adding 1–2 drops detergent to 200 mL distilled water in a container with a cap.
2. Mix well by inverting the container 4–5 times.
3. Add diluted detergent to a Tissue-Tek Staining Dish.

Note: Store diluted detergent at **RT**.

1. Submerge a Tissue-Tek Slide Rack into the Tissue-Tek Staining Dish containing 200 mL diluted detergent.
2. Open the instrument slide drawers and unload slides.

3. Decant solution on the slides into the slide drawer, then *immediately* load slides into the Tissue-Tek Slide Rack submerged in detergent.
4. Rinse oil off the slides by moving the slide rack up and down in the dish 10 times.
5. Replace the detergent with distilled water and rinse slides by moving the slide rack up and down a minimum of **10** times.
6. Repeat Step 8 three to five times.
7. Transfer the slides into a Tissue-Tek Staining Dish containing **200 mL** distilled water.
8. Place slides in a drying oven at **60°C** for at least **30 MIN.**

Mount the samples

1. In a fume hood, fill two clearing agent dishes with ~200 mL fresh xylene.
2. Once slides are dry, move the Tissue-Tek Slide rack into the staining dish containing xylene for **1 MIN** with occasional agitation.
3. Move the Tissue-Tek Slide rack into the staining dish containing xylene for **1 MIN** with occasional agitation.
4. Lay each slide flat with the sections facing up in the fume hood then add 1–2 drops of EcoMount or other chromogen-compatible xylene-based mounting medium. Carefully place a 24 mm x 50 mm coverslip over the section and avoid trapping air bubbles.
5. Air dry slides for at least **15 MIN** before evaluation.

IMPORTANT! mRNA Teal, mRNA Green, DISCOVERY Teal HRP and DISCOVERY Green HRP chromogens are light sensitive and may fade over time. For best results, protect stored slides from the light and image within one week of staining. .

Interpret the results of protein target qualification:

1. Compare the staining patterns with and without RNAscope VS Protease application.
2. If the staining is comparable, proceed to **Chapter 2: Sequential RNA-Protein Co-Detection**.
3. If protein staining intensity, cell positivity rate, or staining distribution is impacted by RNAscope VS Protease treatment, proceed to **Chapter 3: Integrated Co-Detection Workflow.**

Chapter 2: Sequential RNA-Protein Co-Detection

In this workflow, tissue samples undergo RNAscope pretreatment including RNAscope VS Protease digestion, followed by ISH detection, and then undergo IHC detection and counterstaining. First complete **Chapter 1: Protein Target Qualification** to confirm your protein epitope(s) of interest are protease-tolerant prior to proceeding with this workflow.

Materials

ACD reagents for Sequential RNA-Protein Co-Detection

Sequential RNA-Protein Co-Detection on the Roche DISCOVERY ULTRA requires the following reagents from ACD:

- RNAscope VS Universal Sample Prep Reagent Kit v2 (Cat. No. 323740)
- ACD Assay-specific Detection Reagent Kit:
  - RNAscope VS Universal AP Detection Reagents (Cat. No. 323260)
  - RNAscope VS Universal HRP Detection Reagents (Cat. No. 323210)
  - RNAscope VS Duplex Detection Reagents (Cat. No. 323310)
  - BaseScope VS Detection Reagents (Cat. No. 323710)
- VS Co-Detection Inhibitor from VS RNA-Protein Co-Detection Ancillary Kit (Cat. No.323760)

RNAscope VS Universal Sample Prep Reagent Kit v2 (Cat. No. 323740) and RNAscope VS Accessory Kit (Cat. No. 320630) are RTU and stored as indicated in the following tables:

RNAscope VS Sample Prep Reagent Kit v2 (Cat. No. 323740)			
☑	**Reagent**	**Quantity**	**Storage**
	RNAscope VS Universal Target Retrieval v2	10 mL x 2 bottle	Room Temp (15–30°C)
	RNAscope VS Universal Dewax	14 mL x 1 bottle	Room Temp (15–30°C)

Assay-specific Detection Reagents within each Reagent Kit are stored as indicated in the following tables:

RNAscope VS Universal AP Detection Reagents (Cat. No. 323260)			
☑	**Reagent**	**Quantity**	**Storage**
	RNAscope VS Universal AP AMP 1	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal AP AMP 2	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal AP AMP 3	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal AP AMP 4	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal AP AMP 5	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal AP AMP 6	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal AP AMP 7	14 mL x 1 bottle	2–8°C
	RNAscope VS Protease	14 mL x 1 bottle	2–8°C

RNAscope VS Universal HRP Detection Reagents (Cat. No. 323210)			
☑	**Reagent**	**Quantity**	**Storage**
	RNAscope VS Universal HRP AMP 1	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal HRP AMP 2	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal HRP AMP 3	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal HRP AMP 4	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal HRP AMP 5	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal HRP AMP 6	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal HRP AMP 7	14 mL x 1 bottle	2–8°C
	RNAscope VS Protease	14 mL x 1 bottle	2–8°C

RNAscope VS Duplex Detection Reagents (Cat. No. 323310)			
☑	**Reagent**	**Quantity**	**Storage**
	RNAscope VS Duplex AMP 1	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP 2	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP 3	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP 4	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP 5	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP 6	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP 7	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP 8	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP 9	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP Wash	14 mL x 2 bottles	2–8°C
	RNAscope VS Protease	14 mL x 1 bottle	2–8°C

BaseScope VS Detection Reagents (Cat. No. 323710)			
☑	**Reagent**	**Quantity**	**Storage**
	BaseScope VS AMP 1	14 mL x 1 bottle	2–8°C
	BaseScope VS AMP 2	14 mL x 1 bottle	2–8°C
	BaseScope VS AMP 3	14 mL x 1 bottle	2–8°C
	BaseScope VS AMP 4	14 mL x 1 bottle	2–8°C
	BaseScope VS AMP 5	14 mL x 1 bottle	2–8°C
	BaseScope VS AMP 6	14 mL x 1 bottle	2–8°C
	BaseScope VS AMP 7	14 mL x 1 bottle	2–8°C
	BaseScope VS AMP 8	14 mL x 1 bottle	2–8°C
	RNAscope 2.5 VS Pretreat 3 - Protease	14 mL x 1 bottle	2–8°C

VS RNA-Protein Co-Detection Ancillary Kit - Cat No. 323760				
☑	Reagent	Source / Ordering Info	Quantity	Storage
	Co-Detection Antibody Diluent*	ACD/Cat No. 323160	120 mL x 1 bottle	2–8°C
	VS RNA-Protein Co-Detection Protease and Inhibitor	ACD/Cat No. 323190	See the following	
	VS Co-Detection Protease†		14 mL x 1 bottle	2–8°C
	VS Co-Detection Inhibitor‡		14 mL x 1 bottle	2–8°C

*Use of Co-Detection Antibody Diluent is optional for Sequential RNA-Protein Co-Detection

†VS Co-Detection Protease is not needed for Sequential RNA-Protein Co-Detection

‡ VS Co-Detection Inhibitor is a chemical enzyme inhibitor recommended for use between any RNAscope/BaseScope assay and IHC detection. We recommend this reagent for both sequential and integrated co-detection workflows.

Optional ACD reagents for Sequential RNA-Protein Co-Detection

The following components from ACD are optional for Sequential RNA-Protein Co-Detection on the Roche DISCOVERY ULTRA:

- Co-Detection Antibody Diluent from VS RNA-Protein Co-Detection Ancillary Kit (Cat No. 323760)
- RNAscope VS Accessory Kit (Cat. No. 320630)

VS RNA-Protein Co-Detection Ancillary Kit - Cat No. 323760				
☑	Reagent	Source / Ordering Info	Quantity	Storage
	Co-Detection Antibody Diluent	ACD/Cat No. 323160	120 mL x 1 bottle	2–8°C

RNAscope VS Accessory Kit (Cat. No. 320630)			
☑	Reagent	Quantity	Storage
	RNAscope VS Hematoxylin	7 mL x 1 bottle	2–8°C
	RNAscope VS Bluing Reagent	7 mL x 1 bottle	2–8°C

Roche materials for Sequential RNA-Protein Co-Detection:

Sequential RNA-Protein Co-Detection can be performed with either Roche RTU primary antibody or with your choice of primary antibody concentrate diluted in the ACD Co-Detection Antibody Diluent. For a list of available Roche RTU Primary Antibodies and ordering information, please contact your local Roche representative.

The following additional materials from Roche can be used for Sequential RNA-Protein Co-Detection on DISCOVERY ULTRA. Catalog numbers are valid in the United States only. For other regions, please check catalog or ordering numbers with your local lab supplier.

Reagent dispensers

Roche Materials for Sequential RNA-Protein Co-Detection				
☑	**Component**	**Cat. No.**	**Ordering Code**	**Fill with:**
	Probe Dispensers	960-761 to 960-780	Contact local Roche representative	RNAscope VS Probes
	mRNA Sample Prep Kit	760-248	08127166001	RNAscope VS Sample Prep Reagent Kit v2 and VS Protease reagent from Detection Kit
	Antibody Dispensers (*Optional**)	770-001 to 770-099	Contact local Roche representative	User-sourced Primary Antibody Concentrate diluted in Co-Detection Antibody Diluent
	Pretreatment 1 Dispenser	960-901	05280095001	VS Co-Detection Inhibitor
	Counterstain 1 dispenser	771-741	05271720001	VS Hematoxylin
	Counterstain 2 dispenser	771-742	05271738001	VS Bluing Reagent
	Roche RTU Counterstain Reagents	Various	Contact local Roche representative	Pre-filled
	DISCOVERY Antibody Block	760-4204	05268869001	Pre-filled
	Additional ISH Assay-Specific Materials	(See the following tables)		
	Additional IHC Detection Materials	(See the following tables)		

*Sequential RNA-Protein Co-Detection can be performed with either Roche RTU primary antibody or your choice of primary antibody.

Additional ISH assay-specific materials

Additional Roche Materials Required for VS Universal AP ISH				
☑	**Component**	**Cat. No.**	**Ordering Code**	**Fill with:**
	mRNA RED Probe Amplification Kit	760-236	07095341001	RNAscope VS Universal AP Detection Reagents AMP 1-7
	mRNA RED Detection Kit	760-234	07099037001	Pre-filled

Additional Roche Materials Required for VS Universal HRP ISH				
☑	**Component**	**Cat. No.**	**Ordering Code**	**Fill with:**
	mRNA Probe Amplification Kit	760-222	06614337001	RNAscope VS Universal HRP Detection Reagents AMP 1–7
	DISCOVERY Inhibitor*	760-4840	07017944001	Pre-filled
	mRNA Purple HRP Detection Kit*	760-255	08127166001	Pre-filled
	mRNA Green HRP Detection Kit†	760-278	08952612001	Pre-filled
	mRNA Teal HRP Detection Kit†	760-256	08352941001	Pre-filled
	mRNA DAB Detection Kit†	760-224	06614353001	Pre-filled
	DISCOVERY DCC Kit†	760-240	07988192001	Pre-filled
	DISCOVERY FAM Kit†	760-243	07988150001	Pre-filled
	DISCOVERY FITC Kit†	760-232	07259212001	Pre-filled
	DISCOVERY Rhodamine Kit†	760-233	07259883001	Pre-filled
	DISCOVERY Rhodamine 6G Kit†	760-244	07988168001	Pre-filled

Additional Roche Materials Required for VS Universal HRP ISH				
	DISCOVERY Red 610 Kit†	760-245	07988176001	Pre-filled
	DISCOVERY Cy5 Kit†	760-238	07551215001	Pre-filled

* DISCOVERY Inhibitor is not required if using mRNA DAB Detection Kit

† Choose one mRNA Detection Kit for RNAscope HRP detection

Additional Roche Materials Required for VS Duplex ISH				
☑	Component	Cat. No.	Ordering Code	Fill with:
	mRNA Duplex Amp Kit	760-249	08127174001	RNAscope VS Duplex Detection Reagents AMP 1–9, AMP Wash
	mRNA Link (Pre-filled)	760-6014	08127115001	Pre-filled
	mRNA RED Detection Kit	760-234	07099037001	Pre-filled
	mRNA Green HRP Detection Kit*	760-278	08952612001	Pre-filled
	mRNA Teal HRP Detection Kit*	760-256	08352941001	Pre-filled
	mRNA DAB Detection Kit*	760-224	06614353001	Pre-filled

* Choose one mRNA Detection Kit for VS Duplex Channel 1 detection

Additional Roche Materials Required for VS BaseScope ISH				
☑	Component	Cat. No.	Ordering Code	Storage
	mRNA RED Probe Amplification Kit	760-236	07095341001	BaseScope VS Detection Reagents AMP 1–7
	Option 8 dispenser	771-758	05271916001	BaseScope VS AMP 8 reagent
	mRNA RED Detection Kit	760-234	07099037001	Pre-filled

Additional IHC assay-specific materials

Reagent Options for Ventana IHC AP Detection				
☑	Component	Cat. No.	Ordering Code	Storage
	DISCOVERY UltraMap anti-Ms Alk Phos*	760-4312	05269687001	2–8°C
	DISCOVERY UltraMap anti-Rb Alk Phos*	760-4314	05269709001	2–8°C
	DISCOVERY Yellow Kit*	760-239	07698445001	2–8°C
	DISCOVERY Red Kit	760-228	07425333001	2–8°C
	DISCOVERY ChromoMap Red Kit*	760-160	05266653001	2–8°C

Reagent Options for Ventana IHC HRP Detection				
☑	Product	Cat. No.	Ordering Code	Storage
	DISCOVERY UltraMap anti-Ms HRP*	760-4313	05269695001	2–8°C
	DISCOVERY UltraMap anti-Rb HRP*	760-4315	05269717001	2–8°C
	DISCOVERY UltraMap anti-Rat HRP*	760-4456	05891884001	2–8°C
	DISCOVERY UltraMap anti-Gt HRP*	760-4648	06607241001	2–8°C
	DISCOVERY Purple Kit*	760-229	07053983001	2–8°C
	DISCOVERY Green HRP Kit †	760-278	07053983001	2–8°C
	DISCOVERY Teal HRP Kit †	760-247	08254338001	2–8°C
	DISCOVERY ChromoMap DAB Kit †	760-159	05266645001	2–8°C
	DISCOVERY DCC Kit †	760-240	07988192001	2–8°C
	DISCOVERY FAM Kit †	760-243	07988150001	2–8°C
	DISCOVERY FITC Kit†	760-232	07259212001	2–8°C
	DISCOVERY Rhodamine Kit †	760-233	07259883001	2–8°C
	DISCOVERY Rhodamine 6G Kit †	760-244	07988168001	2–8°C
	DISCOVERY Red 610 Kit†	760-245	07988176001	2–8°C
	DISCOVERY Cy5 Kit †	760-238	07551215001	2–8°C

* Choose one secondary detection antibody depending on the primary antibody species and desired IHC chromogen
† Choose one IHC chromogen

Instrument buffers

☑	Component	Cat. No.	Ordering Code	Storage
	10X DISCOVERY Wash (RUO)	950-510	7311079001	Room Temp (15–30°C)
	ULTRA LCS (Predilute)	650-210	5424534001	Room Temp (15–30°C)
	SSC Buffer (10X)	950-110	5353947001	Room Temp (15–30°C)
	Reaction Buffer (10X)	760-107	5266262001	Room Temp (15–30°C)
	DISCOVERY CC1	950-500	6414575001	Room Temp (15–30°C)

User-Sourced Materials

☑	Description	Supplier	Cat. No.
	Primary Antibody (RTU)	User	**Various**
	Primary Antibody Concentrate	User	Various
	SuperFrost Plus Slides (required)	Fisher Scientific	12-550-15
	ProLong Gold Antifade Reagent	Life Technologies	P36930
	EcoMount (if using Red detection)	Biocare	EM897L

☑	Description	Supplier	Cat. No.
	Tissue-Tek Vertical 24 Slide Rack	American Master Tech Scientific/MLS	LWSRA24
	Tissue-Tek Staining Dishes	American Master Tech Scientific/MLS	LWT4457EA
	Cover Glass 24 x 50 mm	Fisher Scientific/MLS	12--545-F
	Distilled water	MLS	—
	Mild liquid dishwashing detergent (Dawn detergent or similar)	MLS	—
	Drying oven, capable of holding temperature at 60 +/– 1°C	MLS	—
	Fume hood	MLS	—
	100% ethanol (EtOH)	MLS	—
	Xylene	MLS	—
	Tissue-Tek Clearing Agent Dishes, xylene-resistant	American Master Tech Scientific/MLS	LWT4456EA
	Optional: Glass beaker (1 or 2 L)	MLS	—
	Optional: Hot plate	Fisher Scientific/MLS	11-300-49SHP

Assay Procedure

Prepare the DISCOVERY ULTRA

Prepare the instrument

If the instrument has not been used for > 1 week, follow guidelines for instrument maintenance from Roche Tissue Diagnostics. Before use, empty the waste carboys if needed.

Dilute instrument bulk reagents

1. Prepare the instrument bulk fluids according to the manufacturer's instructions.
2. Fill bulk solution containers for 1X DISCOVERY Wash, ULTRA LCS (predilute), and CC1 (predilute) to be at least half full. Fully fill bulk solution containers for 2X SSC and 1X Reaction Buffer.

IMPORTANT! Do not use expired reagents.

Register new reagents

Reagent dispensers come with appropriate barcode labels and registration buttons for dispensing RNAscope VS Universal Reagents. Refer to the *Ventana DISCOVERY ULTRA System User Manual* for details. To register reagents:

- Log all ACD reagents and probes into the software as **log user-fillable reagents** and **log user-**

fillable probes, respectively**.**

- Use the reagent registration wand that comes with the instrument to register new reagent kits from Roche Tissue Diagnostics

Prepare user-fillable reagents for RNA-Protein Co-Detection

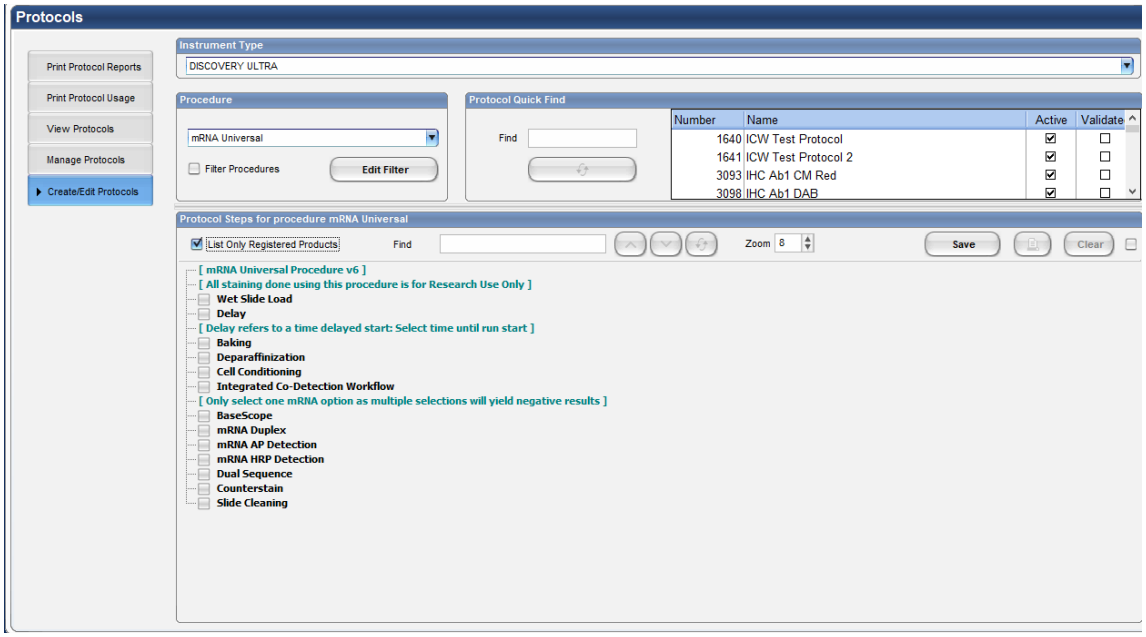
Refer to the table on pages 6–7 to determine the proper dispenser for each reagent.

IMPORTANT! Avoid cross contamination between reagents. Dewax must be warmed to room temperature and be completely in solution before use.

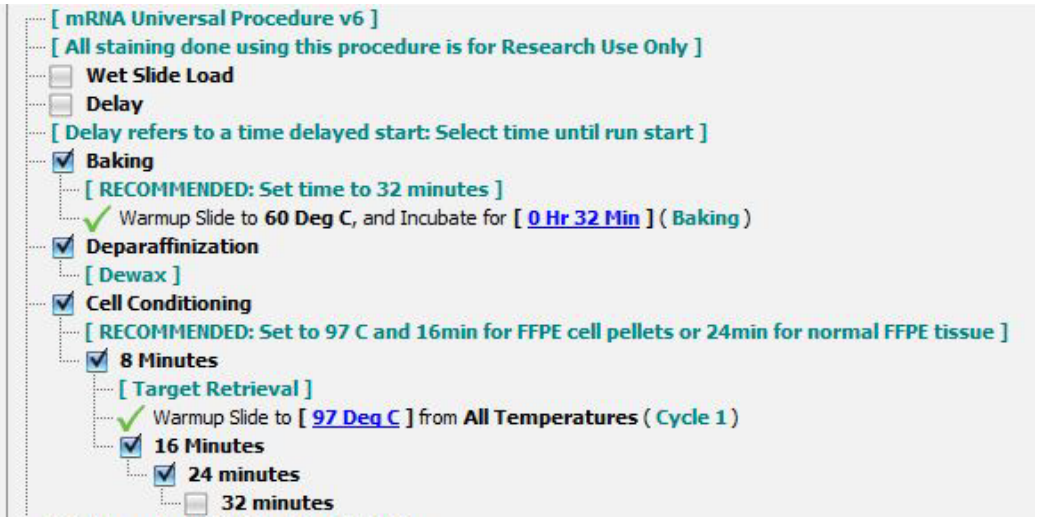
1. Transfer the entire volume of each AMP component of the Detection Kit to the corresponding labeled dispenser from the appropriate mRNA Amplification kit (see pages 6–7 for details)
2. Fill the mRNA Sample Prep Kit:
   a. Transfer the contents of both bottles of VS Target Retrieval v2 from the RNAscope VS Sample Prep Reagent Kit v2 to the mRNA Target Retrieval Dispenser.
   b. Transfer the VS Dewax reagent from the RNAscope VS Sample Prep Reagent Kit v2 to the mRNA Dewax Dispenser.
   c. Transfer the VS Protease from the Detection Kit to the mRNA Protease Dispenser
3. Fill the User Fillable Dispensers:
   a. Transfer the RNAscope 2.5 or BaseScope VS Target Probe and control probes to the corresponding probe dispensers.
   b. Transfer the VS Co-Detection Inhibitor from the VA RNA-Protein Co-Detection Ancillary kit to the Pretreatment 1 dispenser.
   c. Transfer the VS Hematoxylin and VS Bluing to the Counterstain 1 and Counterstain 2 dispensers.
4. Follow the dispenser product insert instructions to properly prime and handle the dispensers.
5. Store tightly capped dispensers (except the mRNA Dewax dispenser) at **4°C** when not in use.
6. Store mRNA Dewax dispenser at room temperature when not in use.

Create an instrument protocol

1. Open the NexES software and click the **Protocol** button.
2. Click **Create/Edit Protocols**, go to the Procedure drop-down menu and select **mRNA Universal.** Main protocol selections appear as shown:



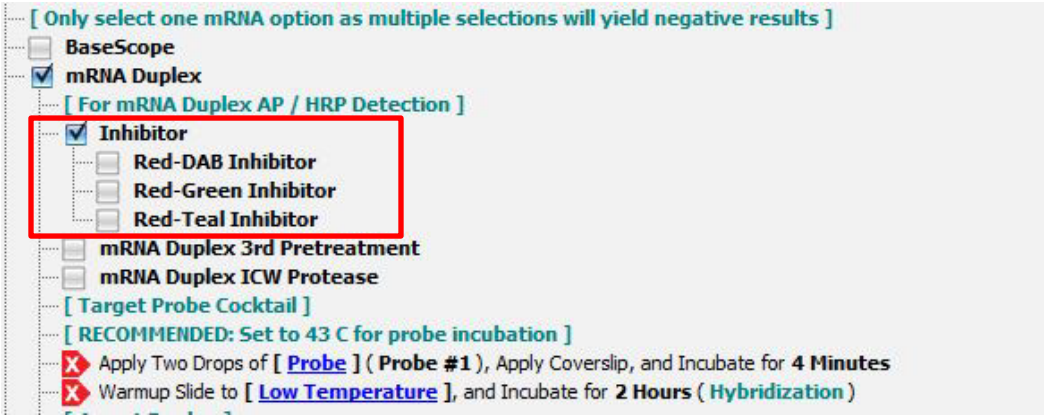
3. Select the appropriate pretreatment conditions as shown in the following screenshot. After the main step selections, drop-down menus become available for further selection. For Cell Conditioning, we recommend the same conditions previously used on your tissue for ISH-only staining.



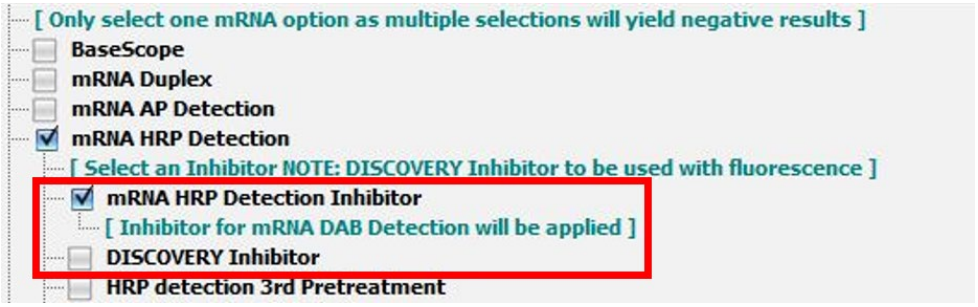
4. Select one mRNA Detection assay of interest.
   - For BaseScope VS, select **BaseScope**.
   - For RNAscope VS Duplex, select **mRNA Duplex**.
   - For RNAscope VS Universal AP, select **mRNA AP Detection**.
   - For RNAscope VS Universal HRP, select **mRNA HRP Detection**.

Select only one mRNA option per protocol. Choosing multiple selections yields negative results.

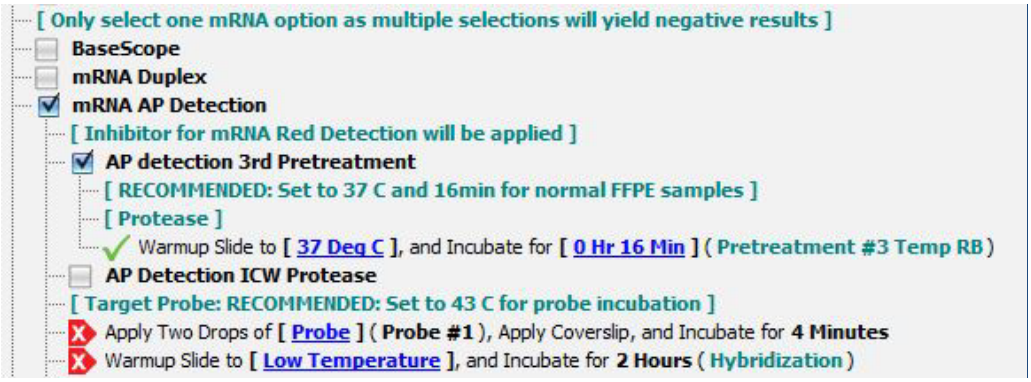
5. Select the appropriate Detection Inhibitor:
   - For **BaseScope** or **mRNA AP Detection**, mRNA Red Inhibitor reagent is applied automatically.
   - For **mRNA Duplex**, select the **Inhibitor** check box to display the available selections. Choose the appropriate inhibitor selection based on the chromogen combination in use for Duplex ISH detection, **Red-DAB Inhibitor**, **Red-Green Inhibitor**, or **Red-Teal Inhibitor**.



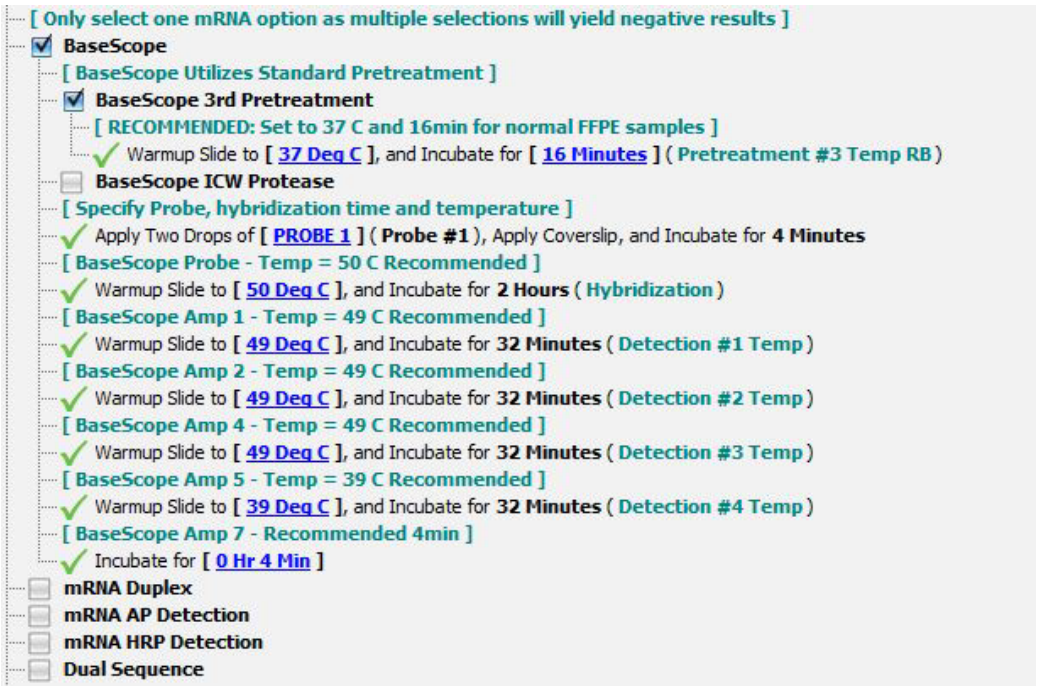
   - For **mRNA HRP Detection,** select mRNA HRP Detection Inhibitor if using the mRNA DAB Detection Kit. Select DISCOVERY Inhibitor if using an alternative HRP-based chromogen such as the mRNA Purple Detection Kit, mRNA Green Detection Kit, or mRNA Teal Detection Kit.



6. Under the mRNA assay of interest, select the corresponding **3rd Pretreatment** check box**.** Do not select the detection ICW Protease option. Recommended incubation is **37°C** for **16 MIN**.



7. Select the ISH staining conditions:
   - If running **BaseScope**, select the following ISH staining conditions:

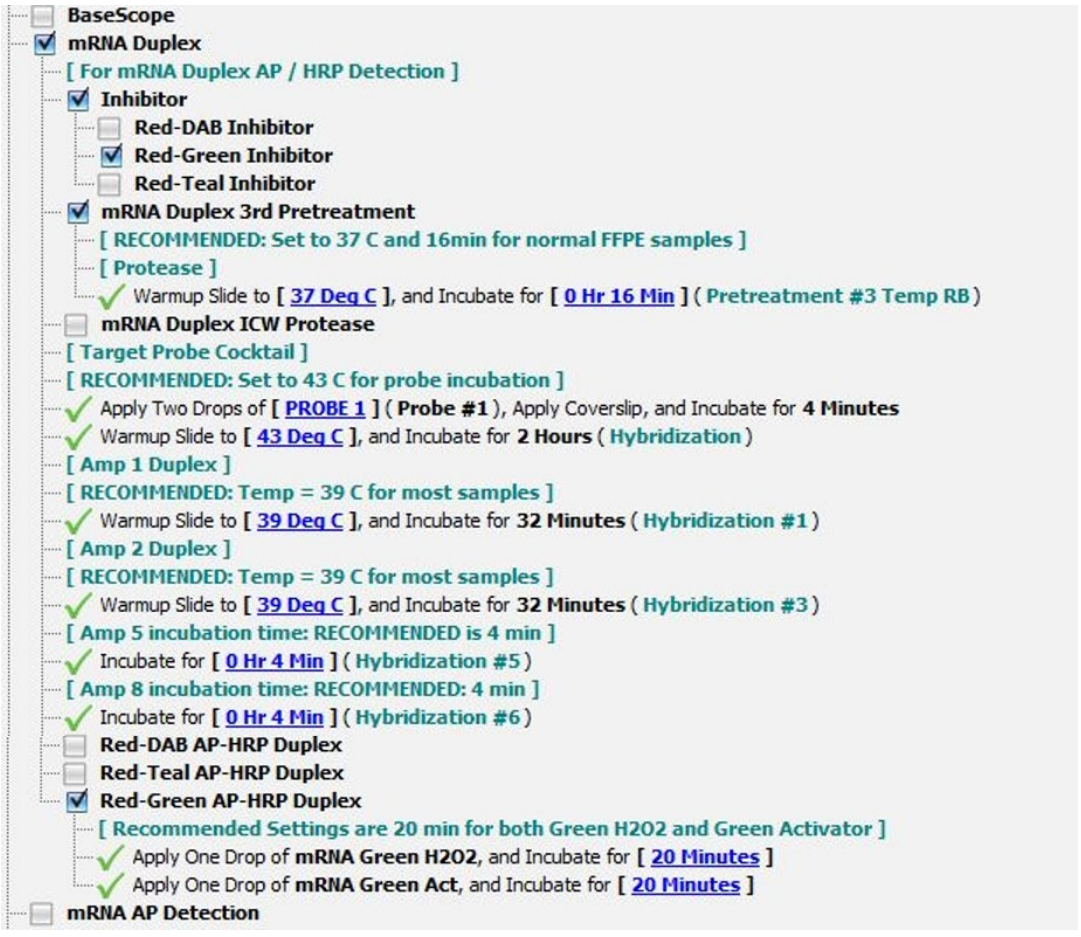


Standard Temperatures/Times for BaseScope VS	
VS Protease temperature and time	37°C, 16 MIN
Standard BaseScope probe temperature	50°C
Standard BaseScope AMP 1 temperature	49°C
Standard BaseScope AMP 2 temperature	49°C
Standard BaseScope AMP 4 temperature	49°C
Standard BaseScope AMP 5 temperature	39°C
Standard BaseScope AMP 7 incubation time*	4 MIN
mRNA Red Chromogen	Default†

* BaseScope Amp 7 incubation time is determined by instrument calibration. Use the instrument setting previously optimized for the mRNA Universal software. For assistance, consult your local ACD FAS for more information.

†For all BaseScope assays, mRNA Red Detection is applied automatically. No chromogen selections are needed.

- If running **mRNA Duplex**, select the following ISH staining conditions. For the purpose of demonstration, selections for the Red-Green chromogen combination are shown:

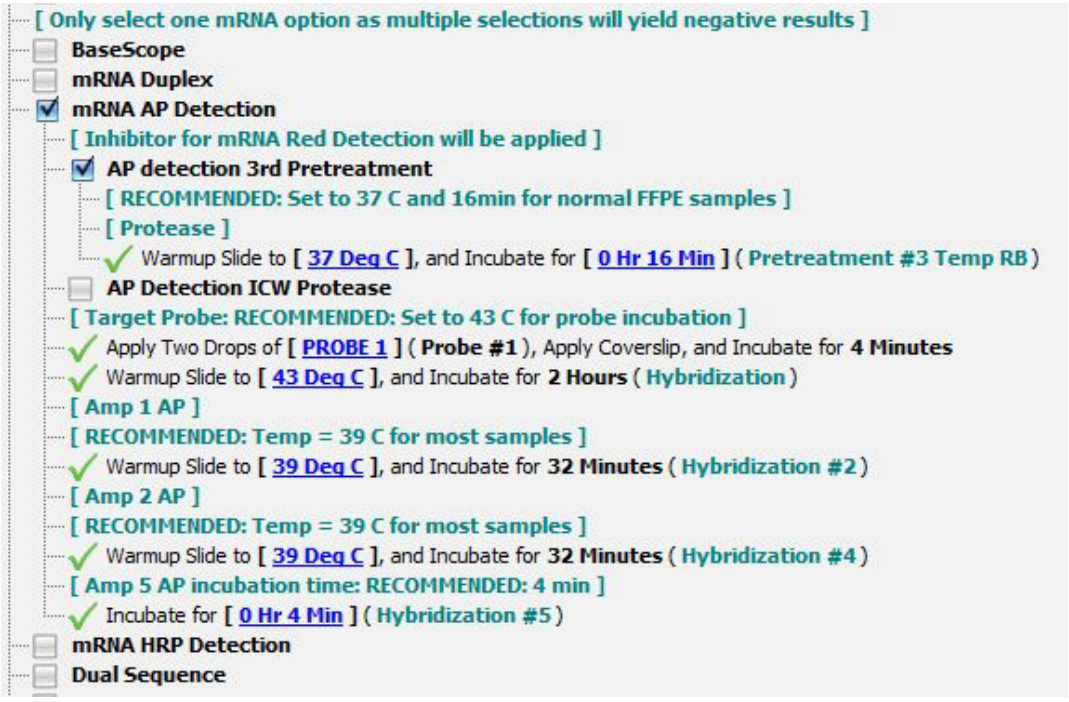


Standard Temperatures/Times for mRNA Duplex Detection	
VS Protease temperature and time	37°C, 16 MIN
Suggested probe temperature	43°C
Suggested RNAscope VS Duplex AMP 1 & AMP 2 temperature	39°C
RNAscope VS Duplex AMP 5 incubation time	4 MIN*
RNAscope VS Duplex AMP 8 incubation time	4 MIN*
Chromogen settings for Red-DAB AP-HRP Duplex†	Preset, no incubation selections
Chromogen settings for Red-Green AP-HRP Duplex†	mRNA Green H_2O_2 — 20 MIN mRNA Green Act — 20 MIN
Chromogen settings for Red-Teal AP-HRP Duplex†	mRNA Teal H_2O_2 — 20 MIN mRNA Teal Act — 20 MIN

* VS Duplex Amp 5 and Amp8 incubation times are determined by instrument calibration. Use the instrument setting previously optimized for the mRNA Universal software. For assistance, consult your local ACD FAS for more information.

†Choose one detection combination for mRNA Duplex. All times/temperatures are preset for Red-DAB Detection.

- If choosing **mRNA AP Detection**, select the following ISH staining conditions:

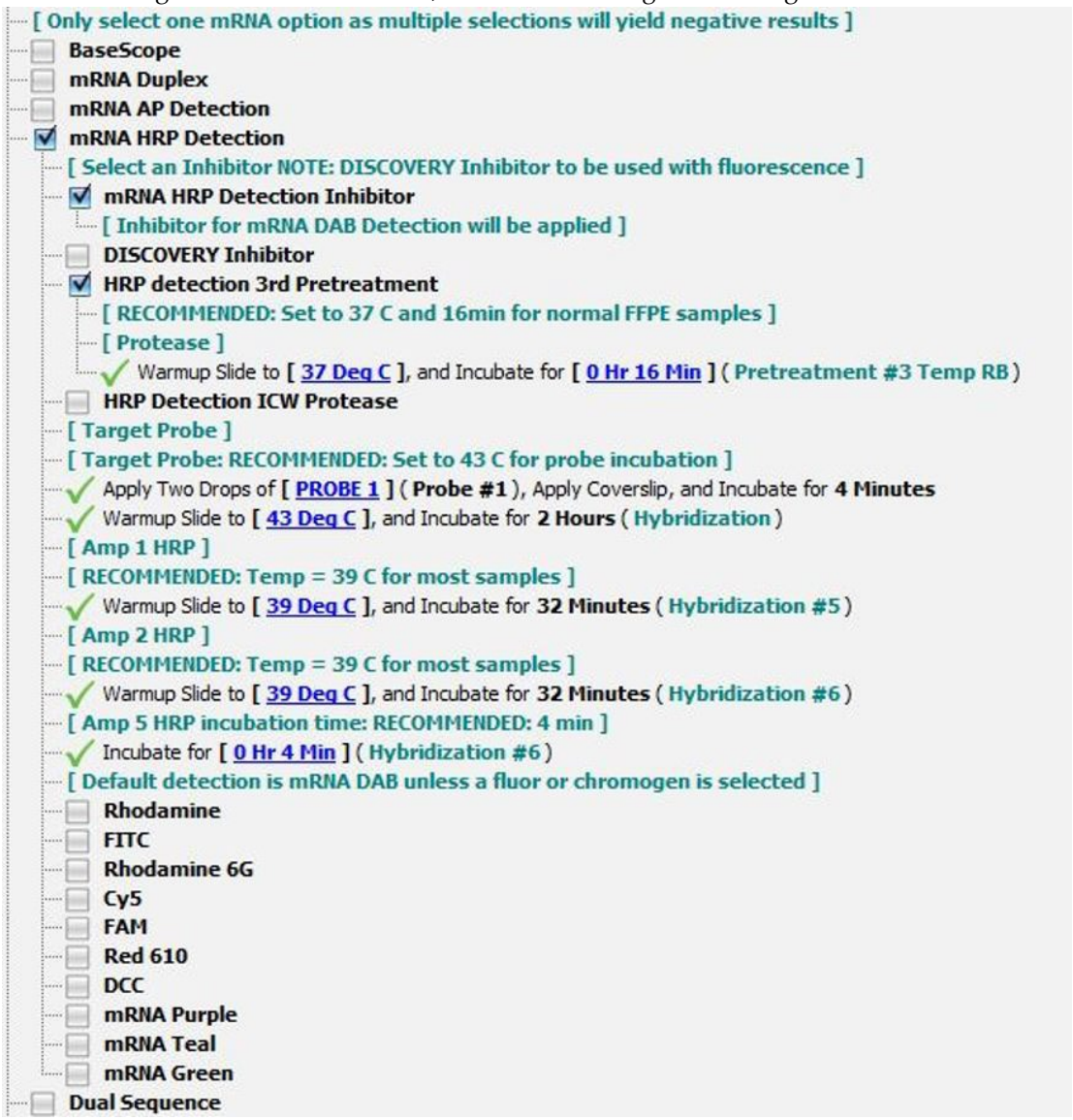


Standard Temperatures/Times for mRNA AP Detection	
VS Protease temperature and time	37°C, 16 MIN
Suggested probe temperature	43°C
Suggested RNAscope AP AMP 1 & AMP 2 temperature	39°C
RNAscope AP AMP 5 incubation time	4 MIN*
Chromogen settings for mRNA Red Detection	Default†

* VS Universal AP Amp 5 incubation time is determined by instrument calibration. Use the instrument setting previously optimized for the mRNA Universal software. For assistance, consult your local ACD FAS for more information.

†For all mRNA AP, mRNA Red Detection is applied automatically. No chromogen selections are needed.

- If choosing **mRNA HRP Detection**, select the following ISH staining conditions:

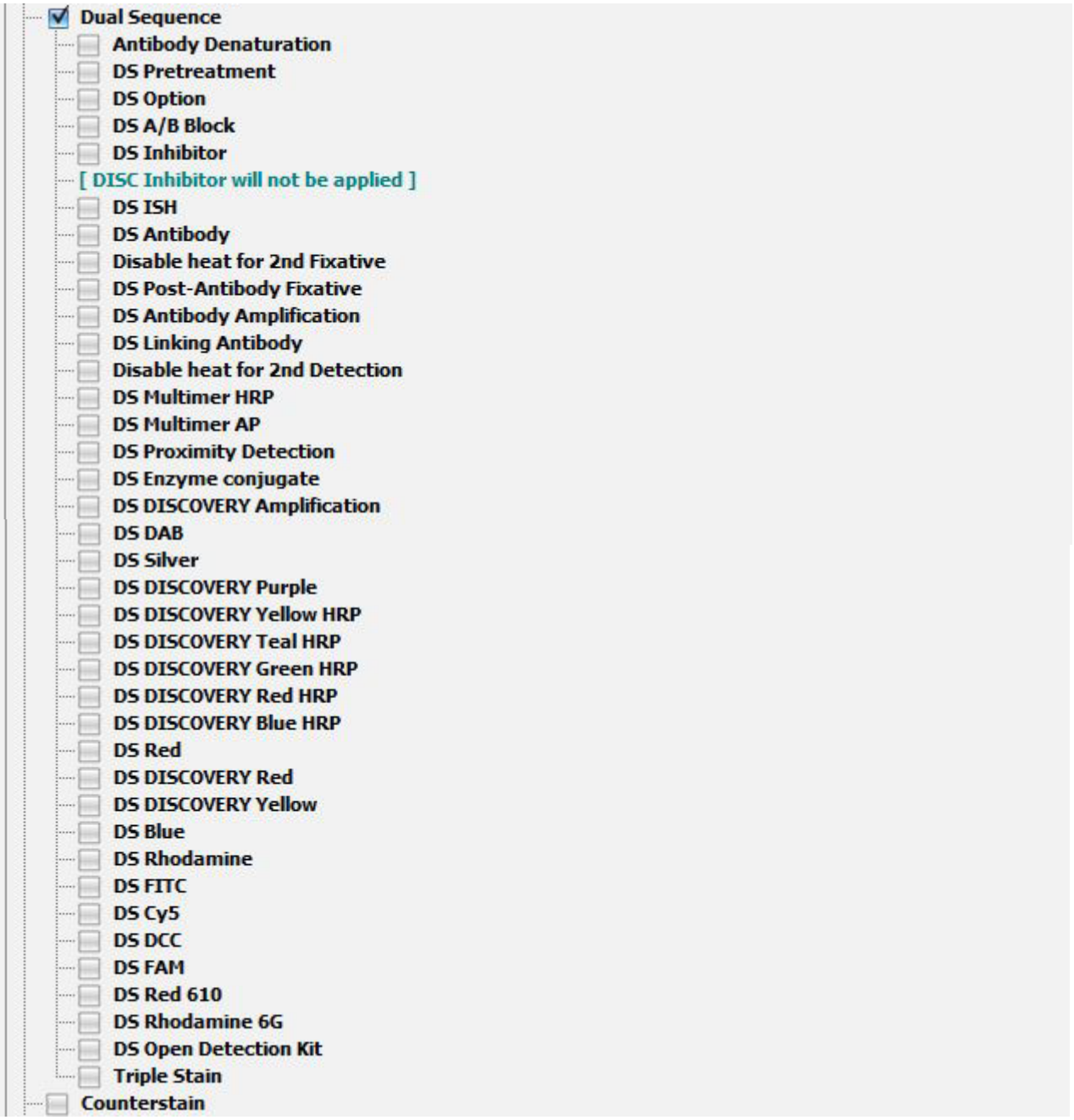


Standard Temperatures/Times for mRNA HRP Detection	
VS Protease temperature and time	37°C, 16 MIN
Suggested probe temperature	43°C
Suggested RNAscope HRP AMP 1 & AMP 2 temperature	39°C
RNAscope HRP AMP 5 incubation time	4 MIN*
Chromogen settings for mRNA DAB†	Default*
Chromogen settings for mRNA Purple	mRNA Purple H_2O_2 — 40 MIN
Chromogen settings for mRNA Teal	mRNA Teal H_2O_2 — 20 MIN mRNA Teal Act — 20 MIN
Chromogen settings for mRNA Green	mRNA Green H_2O_2 — 20 MIN mRNA Green Act — 20 MIN

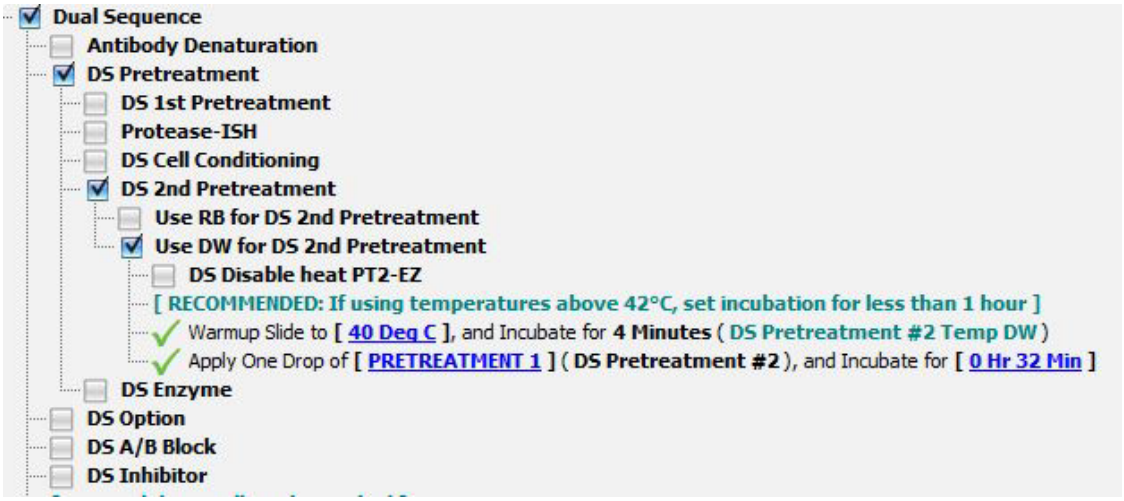
* VS Universal HRP Amp 5 incubation time is determined by instrument calibration. Use the instrument setting previously optimized for the mRNA Universal software. For assistance, consult your local ACD FAS for more information.

†For mRNA HRP, mRNA DAB Detection is applied by default if no other chromogens are selected.

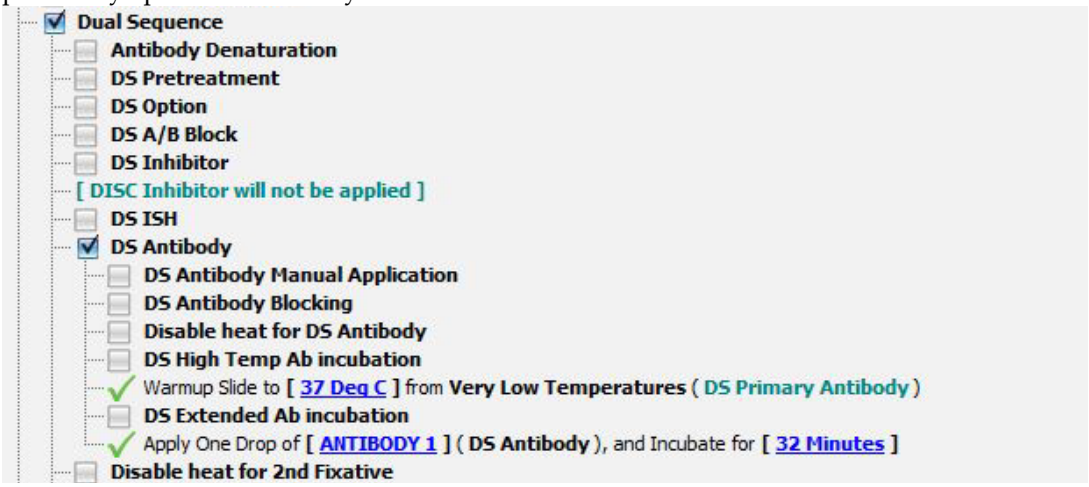
8. After completing ISH assay selections, select **Dual Sequence** to enable selections for secondary antibody detection.



9. To apply VS Co-Detection Inhibitor from the VS RNA-Protein Co-Detection Ancillary Kit, select **DS Pretreatment**, **DS 2nd Pretreatment**, **Use DW for DS 2ns Pretreatment**. Select the Pretreatment 1 barcode to correspond to the VS Co-Detection Inhibitor reagent. Recommended incubation is **40°C** for **32 MIN** prior to protein detection.



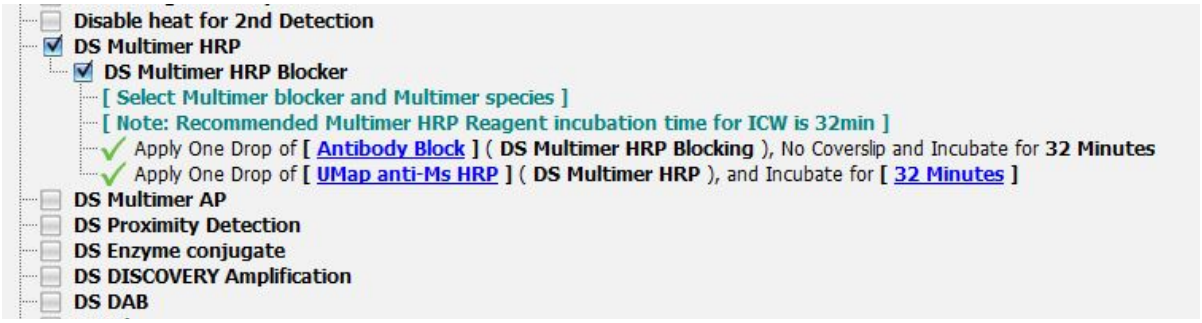
10. To apply Primary antibody, select **DS Antibody** and your desired incubation time and temperature, using previously optimized IHC-only conditions.



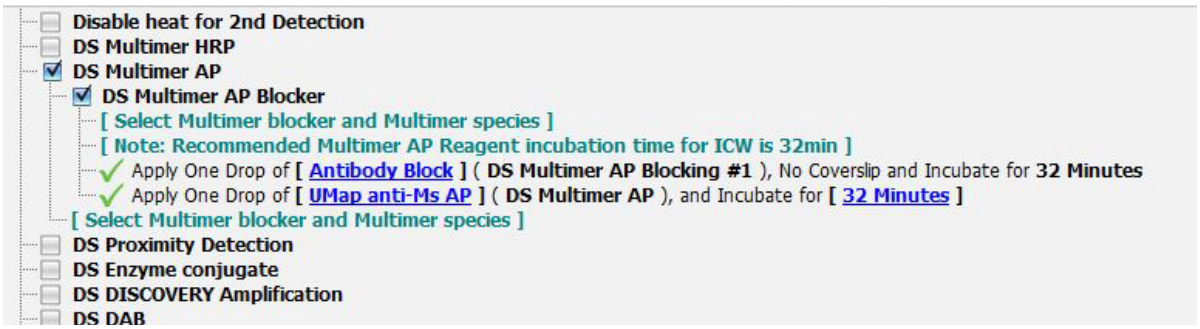
11. Select the settings for secondary detection:

Note: We recommend using Roche's Antibody Block reagent directly before applying the secondary antibody to prevent non-specific cross-detection of RNAscope. RNA-Protein Co-Detection performed without Antibody Block could result in a hue shift of the RNA dots and interfere with interpretation of RNA-protein colocalization.

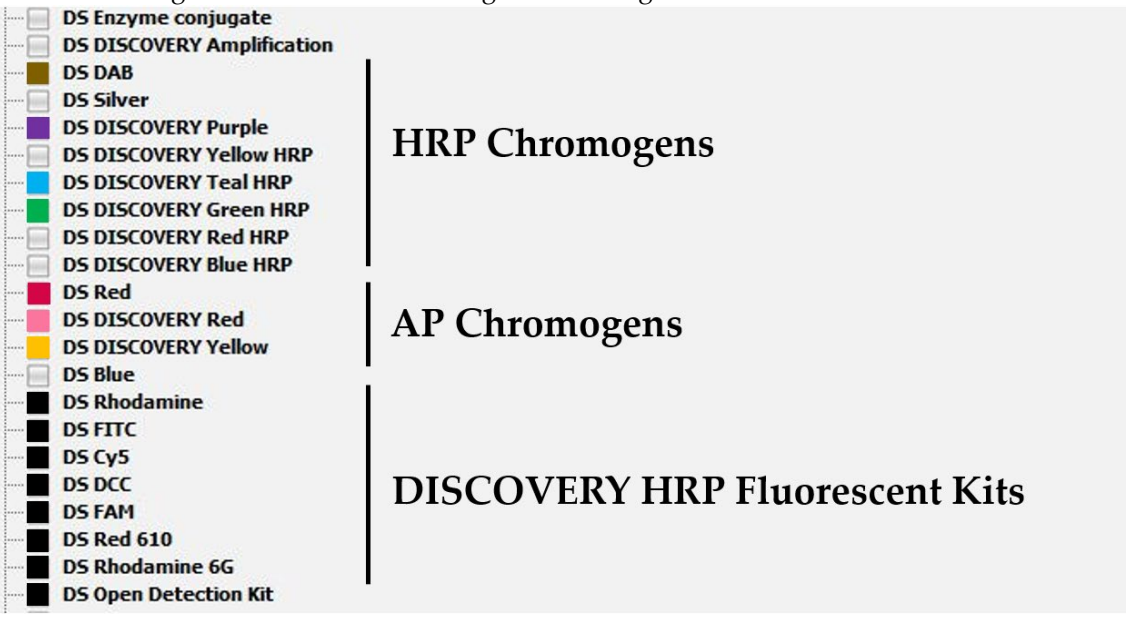
- For **HRP-based secondary detection**, choose a Roche Multimer HRP reagent corresponding to the primary host species. To enable secondary incubation, select **DS Multimer HRP**. To apply Antibody Block, select **DS Multimer HRP Blocker**, then select Antibody Block reagent. Select your Multimer HRP reagent of choice and desired incubation time, using previously optimized IHC-only conditions.



- For AP-based secondary detection, choose a Roche Multimer AP reagent corresponding to the primary host species. To enable secondary incubation, select **DS Multimer AP**. To apply Antibody Block, select **DS Multimer AP Blocker**, then select Antibody Block reagent. Select your Multimer AP reagent of choice and desired incubation time, using previously optimized IHC-only conditions.



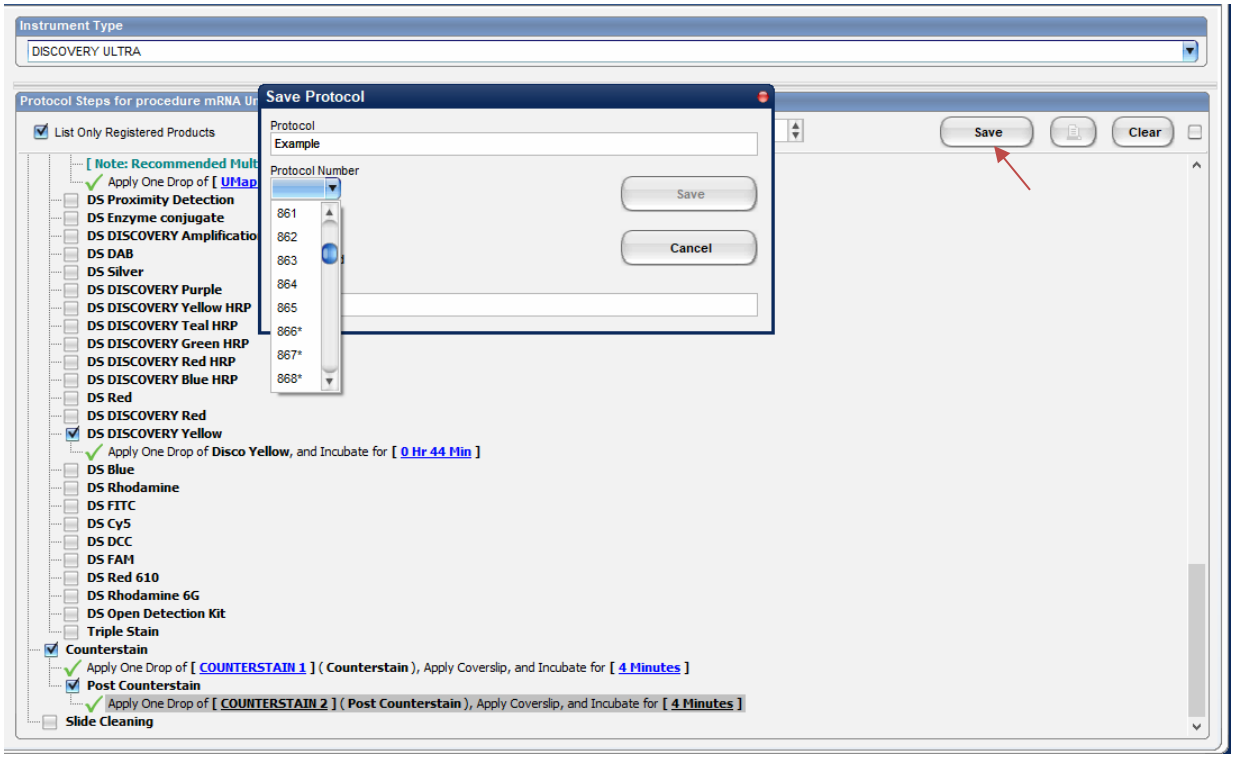
12. Select chromogens for IHC detection using the following recommendations:



IHC Chromogen Selection and Settings		
IHC Enzyme	**IHC Chromogen**	**Recommended Chromogen Settings***
AP	DISCOVERY Yellow	44 MIN – 2 HRS
	DISCOVERY Red	12 MIN†
	DS Red / ChromoMap Red‡	Default
HRP (chromogenic)	DS DAB / ChromoMap DAB	Default
	DISCOVERY Purple	40 MIN
	DISCOVERY Teal HRP	DISCOVERY Teal H_2O_2 — 16–32 MIN DISCOVERY Teal Act – 16 MIN
	DISCOVERY Green HRP	DISCOVERY Green H_2O_2 — 16–32 MIN DISCOVERY Green Act — 16 MIN
HRP (fluorescent)	DISCOVERY DCC Kit	32 MIN
	DISCOVERY FAM Kit	20 MIN
	DISCOVERY FITC Kit	20 MIN
	DISCOVERY Rhodamine Kit	32 MIN
	DISCOVERY Rhodamine 6G Kit	32 MIN
	DISCOVERY Red 610 Kit	32 MIN
	DISCOVERY Cy5 Kit	40 MIN

*We recommend using the same IHC chromogen settings for Sequential ISH-IHC as previously optimized for IHC alone.
†Extending DISCOVERY Red incubation can result in a dot-like background
‡For stronger AP-based Red IHC detection, ChromoMap Red is recommended (select **DS Red**).

13. Select your preferred Counterstain and Post-Counterstain settings. A light counterstain is recommended for best visualization of multiplex chromogenic staining.
14. At the top of the Protocol Steps window, click **Save As**, then select a unique protocol number from the drop-down menu and choose a protocol name. Click **Active**, add relevant comments in the available field, and click **Save**.



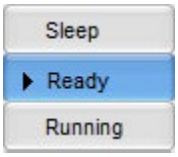
15. Make a new protocol for each probe/antibody/chromogen combination. Click **Save**.

Print the labels

1. Select the **Print Label** icon from the upper right corner of the home screen.
2. Select your preferred template or create a new template. To create a new template, refer to the *Ventana DISCOVERY ULTRA System User Manual* for details.
3. Click **Protocol**.
4. Select the protocols you created in the section above. Click the **Add** button. When the protocols for all slides have been assigned, click **Close/Print**.
5. Fill in the template for each slide. Click **Print** when completed

Load the reagents

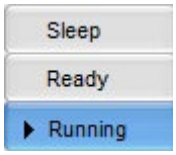
1. Remove the nozzle caps from the filled dispensers and place each cap on the post located on the back of the dispenser.
2. Prime the user-fillable dispensers. For guidance, refer to the instructions provided by Roche Tissue Diagnostics.
3. If needed, remove any air bubbles at the nozzle tip by pushing down on the nozzle until the liquid reaches the tip of the nozzle or forms a small meniscus at the tip of the nozzle.
4. Remove the yellow locking ring from the dispensers in all the prefilled dispensers. Refer to the instructions provided by Roche Tissue Diagnostics.
5. Load the dispensers onto the reagent racks.
6. Load the reagent racks onto the reagent carousel.
7. Select the **Ready** button.



8. Open the slide drawers.
9. Load each slide onto a heater pad with the label facing upward and inward. Ensure that the slides sit securely on the pads.

IMPORTANT! Prior to loading the slides, ensure heater pads are completely dry. Wipe off any liquid using laboratory tissue paper.

10. Close the slide drawers.
11. Select the **Running** button.



12. The assay duration varies based on assay selections, approximately **15 – 20 HRS**.

IMPORTANT! Before leaving the instrument unattended, ensure all reagents and slides are successfully registered and the instrument is running.

Complete the run

1. After the run is complete, remove the Dewax reagent, place nozzle cap on the dispenser, and store at room temperature.
2. For the remaining reagents, place nozzle caps back on the dispensers and place racks onto magnet locking tray.

IMPORTANT! Store reagent racks at **4°C** until next use. Store the Dewax dispenser at room temperature.

Wash and dry the slides

1. Prepare 200 mL of diluted detergent by adding 1–2 drops detergent to 200 mL distilled water in a container with a cap.
2. Mix well by inverting the container 4–5 times.
3. Add diluted detergent to a Tissue-Tek Staining Dish.

Note: Store diluted detergent at **RT**.

4. Submerge a Tissue-Tek Slide Rack into the Tissue-Tek Staining Dish containing 200 mL diluted detergent.
5. Open the instrument slide drawers and unload slides.
6. Decant solution on the slides into the slide drawer, then *immediately* load slides into the Tissue-Tek Slide Rack submerged in detergent.
7. Rinse oil off the slides by moving the slide rack up and down in the dish 10 times.
8. Replace the detergent with distilled water and rinse slides by moving the slide rack up and down a minimum of **10** times.
9. Repeat Step 8 three to five times.
10. Transfer the slides into a Tissue-Tek Staining Dish containing **200 mL** distilled water.
11. Place slides in a drying oven at **60°C** for at least **30 MIN.**

Mount the samples

1. In a fume hood, fill two clearing agent dishes with ~200 mL fresh xylene.
2. Once slides are dry, move the Tissue-Tek Slide rack into the staining dish containing xylene for **1 MIN** with occasional agitation.
3. Move the Tissue-Tek Slide rack into the staining dish containing xylene for **1 MIN** with occasional agitation.
4. Lay each slide flat with the sections facing up in the fume hood then add 1–2 drops of EcoMount or other chromogen-compatible xylene-based mounting medium. Carefully place a 24 mm x 50 mm coverslip over the section and avoid trapping air bubbles.
5. Air dry slides for at least **15 MIN** before evaluation.

IMPORTANT! mRNA Teal, mRNA Green, DISCOVERY Teal HRP and DISCOVERY Green HRP chromogens are light sensitive and can fade over time. For best results, protect stored slides from the light and image within one week of staining.

Chapter 3: Integrated RNA-Protein Co-Detection Workflow (ICW)

This workflow is designed to enable broader compatibility of RNA co-detection with protease-tolerant protein epitopes. Tissue samples undergo deparaffinization and RNAscope Target Retrieval, then primary antibody incubation and post-primary fixation for stabilization prior to protease digestion. After protease digestion, use ACD's probes and ISH detection reagents to detect RNA, followed by secondary antibody detection and counterstaining.

Materials

ACD reagents for Integrated RNA-Protein Co-Detection

Integrated RNA-Protein Co-Detection Workflow requires the use of the following reagents from ACD for use on Roche DISCOVERY ULTRA:

- RNAscope VS Universal Sample Prep Reagent Kit v2 (Cat. No. 323740)
- ACD Assay-specific Detection Reagent Kit:
  - RNAscope VS Universal AP Detection Reagents (Cat. No. 323260)
  - RNAscope VS Universal HRP Detection Reagents (Cat. No. 323210)
  - RNAscope VS Duplex Detection Reagents (Cat. No. 323310)
  - BaseScope VS Detection Reagents (Cat. No. 323710)
- VS RNA-Protein Co-Detection Ancillary Reagent Kit (Cat. No. 323760**)**

RNAscope VS Universal Sample Prep Reagent Kit v2 (Cat. No. 323740) and RNAscope VS Accessory Kit (Cat. No. 320630) are RTU and stored as indicated in the following tables:

RNAscope VS Sample Prep Reagent Kit v2 (Cat. No. 323740)			
☑	**Reagent**	**Quantity**	**Storage**
	RNAscope VS Universal Target Retrieval v2	10 mL x 2 bottle	Room Temp (15–30°C)
	RNAscope VS Universal Dewax	14 mL x 1 bottle	Room Temp (15–30°C)

Assay-specific Detection Reagents within each Reagent Kit are stored as indicated in the following tables:

RNAscope VS Universal AP Detection Reagents (Cat. No. 323260)			
☑	**Reagent**	**Quantity**	**Storage**
	RNAscope VS Universal AP AMP 1	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal AP AMP 2	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal AP AMP 3	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal AP AMP 4	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal AP AMP 5	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal AP AMP 6	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal AP AMP 7	14 mL x 1 bottle	2–8°C
	RNAscope VS Protease	14 mL x 1 bottle	2–8°C

RNAscope VS Universal HRP Detection Reagents (Cat. No. 323210)

☑	Reagent	Quantity	Storage
	RNAscope VS Universal HRP AMP 1	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal HRP AMP 2	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal HRP AMP 3	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal HRP AMP 4	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal HRP AMP 5	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal HRP AMP 6	14 mL x 1 bottle	2–8°C
	RNAscope VS Universal HRP AMP 7	14 mL x 1 bottle	2–8°C
	RNAscope VS Protease	14 mL x 1 bottle	2–8°C

RNAscope VS Duplex Detection Reagents (Cat. No. 323310)			
☑	Reagent	Quantity	Storage
	RNAscope VS Duplex AMP 1	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP 2	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP 3	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP 4	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP 5	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP 6	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP 7	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP 8	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP 9	14 mL x 1 bottle	2–8°C
	RNAscope VS Duplex AMP Wash	14 mL x 2 bottles	2–8°C
	RNAscope VS Protease	14 mL x 1 bottle	2–8°C

BaseScope VS Detection Reagents (Cat. No. 323710)			
☑	Reagent	Quantity	Storage
	BaseScope VS AMP 1	14 mL x 1 bottle	2–8°C
	BaseScope VS AMP 2	14 mL x 1 bottle	2–8°C
	BaseScope VS AMP 3	14 mL x 1 bottle	2–8°C
	BaseScope VS AMP 4	14 mL x 1 bottle	2–8°C
	BaseScope VS AMP 5	14 mL x 1 bottle	2–8°C
	BaseScope VS AMP 6	14 mL x 1 bottle	2–8°C
	BaseScope VS AMP 7	14 mL x 1 bottle	2–8°C
	BaseScope VS AMP 8	14 mL x 1 bottle	2–8°C
	RNAscope 2.5 VS Pretreat 3 - Protease	14 mL x 1 bottle	2–8°C

VS RNA-Protein Co-Detection Ancillary Kit - Cat No. 323760

☑	Reagent	Source / Ordering Info	Quantity	Storage
	Co-Detection Antibody Diluent*	ACD/Cat No. 323160	120 mL x 1 bottle	2–8°C
	VS RNA-Protein Co-Detection Protease and Inhibitor	ACD/Cat No. 323190	See the following	
	VS Co-Detection Protease		14 mL x 1 bottle	2–8°C
	VS Co-Detection Inhibitor		14 mL x 1 bottle	2–8°C

**Use of Co-Detection Antibody Diluent is recommended for best preservation of RNA and maintenance of ISH sensitivity when performing Integrated RNA-Protein Co-Detection*

Optional ACD reagents for Integrated RNA-Protein Co-Detection:

The following additional components from ACD are optional for the Integrated RNA-Protein Co-Detection Workflow on Roche DISCOVERY ULTRA:

- RNAscope VS Accessory Kit (Cat. No. 320630)

RNAscope VS Accessory Kit (Cat. No. 320630)			
☑	Reagent	Quantity	Storage
	RNAscope VS Hematoxylin	7 mL x 1 bottle	2–8°C
	RNAscope VS Bluing Reagent	7 mL x 1 bottle	2–8°C

Roche materials for Integrated RNA-Protein Co-Detection:

Integrated RNA-Protein Co-Detection may be performed with either Roche RTU primary antibody or your choice of primary antibody concentrate diluted in the ACD Co-Detection Antibody Diluent. For a list of available Roche RTU Primary Antibodies and ordering information, please contact your local Roche representative.

The following additional materials from Roche can be used for Integrated RNA-Protein Co-Detection on DISCOVERY ULTRA. Catalog numbers are valid in the United States only. For other regions, please check catalog or ordering numbers with your local lab supplier.

Reagent dispensers

Roche Materials for Integrated RNA-Protein Co-Detection				
☑	Component	Cat. No.	Ordering Code	Fill with:
	Probe Dispensers	960-761 to 960-780	Contact local Roche representative	RNAscope VS Probes
	mRNA Sample Prep Kit	760-248	08127166001	RNAscope VS Sample Prep Reagent Kit v2 and VS Protease reagent from Detection Kit
	Antibody Dispensers (*Optional**)	770-001 to 770-099	Contact local Roche representative	User-sourced Primary Antibody Concentrate diluted in Co-Detection Antibody Diluent
	Enzyme 1 Dispenser	771-721	05271517001	VS Co-Detection Protease
	Pretreatment 1 Dispenser	960-901	05280095001	VS Co-Detection Inhibitor
	Counterstain 1 dispenser	771-741	05271720001	VS Hematoxylin
	Counterstain 2 dispenser	771-742	05271738001	VS Bluing Reagent
	Roche RTU Counterstain	Various	Contact local Roche	Pre-filled

Roche Materials for Integrated RNA-Protein Co-Detection				
☑	Component	Cat. No.	Ordering Code	Fill with:
	Reagents		representative	
	DISCOVERY Antibody Block	760-4204	05268869001	Pre-filled
	Additional ISH Assay-Specific Materials	see the following tables		
	Additional IHC Detection Materials	see the following tables		

*Protein target qualification can be performed with either Roche RTU primary antibody or your choice of primary antibody.

Additional ISH assay-specific materials

Additional Roche Materials Required for VS Universal AP ISH				
☑	Component	Cat. No.	Ordering Code	Fill with:
	mRNA RED Probe Amplification Kit	760-236	07095341001	RNAscope VS Universal AP Detection Reagents AMP 1-7
	mRNA RED Detection Kit	760-234	07099037001	Pre-filled

Additional Roche Materials Required for VS Universal HRP ISH				
☑	Component	Cat. No.	Ordering Code	Fill with:
	mRNA Probe Amplification Kit	760-222	06614337001	RNAscope VS Universal HRP Detection Reagents AMP 1–7
	DISCOVERY Inhibitor*	760-4840	07017944001	Pre-filled
	mRNA Purple HRP Detection Kit*	760-255	08127166001	Pre-filled
	mRNA Green HRP Detection Kit†	760-278	08952612001	Pre-filled
	mRNA Teal HRP Detection Kit†	760-256	08352941001	Pre-filled
	mRNA DAB Detection Kit†	760-224	06614353001	Pre-filled
	DISCOVERY DCC Kit†	760-240	07988192001	Pre-filled
	DISCOVERY FAM Kit†	760-243	07988150001	Pre-filled
	DISCOVERY FITC Kit†	760-232	07259212001	Pre-filled
	DISCOVERY Rhodamine Kit†	760-233	07259883001	Pre-filled
	DISCOVERY Rhodamine 6G Kit†	760-244	07988168001	Pre-filled
	DISCOVERY Red 610 Kit†	760-245	07988176001	Pre-filled
	DISCOVERY Cy5 Kit†	760-238	07551215001	Pre-filled

* DISCOVERY Inhibitor is not required if using mRNA DAB Detection Kit
† Choose one mRNA Detection Kit for RNAscope HRP detection

Additional Roche Materials Required for VS Duplex ISH				
☑	Component	Cat. No.	Ordering Code	Fill with:
	mRNA Duplex Amp Kit	760-249	08127174001	RNAscope VS Duplex Detection Reagents AMP 1–9, AMP Wash
	mRNA Link (Pre-filled)	760-6014	08127115001	Pre-filled
	mRNA RED Detection Kit	760-234	07099037001	Pre-filled

Additional Roche Materials Required for VS Duplex ISH				
☑	Component	Cat. No.	Ordering Code	Fill with:
	mRNA Green HRP Detection Kit*	760-278	08952612001	Pre-filled
	mRNA Teal HRP Detection Kit*	760-256	08352941001	Pre-filled
	mRNA DAB Detection Kit*	760-224	06614353001	Pre-filled

* Choose one mRNA Detection Kit for VS Duplex Channel 1 detection

Additional Roche Materials Required for VS BaseScope ISH				
☑	Component	Cat. No.	Ordering Code	Storage
	mRNA RED Probe Amplification Kit	760-236	07095341001	BaseScope VS Detection Reagents AMP 1–7
	Option 8 dispenser	771-758	05271916001	BaseScope VS AMP 8 reagent
	mRNA RED Detection Kit	760-234	07099037001	Pre-filled

Additional IHC assay-specific materials

Reagent Options for Ventana IHC AP Detection				
☑	Component	Cat. No.	Ordering Code	Storage
	DISCOVERY UltraMap anti-Ms Alk Phos*	760-4312	05269687001	2–8°C
	DISCOVERY UltraMap anti-Rb Alk Phos*	760-4314	05269709001	2–8°C
	DISCOVERY Yellow Kit†	760-239	07698445001	2–8°C
	DISCOVERY Red Kit	760-228	07425333001	2–8°C
	DISCOVERY ChromoMap Red Kit*	760-160	05266653001	2–8°C

Reagent Options for Ventana IHC HRP Detection				
☑	Product	Cat. No.	Ordering Code	Storage
	DISCOVERY UltraMap anti-Ms HRP*	760-4313	05269695001	2–8°C
	DISCOVERY UltraMap anti-Rb HRP*	760-4315	05269717001	2–8°C
	DISCOVERY UltraMap anti-Rat HRP*	760-4456	05891884001	2–8°C
	DISCOVERY UltraMap anti-Gt HRP*	760-4648	06607241001	2–8°C
	DISCOVERY Purple Kit*	760-229	07053983001	2–8°C
	DISCOVERY Green HRP Kit †	760-278	07053983001	2–8°C
	DISCOVERY Teal HRP Kit †	760-247	08254338001	2–8°C
	DISCOVERY ChromoMap DAB Kit †	760-159	05266645001	2–8°C
	DISCOVERY DCC Kit †	760-240	07988192001	2–8°C
	DISCOVERY FAM Kit †	760-243	07988150001	2–8°C
	DISCOVERY FITC Kit†	760-232	07259212001	2–8°C
	DISCOVERY Rhodamine Kit †	760-233	07259883001	2–8°C
	DISCOVERY Rhodamine 6G Kit †	760-244	07988168001	2–8°C
	DISCOVERY Red 610 Kit†	760-245	07988176001	2–8°C
	DISCOVERY Cy5 Kit †	760-238	07551215001	2–8°C

* Choose one secondary detection antibody depending on the primary antibody species and desired IHC chromogen
† Choose one IHC chromogen

Instrument buffers

☑	Component	Cat. No.	Ordering Code	Storage
	10X DISCOVERY Wash (RUO)	950-510	7311079001	Room Temp (15–30°C)
	ULTRA LCS (Predilute)	650-210	5424534001	Room Temp (15–30°C)
	SSC Buffer (10X)	950-110	5353947001	Room Temp (15–30°C)
	Reaction Buffer (10X)	760-107	5266262001	Room Temp (15–30°C)
	DISCOVERY CC1	950-500	6414575001	Room Temp (15–30°C)

User-Sourced Materials

☑	Description	Supplier	Cat. No.
	10% Neutral Buffered Formalin	SigmaAldrich	HT5011
	Primary Antibody (RTU)	User	Various
	Primary Antibody Concentrate	User	Various
	SuperFrost Plus Slides (required)	Fisher Scientific	12-550-15

☑	Description	Supplier	Cat. No.
	ProLong Gold Antifade Reagent	Life Technologies	P36930
	EcoMount mounting medium	Biocare	EM897L
	Tissue-Tek Vertical 24 Slide Rack	American Master Tech Scientific/MLS	LWSRA24
	Tissue-Tek Staining Dishes	American Master Tech Scientific/MLS	LWT4457EA
	Cover Glass 24 x 50 mm	Fisher Scientific/MLS	12-545-F
	Distilled water	MLS	—
	Mild liquid dishwashing detergent (Dawn detergent or similar)	MLS	—
	Drying oven, capable of holding temperature at 60 +/– 1°C	MLS	—
	Fume hood	MLS	—
	100% ethanol (EtOH)	MLS	—
	Xylene	MLS	—
	Tissue-Tek Clearing Agent Dishes, xylene-resistant	American Master Tech Scientific/MLS	LWT4456EA
	Optional: Glass beaker (1 or 2 L)	MLS	—
	Optional: Hot plate	Fisher Scientific/MLS	11-300-49SHP

Assay Procedure

Prepare the DISCOVERY ULTRA

Prepare the instrument

If the instrument has not been used for > 1 week, follow guidelines for instrument maintenance from Roche Tissue Diagnostics. Before use, empty the waste carboys if needed.

Dilute instrument bulk reagents

1. Prepare the instrument bulk fluids according to the manufacturer's instructions.
2. Fill bulk solution containers for 1X DISCOVERY Wash, ULTRA LCS (Predilute), and CC1 (Predilute) to be at least half full. Fully fill bulk solution containers for 2X SSC and 1X Reaction Buffer.

IMPORTANT! Do not use expired reagents.

Register new reagents

Reagent dispensers come with appropriate barcode labels and registration buttons for dispensing RNAscope VS Universal Reagents. Refer to the *Ventana DISCOVERY ULTRA System User Manual* for

details. To register reagents:

- Log all ACD reagents and probes into the software as **log user-fillable reagents** and **log user-fillable probes**, respectively.
- Use the reagent registration wand that comes with the instrument to register new reagent kits from Roche Tissue Diagnostics

Prepare user-fillable reagents for RNA-Protein Co-Detection

Refer to the table on pages 6–7 to determine the proper dispenser for each reagent.

IMPORTANT! Avoid cross contamination between reagents. Dewax must be warmed to room temperature and be completely in solution before use.

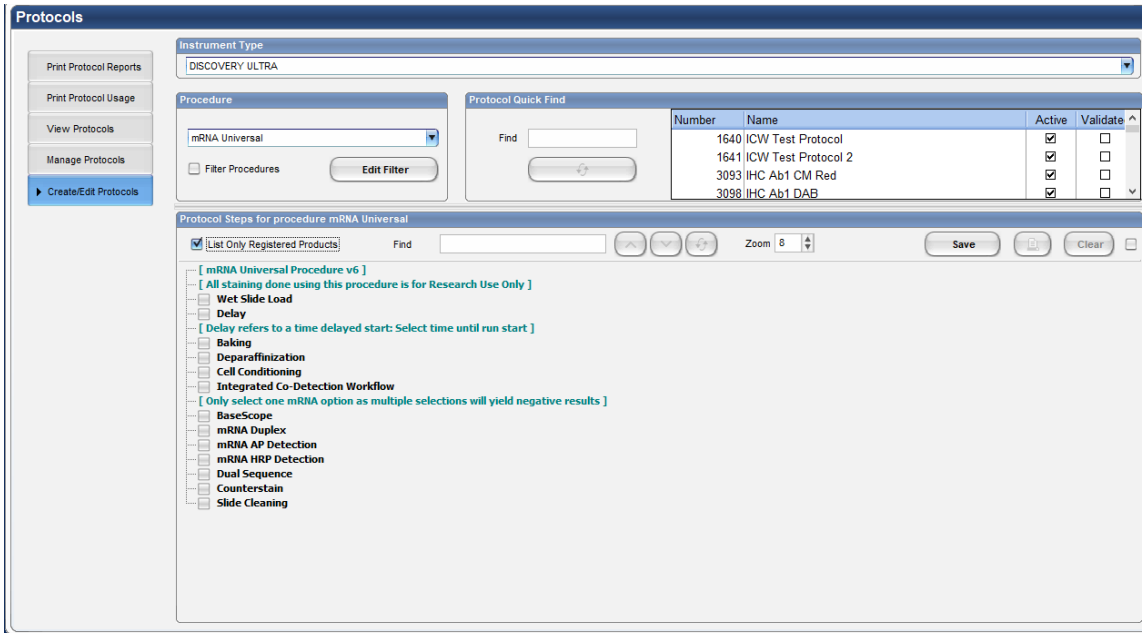
1. Transfer the entire volume of each AMP component of the Detection Kit to the corresponding labeled dispenser from the appropriate mRNA Amplification kit (see pages 6-7 for details)
2. Fill the mRNA Sample Prep Kit:
   a. Transfer the contents of both bottles of VS Target Retrieval v2 from the RNAscope VS Sample Prep Reagent Kit v2 to the mRNA Target Retrieval Dispenser.
   b. Transfer the VS Dewax reagent from the RNAscope VS Sample Prep Reagent Kit v2 to the mRNA Dewax Dispenser.
   c. Transfer the VS Protease from the Detection Kit to the mRNA Protease Dispenser
3. Fill the User Fillable Dispensers:
   a. Transfer the RNAscope 2.5 or BaseScope VS Target Probe and control probes to the correspondingly Probe dispensers.
   b. Transfer the VS Co-Detection Protease to the Enzyme 1 dispenser.
   c. Transfer the VS Co-Detection Inhibitor to the Pretreatment 1 dispenser.
   d. Transfer the VS Hematoxylin and VS Bluing to the Counterstain 1 and Counterstain 2 dispensers.
   e. In a fume hood, carefully transfer 10% Neutral Buffered Formalin (NBF) to the Fixative 1 dispenser. For best results, use freshly aliquoted 10% NBF for each run.

IMPORTANT! Keep the Fixative 1 dispenser filled with 10% NBF capped when not on the instrument.

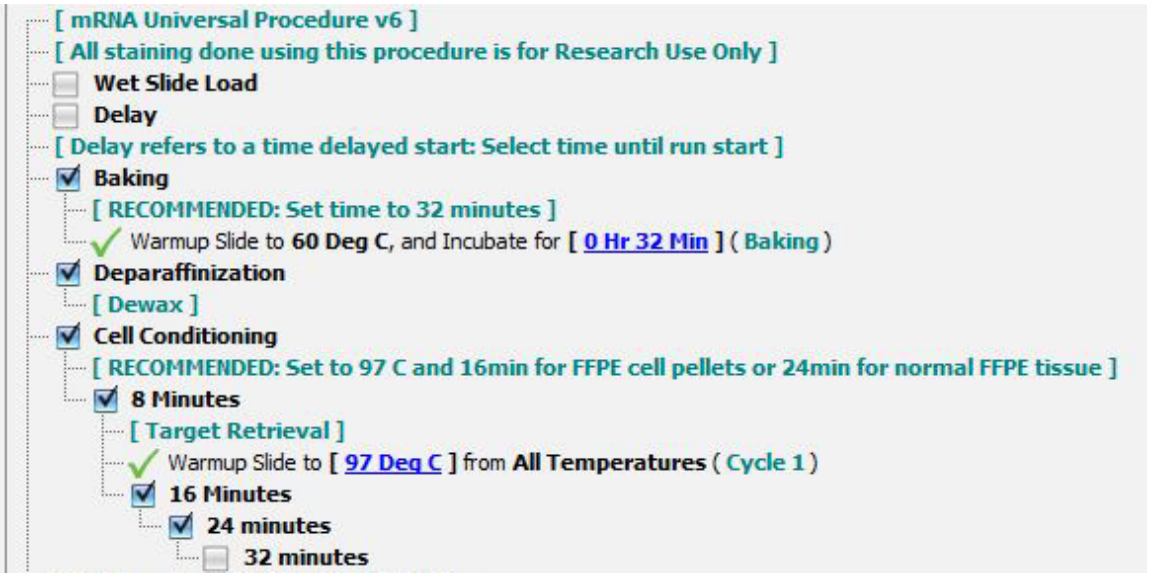
4. Follow the dispenser product insert instructions to properly prime and handle the dispensers.
5. Store tightly capped dispensers (except the Dewax dispenser) at **4°C** when not in use.
6. Store tightly capped mRNA Dewax dispenser at room temperature when not in use.

Create an instrument protocol

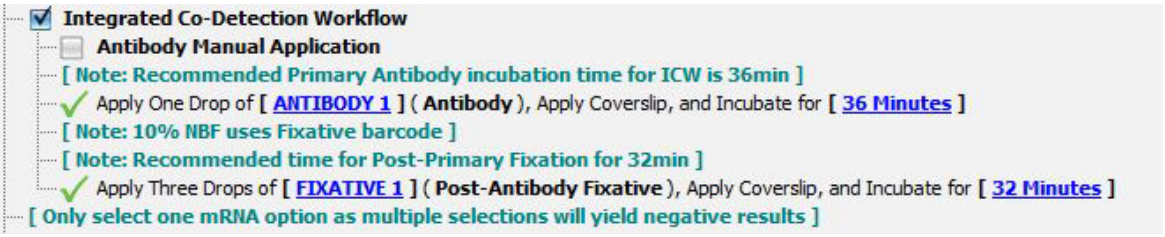
1. Open the NexES software and click the **Protocol** button.
2. Click **Create/Edit Protocols**, go to the Procedure drop-down menu and select **mRNA Universal.** Main protocol selections appear as shown:



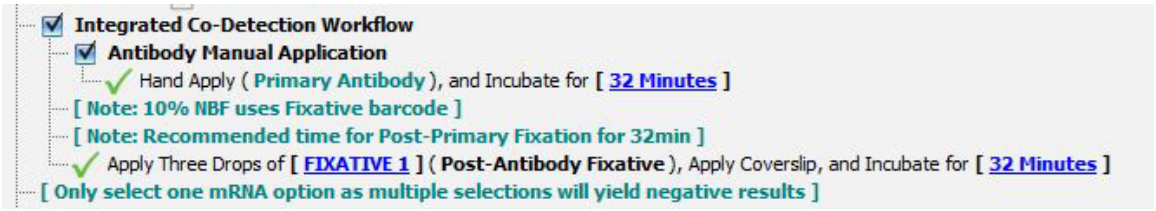
3. Select the appropriate pretreatment conditions as shown in the following screenshot. After the main step selections, drop-down menus become available for further selection. For Cell Conditioning, we recommend starting with the same conditions previously used on your tissue for ISH-only staining.



4. To enable Co-Detection, select **Integrated Co-Detection Workflow**. Select Antibody barcode for primary antibody and Fixative 1 barcode for 10% NBF. Recommended incubation times for ICW Primary Antibody and post-primary fixation incubation is **36 MIN** and **32 MIN**, respectively.



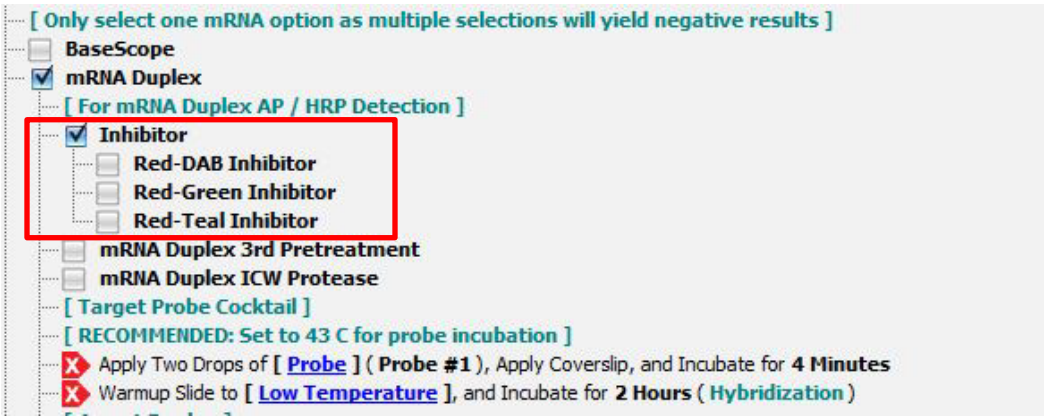
Note: To preserve RNA quality during primary antibody incubation, we recommend using primary antibody concentrate diluted in Co-Detection Antibody Diluent and that the primary antibody incubation occurs at room temperature. You may need to titrate the primary antibody concentration to strengthen protein detection. If titration by manual antibody application is desired instead of by automated dispenser, select **Antibody Manual Application**.



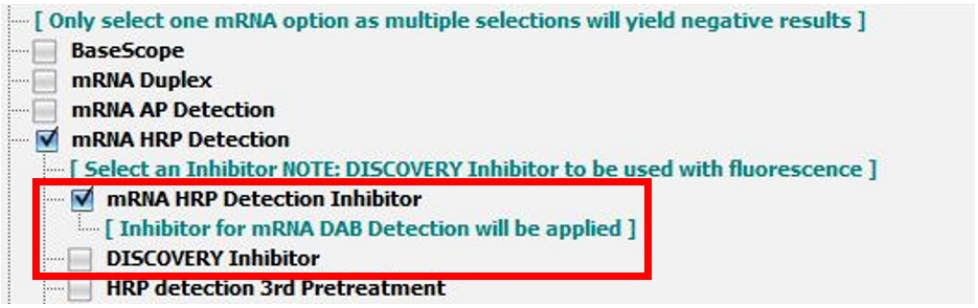
5. Select one mRNA Detection assay of interest.
   - For BaseScope VS, select **BaseScope**.
   - For RNAscope VS Duplex, select **mRNA Duplex**.
   - For RNAscope VS Universal AP, select **mRNA AP Detection**.
   - For RNAscope VS Universal HRP, select **mRNA HRP Detection**.

Select only one mRNA option per protocol. Choosing multiple selections yields negative results.

6. Select the appropriate Detection Inhibitor:
   - For **BaseScope** or **mRNA AP Detection**, mRNA Red Inhibitor reagent is applied automatically.
   - For **mRNA Duplex**, select the **Inhibitor** check box to display the available selections. Choose the appropriate inhibitor selection based on the chromogen combination in use for Duplex ISH detection, **Red-DAB Inhibitor**, **Red-Green Inhibitor**, or **Red-Teal Inhibitor**.



- For **mRNA HRP Detection,** select mRNA HRP Detection Inhibitor if using the mRNA DAB Detection Kit. Select DISCOVERY Inhibitor if using an alternative HRP-based chromogen such as the mRNA Purple Detection Kit, mRNA Green Detection Kit, or mRNA Teal Detection Kit.

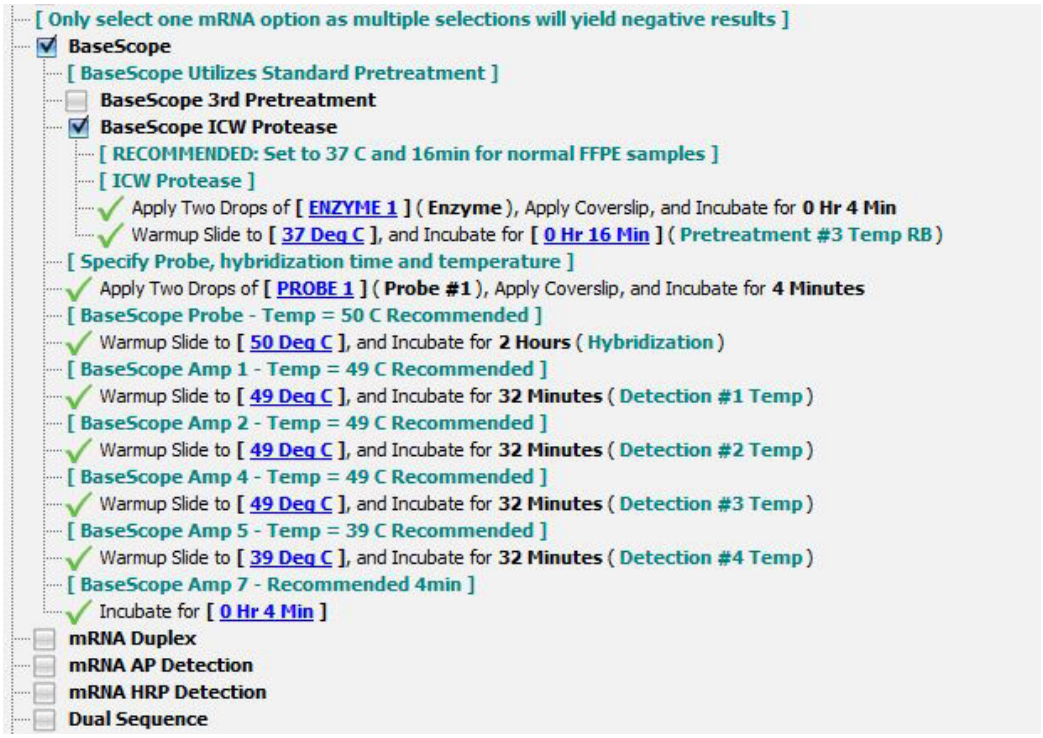


7. Under the mRNA assay of interest, select the corresponding **ICW Protease** check box**.** Do not select the detection 3rd Pretreatment option.
8. Under **ICW Protease** select the **Enzyme 1** barcode to correspond to the open dispenser filled with VS Co-Detection Protease reagent. Recommended incubation is **37°C** for **16 MIN**.

IMPORTANT! Incubating VS Co-Detection Protease above 37°C is not recommended and may negatively impact protein detection.

IMPORTANT! For the ICW protocol to start, the complete mRNA Sample Prep dispenser set including the mRNA Protease dispenser must be present on the reagent carousel even if the **3rd Pretreatment** selection is not checked. We do not recommend filling the mRNA Protease dispenser with VS Co-Detection Protease reagent as the instrument cannot distinguish it from RNAscope VS Protease reagent and this could lead to misapplication of the incorrect protease reagent when using other protocols. Instead, use the **ICW Protease** selection and Enzyme 1 dispenser as described above.

9. Select the ISH staining conditions:
   - If running **BaseScope**, select the following ISH staining conditions:



Standard Temperatures/Times for BaseScope VS	
ICW Protease temperature and time	37°C, 16 MIN
Standard BaseScope probe temperature	50°C
Standard BaseScope AMP 1 temperature	49°C
Standard BaseScope AMP 2 temperature	49°C
Standard BaseScope AMP 4 temperature	49°C
Standard BaseScope AMP 5 temperature	39°C
Standard BaseScope AMP 7 incubation time*	4 MIN
mRNA Red Chromogen	Default†

* BaseScope Amp 7 incubation time is determined by instrument calibration. Use the instrument setting previously optimized for the mRNA Universal software. For assistance, consult your local ACD FAS for more information.

†For all BaseScope assays, mRNA Red Detection is applied automatically. No chromogen selections are needed.

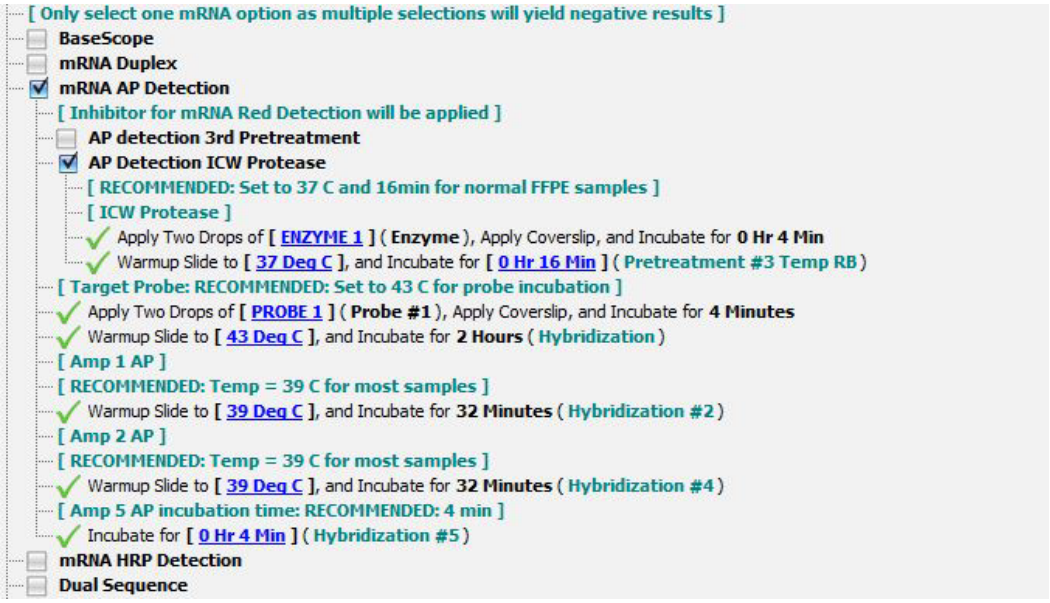
- If running **mRNA Duplex**, select the following ISH staining conditions. In this example, selections are shown for a Red-Green chromogen combination:

Standard Temperatures/Times for mRNA Duplex Detection	
ICW Protease temperature and time	37°C, 16 MIN
Suggested probe temperature	43°C
Suggested RNAscope VS Duplex AMP 1 & AMP 2 temperature	39°C
RNAscope VS Duplex AMP 5 incubation time	4 MIN*
RNAscope VS Duplex AMP 8 incubation time	4 MIN*
Chromogen settings for Red-DAB AP-HRP Duplex†	Preset, no incubation selections
Chromogen settings for Red-Green AP-HRP Duplex†	mRNA Green H_2O_2 — 20 MIN mRNA Green Act — 20 MIN
Chromogen settings for Red-Teal AP-HRP Duplex†	mRNA Teal H_2O_2 — 20 MIN mRNA Teal Act — 20 MIN

* VS Duplex Amp 5 and Amp8 incubation times are determined by instrument calibration. Use the instrument setting previously optimized for the mRNA Universal software. For assistance, consult your local ACD FAS for more information.

†Choose one detection combination for mRNA Duplex. All times/temperatures are preset for Red-DAB Detection.

- If choosing **mRNA AP Detection**, select the following ISH staining conditions:

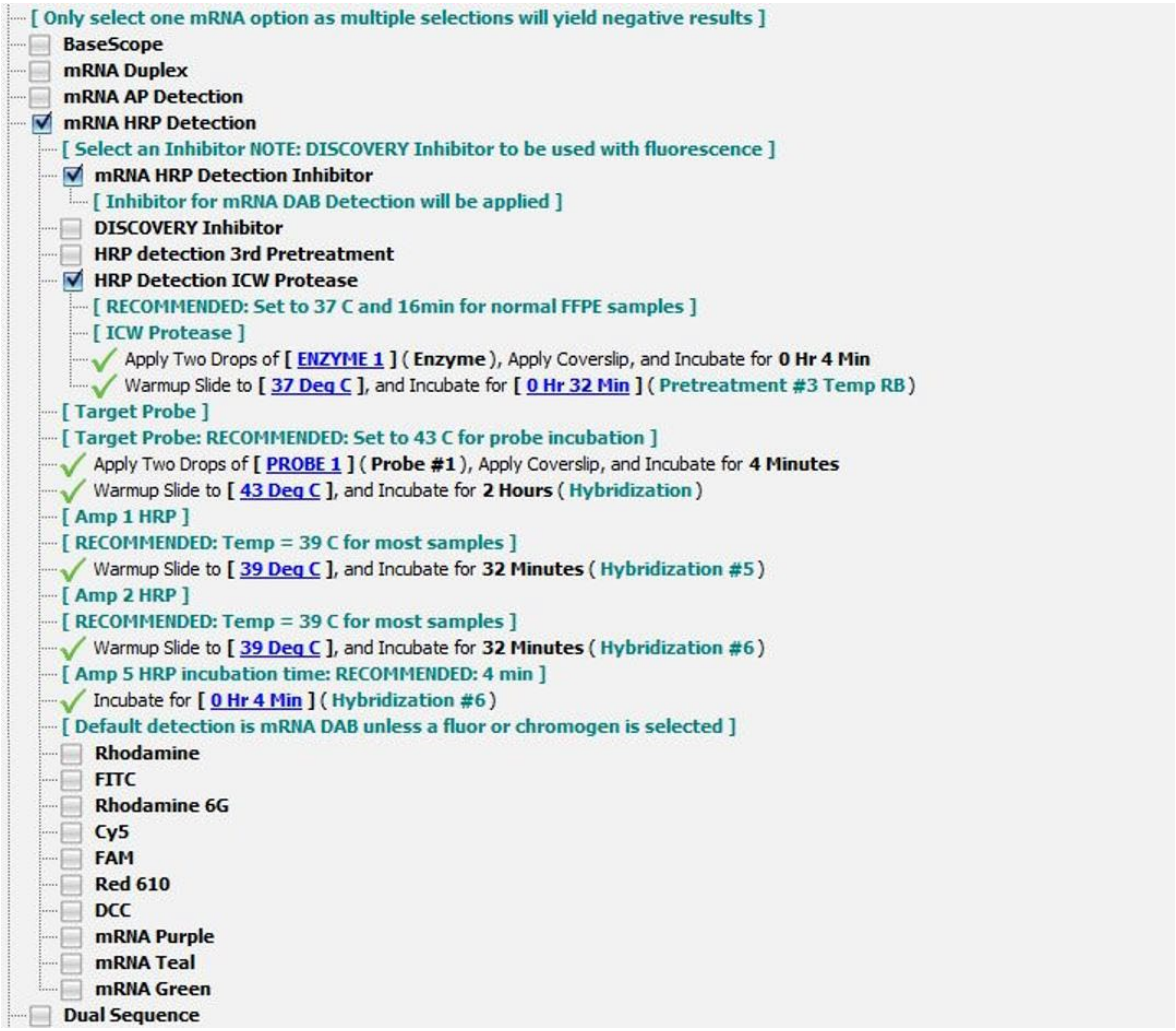


Standard Temperatures/Times for mRNA AP Detection	
ICW Protease temperature and time	37°C, 16 MIN
Suggested probe temperature	43°C
Suggested RNAscope AP AMP 1 & AMP 2 temperature	39°C
RNAscope AP AMP 5 incubation time	4 MIN*
Chromogen settings for mRNA Red Detection	Default†

* VS Universal AP Amp 5 incubation time is determined by instrument calibration. Use the instrument setting previously optimized for the mRNA Universal software. For assistance, consult your local ACD FAS for more information.

†For all mRNA AP, mRNA Red Detection is applied automatically. No chromogen selections are needed.

- If choosing **mRNA HRP Detection**, select the following ISH staining conditions:

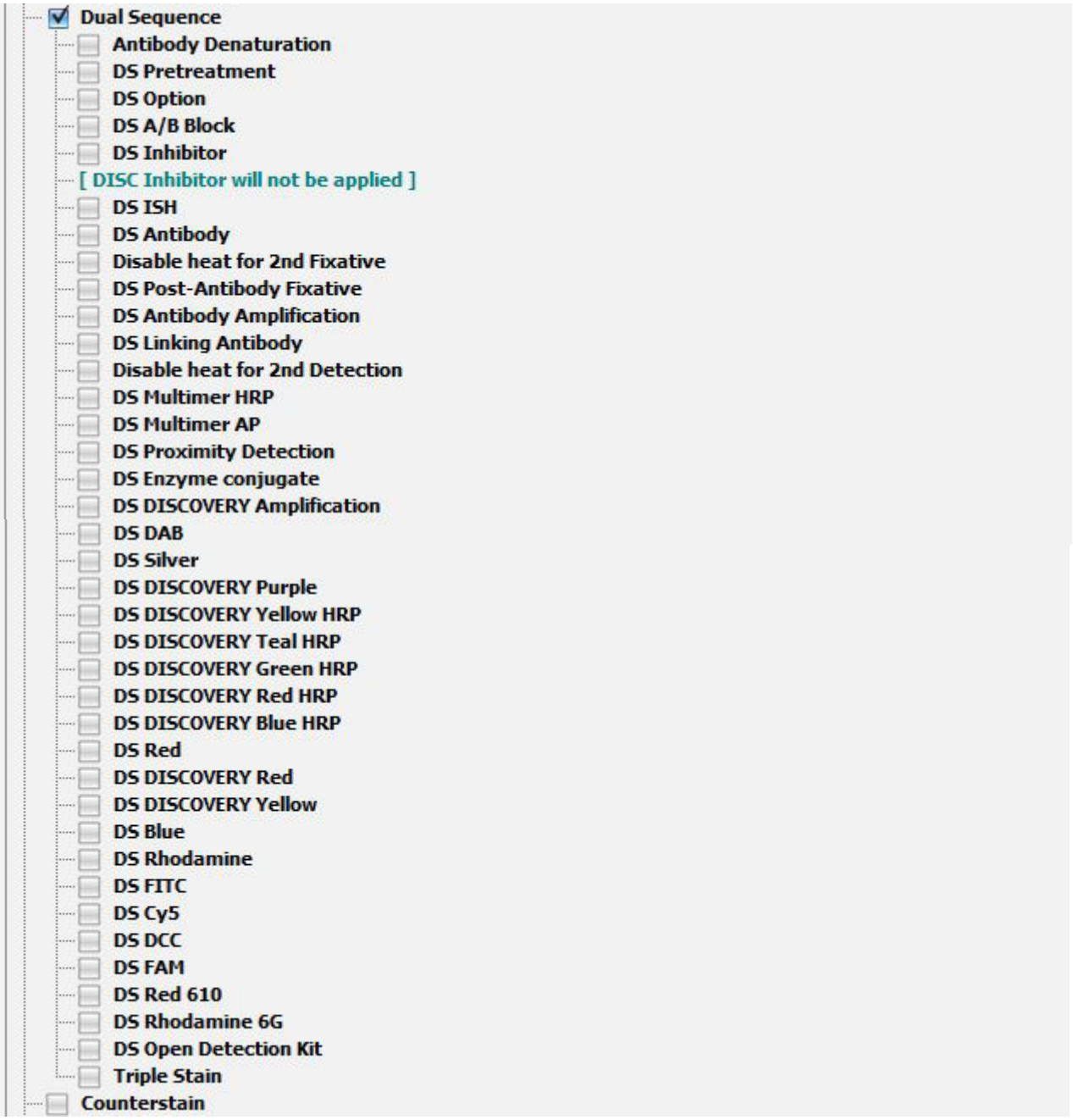


Standard Temperatures/Times for mRNA HRP Detection	
ICW Protease temperature and time	37°C, 16 MIN
Suggested probe temperature	43°C
Suggested RNAscope HRP AMP 1 & AMP 2 temperature	39°C
RNAscope HRP AMP 5 incubation time	4 MIN*
Chromogen settings for mRNA DAB†	Default*
Chromogen settings for mRNA Purple	mRNA Purple H_2O_2 — 40 MIN
Chromogen settings for mRNA Teal	mRNA Teal H_2O_2 — 20 MIN mRNA Teal Act — 20 MIN
Chromogen settings for mRNA Green	mRNA Green H_2O_2 — 20 MIN mRNA Green Act — 20 MIN

* VS Universal HRP Amp 5 incubation time is determined by instrument calibration. Use the instrument setting previously optimized for the mRNA Universal software. For assistance, consult your local ACD FAS for more information.

†For mRNA HRP, mRNA DAB Detection is applied by default if no other chromogens are selected.

10. After completing ISH assay selections, select **Dual Sequence** to enable selections for secondary antibody detection.

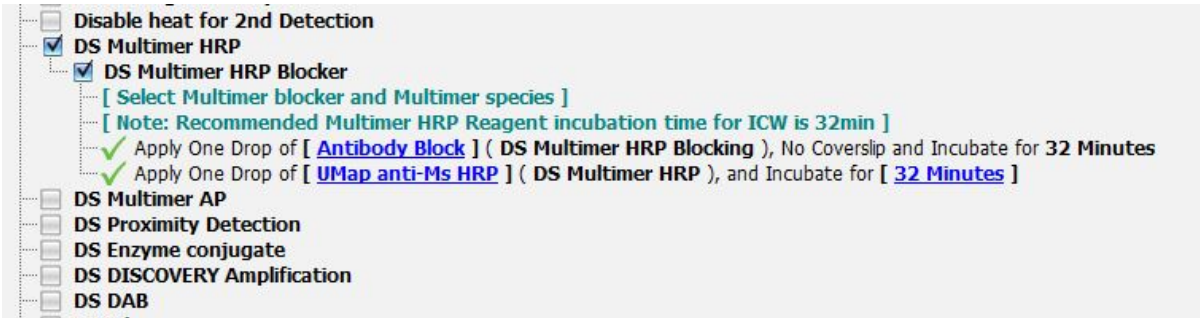


To apply VS Co-Detection Inhibitor, select **DS Pretreatment**, **DS 2nd Pretreatment**, and **Use DW for DS 2nd Pretreatment**. Select the Pretreatment 1 barcode to correspond to the VS Co-Detection Inhibitor reagent. Recommended incubation is **40°C** for **32 MIN** prior to protein detection.

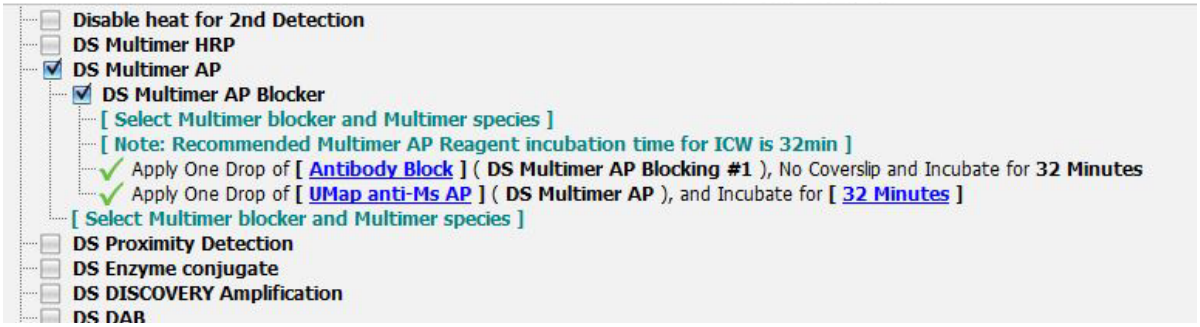
11. Select the settings for secondary detection:

Note: We recommend using Roche's Antibody Block reagent directly prior to secondary application, to prevent non-specific cross-detection of RNAscope. RNA-Protein Co-Detection can be performed without Antibody Block, but this could result in a hue shift of the RNA dots and interfere with interpretation of RNA-protein colocalization.

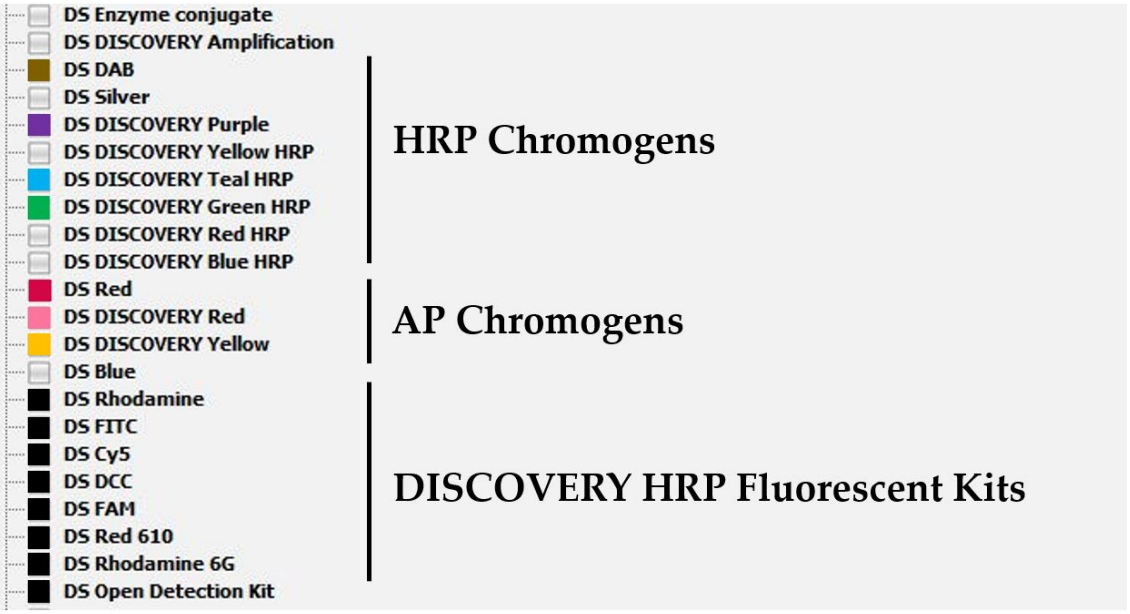
- For **HRP-based secondary detection**, choose a Roche Multimer HRP reagent corresponding to the primary host species. To enable secondary incubation, select **DS Multimer HRP**. To apply Antibody Block, select **DS Multimer HRP Blocker**, then select Antibody Block reagent. Select your Multimer HRP reagent of choice and desired incubation time, using previously optimized IHC-only conditions.



- For AP-based secondary detection, choose a Roche Multimer AP reagent corresponding to the primary host species. To enable secondary incubation, select **DS Multimer AP**. To apply Antibody Block, select **DS Multimer AP Blocker**, then select Antibody Block reagent. Select your Multimer AP reagent of choice and desired incubation time, using previously optimized IHC-only conditions.



12. Select chromogens for IHC detection using the following recommendations:



IHC Chromogen Selection and Settings		
IHC Enzyme	**IHC Chromogen**	**Recommended Chromogen Settings***
AP	DISCOVERY Yellow	44 MIN – 2 HRS
	DISCOVERY Red	12 MIN†
	DS Red / ChromoMap Red‡	Default
HRP (chromogenic)	DS DAB / ChromoMap DAB	Default
	DISCOVERY Purple	40 MIN
	DISCOVERY Teal HRP	DISCOVERY Teal H_2O_2 — 16–32 MIN DISCOVERY Teal Act – 16 MIN
	DISCOVERY Green HRP	DISCOVERY Green H_2O_2 — 16–32 MIN DISCOVERY Green Act — 16 MIN
HRP (fluorescent)	DISCOVERY DCC Kit	32 MIN
	DISCOVERY FAM Kit	20 MIN
	DISCOVERY FITC Kit	20 MIN
	DISCOVERY Rhodamine Kit	32 MIN
	DISCOVERY Rhodamine 6G Kit	32 MIN
	DISCOVERY Red 610 Kit	32 MIN
	DISCOVERY Cy5 Kit	40 MIN

*Protein detection with ICW can benefit from longer chromogen incubation or selection of a stronger chromogen relative to IHC alone

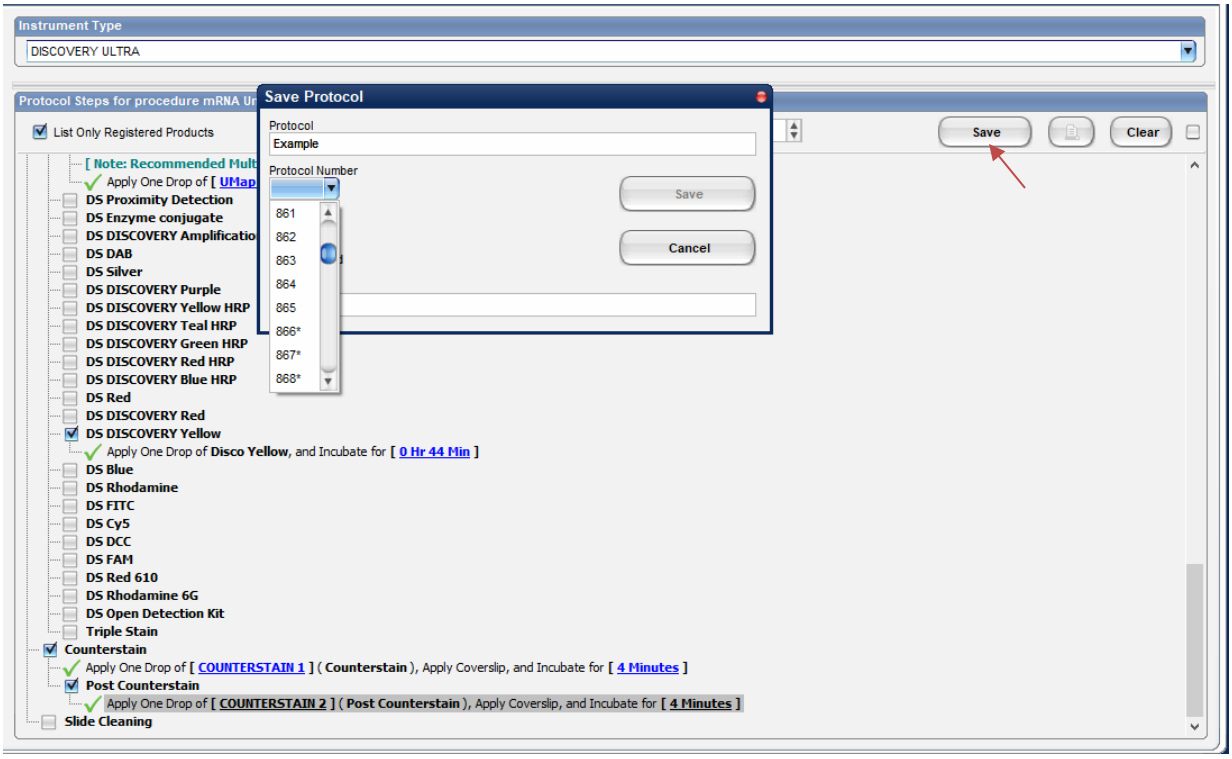
†Extending DISCOVERY Red incubation can result in a dot-like background

‡For stronger AP-based Red IHC detection, ChromoMap Red is recommended (select **DS Red**).

13. Select your preferred Counterstain and Post-Counterstain settings.

Note: Hematoxylin staining can appear darker with ICW than with ISH-alone. Try a lighter counterstain if needed for optimal multiplex chromogenic co-detection.

14. At the top of the Protocol Steps window, click **Save As**, then select a unique protocol number from the drop-down menu and choose a protocol name. Click **Active**, add relevant comments in the available field, and click **Save**.



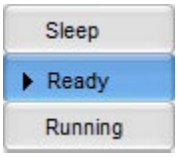
15. Make a new protocol for each probe/antibody/chromogen combination. Save as a unique protocol number.

Print the labels

1. Select the **Print Label** icon from the upper right corner of the home screen.
2. Select your preferred template or create a new template. To create a new template, refer to the *Ventana DISCOVERY ULTRA System User Manual* for details.
3. Click **Protocol**.
4. Select the protocols you created in the section above. Click the **Add** button. When the protocols for all slides have been assigned, click **Close/Print**.
5. Fill in the template for each slide. Click **Print** when completed

Load the Reagents

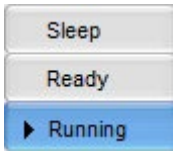
1. Remove the nozzle caps from the filled dispensers and place each cap on the post located on the back of the dispenser.
2. Prime the user-fillable dispensers. For guidance, refer to the instructions provided by Roche Tissue Diagnostics.
3. If needed, remove any air bubbles at the nozzle tip by pushing down on the nozzle until the liquid reaches the tip of the nozzle or forms a small meniscus at the tip of the nozzle.
4. Remove the yellow locking ring from the dispensers in all the prefilled dispensers. Refer to the instructions provided by Roche Tissue Diagnostics.
5. Load the dispensers onto the reagent racks.
6. Load the reagent racks onto the reagent carousel.
7. Select the **Ready** button.



8. Open the slide drawers.
9. Load each slide onto a heater pad with the label facing upward and inward. Ensure that the slides sit securely on the pads.

IMPORTANT! Prior to loading the slides, ensure heater pads are completely dry. Wipe off any liquid using laboratory tissue paper.

10. Close the slide drawers.
11. Select the **Running** button.



12. The assay duration varies based on assay selections, approximately **15 – 20 HRS**.

IMPORTANT! Before leaving the instrument unattended, ensure all reagents and slides are successfully registered and the instrument is running.

Complete the run

1. After the run is complete, remove the Dewax reagent, place nozzle cap on the dispenser, and store at room temperature.
2. For the remaining reagents, place nozzle caps back on the dispensers and place racks onto magnet locking tray.

IMPORTANT! Store reagent racks at **4°C** until next use. Store the Dewax dispenser at room temperature.

Wash and dry the slides

1. Prepare 200 mL of diluted detergent by adding 1–2 drops detergent to 200 mL distilled water in a container with a cap.
2. Mix well by inverting the container 4–5 times.
3. Add diluted detergent to a Tissue-Tek Staining Dish.

Note: Store diluted detergent at **RT**.

4. Submerge a Tissue-Tek Slide Rack into the Tissue-Tek Staining Dish containing 200 mL diluted detergent.
5. Open the instrument slide drawers and unload slides.
6. Decant solution on the slides into the slide drawer, then *immediately* load slides into the Tissue-Tek Slide Rack submerged in detergent.
7. Rinse oil off the slides by moving the slide rack up and down in the dish 10 times.
8. Replace the detergent with distilled water and rinse slides by moving the slide rack up and down a minimum of **10** times.
9. Repeat Step 8 three to five times.
10. Transfer the slides into a Tissue-Tek Staining Dish containing **200 mL** distilled water.
11. Place slides in a drying oven at **60°C** for at least **30 MIN.**

Mount the samples

1. In a fume hood, fill two clearing agent dishes with ~200 mL fresh xylene.
2. Once slides are dry, move the Tissue-Tek Slide rack into the staining dish containing xylene for **1 MIN** with occasional agitation.
3. Move the Tissue-Tek Slide rack into the staining dish containing xylene for **1 MIN** with occasional agitation.
4. Lay each slide flat with the sections facing up in the fume hood then add 1–2 drops of EcoMount or other chromogen-compatible xylene-based mounting medium. Carefully place a 24 mm x 50 mm coverslip over the section and avoid trapping air bubbles.
5. Air dry slides for at least **15 MIN** before evaluation.

IMPORTANT! mRNA Teal, mRNA Green, DISCOVERY Teal HRP and DISCOVERY Green HRP chromogens are light sensitive and can fade over time. For best results, protect stored slides from the light and image within one week of staining.

Appendix A. Sequential ISH-IHC Staining Optimization and Troubleshooting

Prior to using the sequential workflow for RNA-Protein Co-Detection, we recommend establishing working protocols for both ISH-only and IHC-only staining in the mRNA Universal procedure, which uses a different uses a different deparaffinization and cell conditioning method than the RUO Universal procedure. Perform protein target qualification to confirm that your protein epitope target is protease-tolerant by following the protocol setup outlined in **Chapter 1. Protein Target Qualification**. Many epitopes are sensitive to RNAscope protease and are not compatible with sequential staining. If your epitope is protease-sensitive, refer to **Chapter 3. Integrated Co-Detection Workflow**.

For Sequential Co-Detection, begin by applying the same baking, deparaffinization, cell conditioning, and protease conditions as the ISH-only protocol. Apply the same primary and secondary antibody, and IHC chromogen conditions as the IHC-only protocol. IHC optimization varies by tissue and primary antibody clone. If needed, the following parameters can be adjusted:

Reagent	User Selection	Recommended Incubation Temperature	Recommended Incubation Time	Optimization Range
Target Retrieval	Cell Conditioning	97°C	24 MIN	85–97°C 8-56 MIN
RNAscope VS Protease	3rd Pretreatment	37°C	16 MIN	37–50°C 8–32 MIN
Primary Antibody	DS Antibody	37°C	32 MIN	Range available by user selection: DS Antibody Default: • 35–42°C, 16–60 MIN DS Disable Heat: • Room Temperature, 4m–24h DS High Temp Ab Incubation: • 60–75°C, 4–60 MIN DS Extended Antibody Incubation: • 4–120 MIN
Manually Applied Primary Antibody	DS Antibody – DS Antibody Manual Application	37°C	32 MIN	Range available by user selection: Default: • 35–42°C, 16–60 MIN DS Disable Heat: • Room Temperature, 4m–24h DS High Temp Ab Incubation: • 60–75°C, 4–60 MIN
Secondary Antibody / Multimer*	DS Multimer AP -or- DS Multimer HRP	Not Selectable	32 MIN	4–32 MIN
IHC Chromogen		See recommendations on page 39		

* For stronger detection, try an alternative Roche secondary system.

Appendix B. ICW Staining Optimization and Troubleshooting

For optimal RNA and protein detection, we recommend establishing ISH-only and IHC-only protocols before proceeding to RNA-Protein Co-Detection. Begin by applying the same baking, deparaffinization, and cell conditioning conditions as the ISH-only protocol. Since a stronger protease treatment is required with Co-Detection than with ISH alone, we recommend using VS ICW Protease in most cases.

To optimize protein detection, titrate the antibody concentration for the co-detection workflow. You may need to use a higher primary antibody concentration for the co-detection workflow than you would normally use for IHC alone. For best preservation of RNA in the Integrated Co-Detection Workflow, we recommend diluting primary antibody concentrate in the ACD VS Co-Detection Antibody diluent. If further optimization is needed for a specific sample or target of interest, the following parameters can be adjusted:

Reagent	User Selection	Recommended Incubation Temperature	Recommended Incubation Time	Optimization Range
Target Retrieval	Cell Conditioning	97°C	24 MIN	85-97°C 8-56 MIN
Primary Antibody	Integrated Co-Detection Workflow	Not Selectable	36 MIN	16-60 MIN
Primary Antibody (Manual Application)	Integrated Co-Detection Workflow / Manual Antibody Application	Not Selectable	32 MIN	16-60 MIN
10% NBF	Integrated Co-Detection Workflow	Not Selectable	32 MIN	16-60 MIN
VS Co-Detection Protease	ICW Protease	37°C	16 MIN	37°C* 8-32 MIN
Secondary Antibody / Multimer**	DS Multimer AP -or- DS Multimer HRP	Not Selectable	32 MIN	4–32 MIN
IHC Chromogen		See recommendations on page 62		

*For best ISH sensitivity with ICW, do not increase VS Co-Detection Protease beyond 37°C

** For stronger detection, try an alternative Roche secondary system.

† Some samples, such as cell pellets, can require reduced protease treatment. For these samples, use RNAscope VS Protease by selecting **3rd Pretreatment** rather than **ICW Protease**. If a sample requires stronger protease treatment than the maximum 32 minutes of VS Co-Detection Protease, we recommend two protease reagent applications. Select both **3rd Pretreatment** and **ICW Protease**. Heating either protease beyond 37°C during the integrated workflow is not recommended because this can result in decreased ISH sensitivity.

For Research Use Only. Not for use in diagnostic procedures.

Advanced Cell Diagnostics, Inc. and/or its affiliate(s) warrant their products as set forth in the ACD General Terms and Conditions of Sale found on the ACD website. Advanced Cell Diagnostics, Inc. reserves the right to change its products and services at any time to incorporate technological developments. This document is subject to change without notice. Although this document has been prepared with every precaution to ensure accuracy, Advanced Cell Diagnostics, Inc. assumes no liability for any errors, omissions, or for any damages resulting from the use of this information.

Headquarters
7707 Gateway Blvd Suite 200, Newark, CA 94545 Phone 1-510-576-8800 Toll Free 1-877-576-3636 For support, email **support.acd@bio-techne.com**
www.acdbio.com