

RNAscope[®] 2.5 LS Assay- RED Combined with Immunohistochemistry (IHC)

Introduction

This Technical Note provides guidelines for performing automated *in situ* hybridization (ISH) using an RNAscope[®] 2.5 LS Reagent Kit- RED (Cat. No. 322100) combined with semi-automated Green immunohistochemistry (IHC) on the Leica BOND RX System. This procedure is based on the standard RNAscope[®] 2.5 LS Red Assay and requires the Leica BOND Detection Kit for immunohistochemistry. Before starting the procedure, create a protocol for the RNAscope[®] assay combined with IHC on the RX controller with the help of your ACD FAS. For every chemical, read the Material Safety Data Sheet (MSDS) and follow handling instructions. Wear appropriate protective eyewear, clothing, and gloves. For the latest service and support information, go to **www.acdbio.com/support**.

Consult www.leicabiosystems.com/ihc-ish-fish/immunohistochemistry-ihc-antibodies-novocastra-reagents/primaryantibodies/ for Ready-To-Use (RTU) compatible antibodies with the BOND RX.

Note: RNAscope[®] uses a proprietary protease that may not be compatible with all antibodies. Please validate your antibody for use with the RNAscope[®] Assay.

ISH - IHC Chromogen Combinations

RNAscope® ISH	Sequential IHC	Automated RNAscope® Detection Kit	IHC Detection System/Reagents
Red	Green	RNAscope [®] 2.5 LS Reagent Kit - RED	Leica BOND Refine Detection Kit, RNAscope® 2.5 LS Green Accessory Pack
Brown (DAB)*	Red	RNAscope® 2.5 LS Reagent Kit - BROWN or RNAscope® LSx Reagent Kit	Leica BOND Red Refine Detection Kit
Brown (DAB)*	Green	RNAscope® 2.5 LS Reagent Kit - BROWN or RNAscope® LSx Reagent Kit	Leica BOND Refine Detection Kit, RNAscope® 2.5 LS Green Accessory Pack

For optimal results using ISH – IHC chromogen combinations, see the following table:

* To perform BROWN ISH - RED IHC or BROWN ISH - GREEN IHC combinations, refer to the RNAscope® 2.5 LS Assay – BROWN Combined with Immunohistochemistry (IHC) Technical Note available at www.acdbio.com/support.

Note: We do not recommend the RED ISH - BROWN IHC combination because of low contrast.

Note: We do not recommend using the green chromogen to perform ISH because of its instability.

Materials Required

RNAscope® 2.5 LS Reagent Kit- RED

The RNAscope[®] 2.5 LS Reagent kit- RED (Cat. No. 322150) provides enough reagents to stain ~60 standard slides on the Leica Biosystems' BOND RX System. The RNAscope[®] 2.5 LS Probes are available separately. The reagents are Ready-To-Use (RTU) and are stored as indicated in the following table:



	RNAscope [®] 2.5 LS Reagent Kit - BROWN (Cat. No. 322100)								
\checkmark	Reagent	Quantity	Storage						
	RNAscope® 2.5 LS Hydrogen Peroxide	21 mL x 1 bottle	2–8°C						
	RNAscope® 2.5 LS Protease III	21 mL x 1 bottle	2–8°C						
	RNAscope® 2.5 LS AMP 1	21 mL x 1 bottle	2–8°C						
	RNAscope® 2.5 LS AMP 2	21 mL x 1 bottle	2–8°C						
	RNAscope® 2.5 LS AMP 3	21 mL x 1 bottle	2–8°C						
	RNAscope® 2.5 LS AMP 3	21 mL x 1 bottle	2–8°C						
	RNAscope® 2.5 LS AMP 4	21 mL x 1 bottle	2–8°C						
	RNAscope® 2.5 LS AMP 5 – RED	21 mL x 1 bottle	2–8°C						
	RNAscope® 2.5 LS AMP 6 – RED	21 mL x 1 bottle	2–8°C						
	RNAscope® 2.5 LS Rinse	30 mL x 2 bottles	2–8°C						

IMPORTANT! Use only RNAscope[®] 2.5 LS Probes. Do not substitute the reagent components of the RNAscope[®] 2.5 LS Reagent Kit with those of other RNAscope[®] Reagent Kits, including the RNAscope[®] 2.0 LS Reagent Kit.

RNAscope® 2.5 LS Green Accessory Pack (Cat. No. 322550)							
Reagent	Quantity	Storage					
RNAscope [®] 2.5 LS Duplex Green A	12 mL x 1 bottle	2–8°C					
RNAscope [®] 2.5 LS Duplex Green A	240 µL x 1 tube	2–8°C					
RNAscope [®] 50X Wash Buffer	60 mL x 1 bottle	Room temp (20–25°C)					

Required Materials from Leica BOND RX

The RNAscope® 2.5 LS Red Assay combined with semi-automated Green IHC requires specific materials and equipment available *only* from Leica Biosystems.

V	Component	Cat. No.	Storage
	BOND 30 mL Open containers	OP309700	Room temp (20–25°C)
	BOND Universal Covertiles 100 pack	S21.2001	Room temp (20–25°C)
	BOND Polymer Refine Detection (DAB) and Hematoxylin *	DS9800	2–8°C
	BOND Epitope Retrieval Solution 1-1L (RTU)	AR9961	2–8°C
	BOND Epitope Retrieval Solution 2-1L (RTU)	AR9640	2–8°C
	BOND Dewax Solution – 1L (RTU)	AR9222	2–8°C
	BOND Wash Solution 10X Concentrate – 1L	AR9590	2–8°C
	BOND Aspirating Probe Cleaning System	CS9100	2–8°C
	BOND Mixing Stations	S21.1971	Room temp (20–25°C)
	BOND Polymer Refine Red Detection and Hematoxylin *	CS9390	2–8°C

* Do not substitute with any other chromogen kit.



Workflow

Part 1: Create software protocols to perform in situ hybridization (ISH)

This section provides instructions for creating *in situ* hybridization (ISH) software protocols compatible with sequential immunohistochemistry (IHC) on the Leica BOND RX System.

Create a ISH protocol for sequential IHC

- 1. In the Protocol setup screen, select Staining under the Protocol group menu.
- 2. Highlight the ***ACD 2.5 Red Rev B** protocol. Select **Copy**.

Note: If you are using software version BDZ 9, select the standard RNAscope[®] 2.5 LS BROWN Assay protocol set up by your ACD FAS.

 Change the protocol name for your first probe to ACD 2.5 Red Rev B P1 no Hematoxylin in the Name text box, 25RP1nH in the Abbreviated name text box, and ACD 2.5 Red Rev B protocol Probe 1 no Hematoxylin in the Description text box.





4. Select Show wash steps. Delete the Hematoxylin step (step 98) and the following wash steps.

Note: You may not be able to delete some wash steps in software version BDZ 9. Change these steps to Bond wash for 0 minutes.

5. To perform a sequential dual stain (ISH-IHC), make sure that **First** is selected under Double-staining status.



Note: (Optional) Select the **Single** button if you are running a single stain assay with no Hematoxylin counterstaining.

- 6. Select Save.
- 7. Click **Next** to proceed. Ignore any pop ups that may appear on the screen.



- 8. To create a protocol for each additional probe, follow these steps:
 - a. Highlight the ACD 2.5 Red Rev B P1 no Hematoxylin protocol. Select Copy.
 - b. Change the protocol name by changing P1 to your probe name (for example, ACD 2.5 Red RevB TGFB1 no Hematoxylin) in the Name text box. Change the Abbreviated name text and Description text box accordingly.
 - c. Select *ACD 2.5 P1. Change the Reagent to your registered probe (for example, TGFB1).

IMPORTANT! Make sure to change all three probe steps.



Part 2: Register the reagents for immunohistochemistry (IHC)

To perform IHC using a Leica Ready-to-Use (RTU) primary antibody on the Leica BOND RX instrument, you must set up the antibody as a **Preferred** reagent. Follow the steps in *Change the Preferred statues of existing reagents* on page 6.

To perform IHC using a diluted antibody or an antibody from a third vendor, you must add the antibody to an open container and register it as a new reagent. Follow the steps in *Register the reagents* on page 7.



Change the Preferred status of existing reagents

1. Select the **Reagent Setup** icon at the top of the screen.

LBOND RX -	System status screen (processing modu	le 1)			
File Window	Item ID Configuration Maintenance Help				
L		۵		eica BOND RX	
RX 1	System status			System	Protocol
	Run 254: Unitocked	Run 255. Unlocked	Run 256: Unlocked		

2. On the bottom, select **Primaries** for **Reagent type** and **All** for the **Preferred status**. All Leica's primary antibody will be displayed.

							0-4			Denela
	Reagent setup					U	Setup			Panels
(Add	Open								
					_					
	Name	Abb name	Type	Supplier	Pref	Staining	HIER	Enzyme	Depaturation	Hybridization
	*Negative	*Neg	Primary	Laboratory S	v	*IHC Protocol F	*	*	Donataration	() Difference of the
	*CD4 (4B12)	*CD4	Primary	Leica Micros		*IHC Protocol F	*HIER 30 min	*		
I	*CD8 (4B11)	*CD8	Primary	Leica Micros	V	*IHC Protocol F	*HIER 20 min	*		
I	*Glial Fibrillary Acidic Protein	*GFAP	Primary	Leica Micros	~	*IHC Protocol F	*HIER 20 min_	*		
I	*CD3 (LN10)	*CD3	Primary	Leica Micros	•	*IHC Protocol F	*HIER 20 min_	*		
I	*CD19 (BT51E)	*CD19	Primary	Leica Micros	•	*IHC Protocol F	*HIER 20 min_	*		
I	*Negative (Mouse)	*Neg MAb	Primary	Leica Micros	V	*IHC Protocol F	*	*		
l	*AccuOyte CTC Pan-Oytoker	*AC-pCK	Primary	RareOyte	~	*AccuOyte CT	*AccuCyte CT_	*		
l	CD4	CD4	Primary	Leica	~	Refine IF Proto.	. *	*		
l	Negative	Neg	Primary		~	*IHC Protocol F	*	*		
l	Antibody 1	Antibod1	Primary		V	*IHC Protocol F	*	*		
l	Antibody 2	Antibod2	Primary			*IHC Protocol F	*	*		
l	Antibody 3	Antibod3	Primary			*IHC Protocol F	*	*		
I	Antibody 4	Antibod4	Primary			*IHC Protocol F	*	*		
I	Antibody 5	Antibod5	Primary		~	*IHC Protocol F	*	*		
I	Antibody 6	Antibod6	Primary		~	*IHC Protocol F	*	*		
l	Antibody 7	Antibod7	Primary		V	*IHC Protocol F	*	*		



- 3. Select the antibody to be used (for example, *CD4). Double click to edit reagent properties.
- 4. Select **Preferred** then **Save**.

📕 BOND RX - R	eagent screen			1	
File Window I	tem ID Configu	🕰 Edit reagent properties	×		
L			(*CD4 (4B12)	BOND RX)
PENNY	Reager		(*CD4		Panels
8:03 AM 8:19 AM 7:53 AM	Ad(Primary Cleica Microsystems	yme Denaturation	Hybridization
LEONAR	*CD20 (M *CD21 (2 *CD23 (1 *CD30 (1	Single/double stain Single Single	Restore factory default protocols		
	*CD31 (1 *CD4 (4E *CD43 (N	Default staining protocol: Default HER protocol:	*IHC Protocol F *HIER 30 min with ER2		
	*CD45RC *CD5 (4C *CD56 (C	Default enzyme protocol:	(* v)		
	*CD68 (5 *CD8 (4B *Chromo				
	*Cytokera *Cytokera *Cytokera				
	*Epithelia *Estroger		Compatible bulks:		
	*Galectin *Glial Fib				
	Package ty All reage	Preferred.	Hazardous:	Preferm	ed status:

Register the reagents

ደ BOND RX - System status screen (processing modu	le 1)			X
File Window Item ID Configuration Maintenance Help	X.	Œ	· DONID DX	
	<i>></i>	L	eica BUND RA	
RX 1 System status			System	Protocol
Fun 254. Unlocked	Run 255: Unlocked	Run 256: Unlocked		
		<u> </u>	*Dewax *DI *BWash *Alcoho	BikWast *BikWast *HazWast

1. Select the **Reagent Setup** icon at the top of the screen.



- 2. Select **Add** to register a new primary antibody.
- 3. Enter a name for the antibody (for example, **Antibody 1**) in the Name text box. Enter the Abbreviated name text box (for example, **Ab1**).

尾 BOND RX - R	eagent screen						_	
File Window I	Item ID Configuration M	🕰 Add reagent			×			
L				Antibody 1	\square	ND RX		
PENNY	Reagent setu	A		(Ab1		ntory	Panels	
	Add			Primary	~			
	Name *Negative					Denaturation	Hybridization	
	*CD4 (4B12)	Single/double stain						ň
LEONAR	*CD8 (4B11)	Single						Ĭ
	*Bond DAB Enhan	Single						
	*CD3 (LN10)	Default		(IHC Protocol J sequential	-			
	*CD19 (B151E) *Negative (Mouse)	Defa		(*	-			
	Enzyme 5	Default		(-			
	*LSI HER2/CEP17					[•] Denaturation (*ISH Hybridiza	
	*1:20 Part B							
	*8:1:1:60 Part A							
	*8:1:1:60 Part B *8:1:1:60 Part C							
	*8:1:1:60 Part D							
	*1:1 Part A *1:1 Part B			Compatible to the				
	*Mixed 1A:20B			Companiore builts.				
	*Mixed 8A:1B:1C:8			*BWash				
	*Open 1				J			
	Package type:		Preferred:	Hazard	ous: 🕚	Preferm	ed status:	
	All reagents		Save	Cancel		Prete	rrea	
						1		

4. Select **Primary** in the Type drop-down menu. Add the Supplier name.

Note: You may leave the Supplier text box empty.

5. Select Save.



Part 3: Create the protocol for sequential immunohistochemistry (IHC)

We recommend combining the RNAscope® Red ISH assay with semi-automated Green IHC.

Create a semi-automated Green IHC protocol using the Leica BOND Refine Detection Kit

1. To create a semi-automated Green IHC protocol, highlight ***IHC Protocol F** in the protocol set up page and select **Copy**.

BOND RX - Pro	otocol screen em ID Configuration Maintenance	Help					<u>- ×</u>
Ł	i 🗎 👔		Leic	a BONI	O RX		
PENNY	Protocol setup	Open	Delete				
	Protocol name	Protocol type	Description	Modified by	Mod. date	Pref.	
	*AccuOyte CTC IF Protocol	IHC staining	AccuCyte CTC IF protocol	Leica	18/05/2016	◄	
	*AccuOyte CTC IHC Protocol	IHC staining	AccuCyte CTC IHC protocol	Leica	18/05/2016	V	Ō
LEONAR	*IF Protocol	IHC staining	IF protocol	Leica	18/05/2016	~	Ť
	*IHC Open Dispense Templ	IHC staining	IHC template with Open Ancillary and Chromogen dispenses	Leica	18/05/2016	~	
	*IHC Protocol B	IHC staining	Bond Intense R IHC protocol	Leica	26/02/2016	Γ	
	*IHC Protocol F	IHC staining	Bond Polymer Refine IHC protocol	Leica	26/02/2016		
	*IHC Protocol FRX 37M	IHC staining	IHC Protocol F with marker step at 37C	Leica	18/05/2016	v	
	*IHC Protocol FRX 40M	IHC staining	IHC Protocol F with marker step at 40C	Leica	18/05/2016	~	
	*IHC Protocol J	IHC staining	Bond Polymer Refine Red IHC protocol	Leica	26/02/2016	~	
	*IHC Protocol J RX 37M	IHC staining	IHC Protocol J with marker step at 37C	Leica	18/05/2016	V	
	*IHC Protocol J RX 40M	IHC staining	IHC Protocol J with marker step at 40C	Leica	18/05/2016	v	
	*IHC Protocol K	IHC staining	ChromoPlex 1 Dual IHC protocol	Leica	18/05/2016	v	
	*IHC Protocol K - 50 Test	IHC staining	ChromoPlex 1 Dual IHC protocol	Leica	18/05/2016	◄	
	*Opal 7-Color (v5.2 plus)	IHC staining	Perkin Elmer Opal multiplex protocol for v5.2 plus	Leica	8/06/2017		
	*Top-up Dispenses Template	IHC staining	Template with multiple dispense types	Leica	8/06/2017		
	Protocol group: Staining	Protocol type: IHC staining	Staining status: All Cleica Micro	systems 🍼	Preferred st	atus:	



- 2. Enter IHC Protocol Offline Green in the Name text box, IHCgreen in the Abbreviated name text box, and Bond Polymer Refine IHC Protocol for offline Green in the Description text box.
- 3. Select **Second** in the Double-staining status menu. Other buttons are optional.

Description: Bond Polymer Re	fine IHC protocol for Off	ine Green			
BOND RX					
Insert reagent Insert wash	Duplicate	Delete wash			Import
Step N* Beagent	Supplier	Inc. (min)	Υ Pr	referred detection system:	
8 *Bond Wash Solution	Leica Microsystems	0:00 (Bond Polymer Refine Detection	1
9 *Post Primary	Leica Microsystems	8:00	YI Y		
10 *Bond Wash Solution	Leica Microsystems	2:00		Step details	
11 *Bond Wash Solution	Leica Microsystems	2:00		The instant Water	
12 *Bond Wash Solution	Leica Microsystems	2:00		Reagent. Defonized Water	
13 *Polymer	Leica Microsystems	8:00			0:00
14 *Bond Wash Solution	Leica Microsystems	2:00			
15 *Bond Wash Solution	Leica Microsystems	2:00			
16 *Deionized Water		0:00			
17 *Mixed DAB Refine	Leica Microsystems	0:00			
18 *Mixed DAB Refine	Leica Microsystems	10:00			
19 *Deionized Water		0:00			
20 *Deionized Water		0:00			
21 *Deionized Water		0:00			
22 *Hernatoxylin	Leica Microsystems	5:00			
23 *Deionized Water		0:00	Y		
24 *Bond Wash Solution	Leica Microsystems	0:00			
25 *Deionized Water		0:00			
		(
Show wash stens					

4. Modify the protocol according to the following table. Delete the steps from *Mixed DAB Refine to *Hematoxylin. Select Show wash steps to display the wash steps, and delete the * Deionized Water wash steps.

Step No.	Reagent	Step Type	Incubation Time	Temperature
1	*Peroxide Block	Reagent	5 MIN	Ambient
2	*Bond Wash Solution	Wash	0 MIN	Ambient
3	*Bond Wash Solution	Wash	0 MIN	Ambient
4	*Bond Wash Solution	Wash	0 MIN	Ambient
5	*MARKER	Reagent	15 MIN/30 MIN	Ambient
6	*Bond Wash Solution	Wash	0 MIN	Ambient
7	*Bond Wash Solution	Wash	0 MIN	Ambient
8	*Bond Wash Solution	Wash	0 MIN	Ambient
9	*Post Primary	Reagent	16 MIN	Ambient
10	*Bond Wash Solution	Wash	2 MIN	Ambient
11	*Bond Wash Solution	Wash	2 MIN	Ambient
12	*Bond Wash Solution	Wash	2 MIN	Ambient
13	*Polymer	Reagent	16 MIN	Ambient
14	*Bond Wash Solution	Wash	2 MIN	Ambient
15	*Bond Wash Solution	Wash	2 MIN	Ambient
16	*Bond Wash Solution	Wash	2 MIN	Ambient



- 5. Adjust the incubation times for ***Post primary** and ***Polymer** to **16 MIN**.
- 6. (Optional) Change the incubation time for *MARKER from 15 MIN to 30 MIN.
 Note: Increasing the primary antibody (*MARKER) incubation time can increase sensitivity.
- 7. Select Save.

Part 4: Set up a study for sequential Red ISH- Green IHC

Build a study

1. Select the **Slide setup** icon at the top of the screen.

🔏 BOND RX - System	m status screen (pro	cessing module 1)			
File Window Item II	D Configuration Mair	ntenance Help				
£	e D	😻 🍐)		Leica BON	D RX
RX 1 S	System status				Syste	m Protocol
	Run 254: University		Run 255. Uhiocked	Run 255. Uhlocked		



2. Select **Add study** and enter a name in the Study ID field (keep the Dispense volume at **150** µL as shown.



For FFPE tissues, select *Bake and Dewax as the Preparation protocol (leave blank for other tissue types).
 Select OK.



Add an ISH and IHC protocol to each slide

1. Select Add slide.



- 2. Enter the tissue type and probe name under the Comments field.
- 3. Select **Sequential DS** from the Staining mode drop down menu.

🖊 Add slide	×
	Slide ID: OAIC
	Study N*: 852
Study name:	
Study comments:	
Study ID:	(ISH-DAB/IHC-Red
Comments:	Tissue TGFB1/CD4
Tissue type: Test tissue Negative tissue Positive tissue	Dispense volume: 100 µL 150 µL
Staining mode: Single Researc Single Parallel DS: Parallel DS: Process: I HC	h ISH
Marker:	
Protocols	
Preparation: *Bake a	and Dewax
Add slide	Close

4. Add the ISH staining protocol by selecting the **First** tab.



5. Select ISH under Process, and mock probe (ACD) from the Marker drop down menu.

尾 Add slide	×
	Silde ID: (DAIF
	Study N*: 853
	Ay name:
Study co	mments:
	Study ID: (ISH-Red/IHC-Green
	mments: tissue TGFB1/CD4
Tissue type: Test tissue Negative tissue Positive tissue	Dispense volume: ● 100 µL ● 150 µL
Staining mode: Sequential DS	Research
First	Second
Process:	🔿 інс 🛛 💿 ізн
Marker:	(Mock Probe (ACD)
Protocols	
	ACD 2.5 Red Rev B TGFB1 no Hematoxlyin
Preparation:	*Bake and Dewax
HIER:	*ACD HIER 15 min with ER2 (95)
Enzyme:	*ACD 15 min Protease
Denaturation:	*
Hybridization:	ISH Hybridization 1 min
Add slide	Close

6. Under Protocols:

- Select a protocol from the Staining drop down menu for each probe. Make sure that each probe is associated with a different protocol (for example, ACD 2.5 Red RevB TGFB1 no Hematoxylin) for the RNAscope[®] ISH Red Assay).
- b. Select ***ACD HIER 15 min with ER2 (95)** as the HIER protocol, or the appropriate HIER protocol for your tissue.
- c. Select ***ACD 15 min Protease** for Enzyme, or the appropriate protease protocol for your tissue.
- d. Select ACD 1 min Hybridization for Hybridization.
- 7. Add the IHC protocol by selecting the **Second** tab.
- Select IHC under Process and the antibody of interest (for example, *CD4) from the Marker menu.
 Note: For antibodies to be available from the menu, you must first register any antibodies not already available through Leica.
- 9. Select IHC Protocol Offline Green under Protocols. Leave HIER and Enzyme blank.

IMPORTANT! Including additional HIER or Enzyme steps following the ISH staining may decrease the intensity of the ISH markers.



🗶 Add slide 🛛 🗶						
Study N*: (853						
Study name:						
Study comments:						
Study ID: (Iannewine-oreen						
Comments: tissue TGFB1/CD4						
Tissue type: Dispense volume: Test tissue 100 µL Negative tissue 150 µL Positive tissue 150 µL						
Staining mode: Sequential D® (Research						
First Second						
Process: O IHC ISH						
Marker: *CD4 (4B12)						
Protocolo						
Staining: (HC Protocol Offline Green						
HIER: •••••• ••						
Add slide Close						

10. Repeat steps 1–9 for each slide.

Note: To use a different probe on the new slide, change the staining protocol in the **First** tab. To use a different antibody on the new slide, change the marker selection in the **Second** tab.

Complete the study

- 1. After adding all the slides to the study, select **Close** to return to the Slide setup screen.
- 2. Select **Print labels** to print barcodes to attach to the slides.
- 3. Place the tray in the Leica BOND RX, and press the button to load the tray onto the machine.

IMPORTANT! Slides for RNAscope[®] BROWN ISH - RED IHC and RNAscope[®] BROWN ISH - GREEN IHC cannot be placed on the same tray.

4. Once the slides have been scanned, select the PLAY (triangular) button on the screen located under the start tray to start the run. Alternatively, right-click on the scanned label images and select Delayed Start to start the run at a future time.

Part 5: Detect green IHC staining off the instrument for RED

Prepare reagents and equipment

1. Before the run completes, remove the Green A and Green B reagents from the refrigerator and warm to ambient temperature.

IMPORTANT! View the wash step video at www.acdbio.com/technical-support/learn-more before proceeding.



Detect Green staining off the instrument

1. As soon as the run is complete, press the button on the front of the instrument and unload the slides immediately.

IMPORTANT! If you do not perform the Green assay immediately, store the slides in 4X SSC at 4°C. After the run is completed, do not leave the slides loaded on the instrument. The slides are automatically rehydrated by the instrument. Do not store the slides in water or wash buffer.

IMPORTANT! Do not let sections dry out between incubation steps. Work quickly and make sure the sections are hydrated at all times.

- 2. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Agitate slides by moving the slide rack up and down in the staining dish.
- 3. Repeat Step 2 with fresh 1X Wash Buffer.
- 4. Briefly spin down the contents of the Green B tube to be sure content is at the bottom of the tube before opening the cap.
- Prepare 200 µL of GREEN working solution per slide using a 1:50 ratio of Green B to Green A. Mix well.
 IMPORTANT! Use the GREEN solution within 5 MIN. Do not expose to direct sunlight or UV light.
- 6. Take each slide one at a time from the Tissue-Tek® Slide Rack and tap and/or flick to remove the excess liquid.
- 7. Pipette ~200 µL GREEN solution onto each tissue section. Ensure sections are covered.
- 8. Incubate the slides for **15 MIN** at **RT**.
- 9. To remove the GREEN working solution from the slides, tilt each slide one at a time over a waste container and tap the corner on the edge of the container. Immediately insert the slide into a Tissue-Tek[®] Slide Rack submerged in a Tissue-Tek[®] Staining Dish filled with distilled water.
- 10. Quickly rinse the slides with fresh distilled water for less than 30 seconds.

IMPORTANT! Proceed quickly to the next step. GREEN substrate may fade if stored in water for too long.

Counterstain the slides

 Move the Tissue-Tek® Slide Rack into the staining dish containing 50% Hematoxylin I staining solution for 30 SEC at RT. Slides will be purple.

IMPORTANT! Proceed quickly to the next step. GREEN substrate may fade if in Hematoxylin for longer than 30 seconds.

- 2. Immediately transfer the slide rack into a staining dish filled with tap water. Do not let the slides remain in the water for more than 30 seconds.
- 3. Repeat Step 2 once or twice.

Dry and mount the samples

1. Remove the slide rack from the staining dish and dry slides in a 60°C dry oven for 30 MIN.

IMPORTANT! The RED and GREEN substrates are alcohol sensitive. Do not dehydrate the slides in alcohol.

- 2. Cool the slides for **5 MIN** at **RT**.
- 3. Briefly dip one slide into fresh pure xylene and immediately place 1−2 drops of VectaMount[™] Mounting Medium on the slide before the xylene dries.
- 4. Carefully place a 24 mm x 50 mm coverslip over the tissue section. Avoid trapping air bubbles.
- 5. Repeat steps 2 and 3 for each slide.
- 6. Air dry the slides for **5 MIN**.



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