

RNAscope[®] 2.5 LS Assay- BROWN 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-BROWN (Cat. No. 322100) combined with immunohistochemistry (IHC) on the Leica BOND RX System. This procedure is based on the standard RNAscope[®] 2.5 LS BROWN Assay and requires the Leica BOND Refine Detection Kit or Leica BOND Red Refine 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 |
|---------------------------|-------------------|---|---|
| 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 |
| Red* | Green | RNAscope [®] 2.5 LS Reagent Kit - RED | 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 RED ISH - GREEN IHC, refer to the RNAscope[®] 2.5 LS Assay – RED Combined with Immunohistochemistry (IHC) Technical Note 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- BROWN

The RNAscope[®] 2.5 LS Reagent kit - BROWN (Cat. No. 322100) 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) | | | | | | |
|---|--|-------------------|---------|--|--|--|--|
| V | 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 – BROWN | 21 mL x 1 bottle | 2–8°C | | | | |
| | RNAscope® 2.5 LS AMP 6 – BROWN | 21 mL x 1 bottle | 2–8°C | | | | |
| | RNAscope [®] 2.5 LS Rinse | 30 mL x 2 bottles | 2–8°C | | | | |
| | RNAscope [®] 2.5 LS Bluing Reagent | 21 mL x 1 bottle | 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) | | | |

* This kit is only required for semi-automated Green IHC. To perform automated Red IHC, use the BOND Polymer Refine Red Detection and Hematoxylin Kit.

Required Materials from Leica BOND RX

The RNAscope® 2.5 LS Brown Assay combined with IHC requires specific materials and equipment available *only* from Leica Biosystems.

| \checkmark | 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.

† This kit is only required for Red IHC. To perform semi-automated Green IHC, use the materials in the RNAscope® 2.5 LS Green Accessory Pack.



Workflow

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

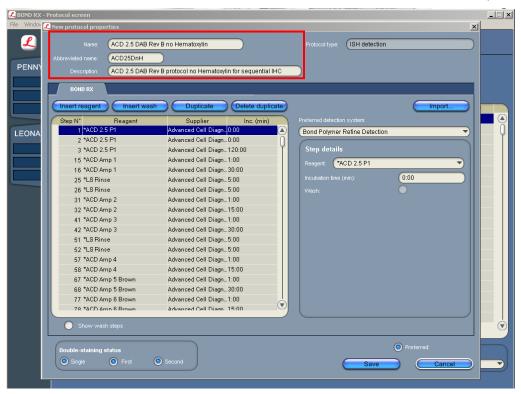
This section provides instructions for creating *in situ* hybridization (ISH) software protocols compatible with sequential immunohistochemistry (IHC) on the Leica BOND RX System.

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 DAB 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.

3. Change the protocol name for your first probe to ACD 2.5 DAB Rev B no Hematoxylin in the Name text box, ACD25DnH in the Abbreviated name text box, and ACD 2.5 DAB Rev B protocol no Hematoxylin in the Description text box.



4. Select **Show wash steps**. Delete the **Hematoxylin** step (step 98) and all the following steps including the **ACD Blue** step (step 105).

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.



| 📕 BOND RX | Protocol screen | | | × |
|-------------|--|--|-------------------------------|---|
| File Windov | L New protocol properties | | × | |
| ſ | Name: ACD 2.5 DAB Rev | B no Hematovivin | Protocol type: (ISH detection | |
| <u> </u> | | | | |
| PENN | Abbreviated name: ACD25DnH |) | _ | |
| | Description: ACD 2.5 DAB Rev | B protocol no Hematoxylin for sequential IHC | | |
| | BOND RX | | | |
| | | | | |
| | (Insert reagent) (Insert wash | Duplicate Delete duplicate | Import | |
| | Step N* Reagent | Supplier Inc. (min) | Preferred detection system: | |
| LEONA | 94 *Deionized Water | 0:00 | Bond Polymer Refine Detection | J |
| | 95 *Deionized Water | 0:00 | | |
| | 96 *Deionized Water | 0:00 | Step details | |
| | 97 *Deionized Water | 0:00 | Reagent: (*Hematoxylin | |
| | 98 *Hematoxylin | Leica Microsystems 5:00 | Incubation time (min): 5:00 | |
| | 99 *Deionized Water | 0:00 | | |
| | 100 *Deionized Water | 0:00 | Wash: | |
| | 101 *Deionized Water 102 *Deionized Water | 0:00 | | |
| | 102 Defonized Water | 0:00 | | |
| | 104 *Deionized Water | 0:00 | | |
| | 105 *ACD Blue | Advanced Cell Diagn 2:00 | | |
| | 106 *Deionized Water | 0:00 | | |
| | 107 *Deionized Water | 0:00 | | |
| | 108 *Deionized Water | 0:00 | | |
| | 109 *Deionized Water | 0:00 | | |
| | 110 *Deionized Water | 0:00 | | |
| | 111 *Deionized Water | 0:00 | | |
| | | | | |
| | Show wash steps | | | |
| | Show wash steps | | | 2 |
| | | | Preferred: | |
| | Double-staining status | | Protonow. | |
| | 💿 Single 💿 First 🤇 | Second | Save Cancel |) |
| | | | | |
| | | | | |

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

| 📕 BOND RX | Protocol screen | | |
|-------------|--|---|-----------|
| File Windov | L New protocol properties | | × |
| L | Name: ACD 2.5 DAB Rev B no Herr Abbreviated name: ACD25DnH | natoxylin Protocol type: (ISH detecti | on |
| | BOND RX Insert wash Step N* Reagent | I no Hernatoxylin for sequential IHC Dupticate Delete wash Supplier Inc. (min) Preferred detection system. dicrosystems 1:00 Bond Polymer Refine Detect | |
| | 83 "Bond Wash Solution Leica N 84 "Bond Wash Solution Leica N 85 "Bond Wash Solution Leica N 86 "Bond Wash Solution Leica N 87 "LS Rinse Advant 89 "Mixed DAB Refine Leica N | Alicrosystems 1:00 Alicrosystems 1:00 Alicrosystems 1:00 Alicrosystems 1:00 Alicrosystems 1:00 Alicrosystems 1:00 Alicrosystems 1:00 Alicrosystems 1:00 Alicrosystems 20:00 | |
| | Show wash steps Double-staining status | | Preferred |
| | Single O First O Second | Save | Cancel |

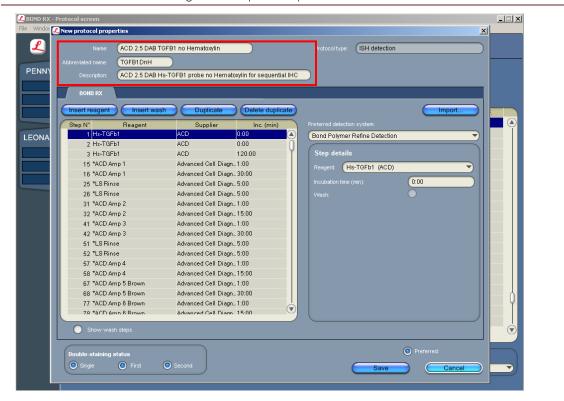
Note: (Optional) Select the **Single** button if you are running a single stain assay with no Hematoxylin counterstaining.

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 DAB Rev B no Hematoxylin protocol. Select Copy.
 - b. Change the protocol name by adding your probe name (for example, **ACD 2.5 DAB 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)

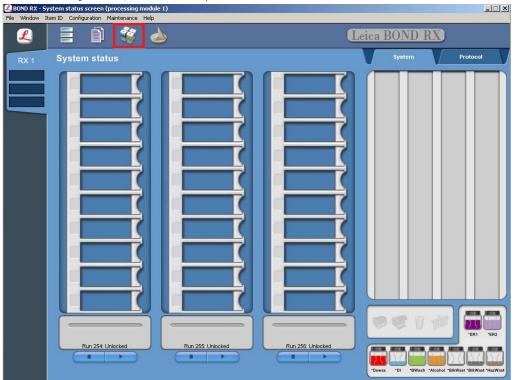
To perform IHC using a Leica Ready-to-Use (RTU) primary antibody on the Leica BOND RX instrument, you must set up the antibody as a **Preferred** reagent. Follow the steps in *Change the Preferred status of existing reagents* on page 6.

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.



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

| BOND RX - Re | - | | | | | | | | | _ | . 🗆 |
|--------------|----------------------------------|------------|---------|--------------|----------|------------------|---------------|----------|------------------------|---------------|-----|
| e Window It | em ID Configuration Maintenance | Help | | | | | | | | | |
| ٤ | 📑 🗊 🐳 | | | | | | | Leica BC | OND RX | l | |
| PENNY | Reagent setup | | | | | | Setup | | entory | Panels | |
| 8:03 AM | Add | Open | | | | | | | | | |
| 8:19 AM | | | | | | | | | | | |
| 7:53 AM | Name | Abb. name | Туре | Supplier | Pref. | Staining | HIER | Enzyme | Denaturation | Hybridization | |
| | *Negative | *Neg | Primary | Laboratory S | ~ | *IHC Protocol F | * | * | | | (|
| | *CD4 (4B12) | *CD4 | Primary | Leica Micros | V | *IHC Protocol F | *HIER 30 min_ | * | | | |
| EONAR | *CD8 (4B11) | *CD8 | Primary | Leica Micros | v | *IHC Protocol F | *HIER 20 min | * | | | 1 |
| | *Glial Fibrillary Acidic Protein | *GFAP | Primary | Leica Micros | ~ | *IHC Protocol F | *HIER 20 min | * | | | |
| | *CD3 (LN10) | *CD3 | Primary | Leica Micros | ~ | *IHC Protocol F | *HIER 20 min | * | | | |
| | *CD19 (BT51E) | *CD19 | Primary | Leica Micros | • | *IHC Protocol F | *HIER 20 min | * | | | |
| | *Negative (Mouse) | *Neg MAb | Primary | Leica Micros | V | *IHC Protocol F | * | * | | | |
| _ | *AccuCyte CTC Pan-Cytoker | *АС-рСК | Primary | RareCyte | ~ | *AccuCyte CT | *AccuCyte CT_ | * | | | |
| | CD4 | CD4 | Primary | Leica | ~ | Refine IF Proto. | .* | * | | | |
| | Negative | Neg | Primary | | ~ | *IHC Protocol F | * | * | | | |
| | Antibody 1 | Antibod1 | Primary | | V | *IHC Protocol F | * | * | | | |
| | Antibody 2 | Antibod2 | Primary | | V | *IHC Protocol F | * | * | | | |
| | Antibody 3 | Antibod3 | Primary | | • | *IHC Protocol F | * | * | | | |
| | Antibody 4 | Antibod4 | Primary | | 1 | *IHC Protocol F | * | * | | | |
| | Antibody 5 | Antibod5 | Primary | | 1 | *IHC Protocol F | * | * | | | |
| | Antibody 6 | Antibod6 | Primary | | ~ | *IHC Protocol F | * | * | | | |
| | Antibody 7 | Antibod7 | Primary | | ~ | *IHC Protocol F | * | * | | | |
| | Package type: | Reagent ty | pe: | _ | | | Suppl | ier: | Preferr | ed status: | |
| | All reagents | Primarie | s | ~ | | | All | | Prefe Prefer All | | _ |

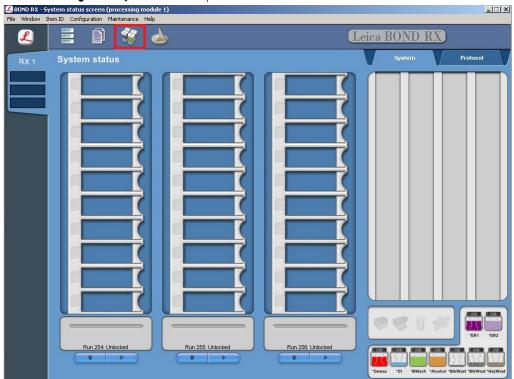


- 3. Select the antibody to be used (for example, *CD4). Double click to edit reagent properties
- 4. Select Preferred then Save.

| 尾 BOND RX - R | | Ledit reagent properties | × | d | | _ | |
|-------------------------------|--|--|--|------|----------------|---------------|---|
| File Window I | ltem ID Configu | | <u>^</u> | | | | |
| L | | | (*CD4 (4B12) | BO | ND RX | | |
| PENNY | Reager | | (*CD4 | Inve | entory | Panels | V |
| 8:03 AM 8:19 AM 7:53 AM | *CD2 (11 *CD20 (1/ *CD21 (2 *CD23 (1 *CD33 (1 *CD33 (1 *CD33 (1 *CD43 (1/ *CD45 (1/ *CD45 (1/ *CD45 (1/ *CD56 (1/ *CD56 (1/ *CD56 (1/ *CD56 (1/) *CD56 (1/) *CD56 (1/) | Type: Suppler: Single/double stain Single Single Default staining protocot Default HER protocot Default enzyme protocot | Primary Leica Microsystems Restore factory default protocols THC Protocol F THIER 30 min with ER2 | yme | Denaturation | Hybridization | |
| | *Oytokere *Oytokere *Eytholiz *Estroger *On Wille *Galectin *Glial Fib Package ty All reage | Preferred C | Competible bulks: *BWash Hazerdous: | | Preferr All | ed status: | |

Register the reagents

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



RNAscope[®] 2.5 LS Assay- BROWN Combined with IHC



- 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**).

| ደ BOND RX - R | leagent screen | | | × |
|---------------|--------------------------------------|----------------------------|---------------------------|---|
| File Window I | Item ID Configuration M | 🛃 Add reagent | × | |
| ſ | E N | Name: | (Antibody 1 | ND RX |
| ~ | | | | |
| PENNY | Reagent setu | Abbreviated name: | Ab1 | ntory Panels |
| | Add | | | |
| | - ridd | | | |
| | Name | Туре: | Primary | Denaturation Hybridization |
| | *Negative | Supplier: | | |
| LEONAR | *CD4 (4B12) | Single/double stain | | Ō |
| | *CD8 (4B11) *Glial Fibrillary Aci | Single 🔻 | | |
| | *Bond DAB Enhan | Single | | |
| | *CD3 (LN10) | | | |
| | *CD19 (BT51E) | Default staining protocol: | IHC Protocol J sequential | |
| | *Negative (Mouse) | Default HIER protocol: | * | |
| | *Enzyme 5 | Default enzyme protocol: | * | |
| | *LSI HER2/CEP17 | | | [•] Denaturation (<mark>*ISH Hybridiza</mark> |
| | *1:20 Part A *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 | | | |
| | *Mixed 1A:20B | | Compatible bulks: | |
| | *Mixed 8A:1B:1C:8 | | *BWash | |
| | *Mixed 1A:1B | | | |
| | *Open 1 | | | |
| | | Preferred: O | | |
| | Package type: | Preferred: | Hazardous: 🔘 | Preferred status: |
| | All reagents | Save | Cancel | Preferred |
| | | | | |

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)

You can combine the RNAscope[®] Brown ISH assay with fully automated Red IHC or semi-automated Green IHC. To combine the RNAscope[®] Brown ISH assay with fully automated Red IHC, follow the steps in *Create a sequential Red immnunohistochemistry (IHC) protocol using the Leica BOND Red Refine Detection kit* on page 9.

To combine the RNAscope[®] Brown ISH assay with semi-automated Green IHC, follow the steps in *Create a semi-automated Green IHC protocol using the Leica BOND Refine Detection kit* on page 10.

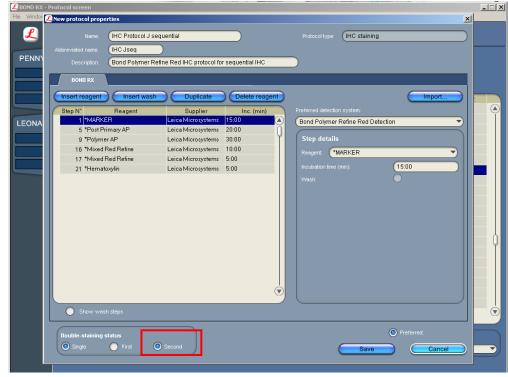


Create a sequential Red immnunohistochemistry (IHC) protocol using the Leica BOND Red Refine Detection Kit

1. To create a sequential Red IHC protocol, highlight *IHC Protocol J in the protocol set up page and select Copy.

| L | em ID Configuration Maintenance | | Leio | ea BONI | D RX | | |
|--------|---------------------------------|-------------------|--|-------------|--------------|----------|---|
| ENNY | Copy | Open | Delete Report | | | | |
| | Protocol name | Protocol type | Description | Modified by | Mod. date | Pref. | |
| _ | *AccuCyte CTC IF Protocol | IHC staining | AccuQ/te CTC IF protocol | Leica | 18/05/2016 | | (|
| 0.11.0 | *AccuCyte CTC IHC Protocol | IHC staining | AccuCyte CTC IHC protocol | Leica | 18/05/2016 | V | Ì |
| ONAR | *IF Protocol | IHC staining | IF protocol | Leica | 18/05/2016 | 1 | |
| | *IHCOpen Dispense Templ | IHC staining | IHC template with Open Ancillary and Chromogen dispenses | Leica | 18/05/2016 | 1 | |
| | *IHC Protocol B | IHC staining | Bond Intense R IHC protocol | Leica | 26/02/2016 | Г | |
| | *IHC Protocol F | IHC staining | Bond Polymer Refine IHC protocol | Leica | 26/02/2016 | 2 | |
| | *IHC Protocol FRX 37M | - IHC staining | IHC Protocol F with marker step at 37C | Leica | 18/05/2016 | 2 | |
| | *IHC Protocol F RX 40M | IHC staining | IHC Protocol F with marker step at 40C | Leica | 18/05/2016 | 2 | |
| | *IHC Protocol J | IHC staining | Bond Polymer Refine Red IHC protocol | Leica | 26/02/2016 | | |
| | | IHC staining | IHC Protocol J with marker step at 37C | Leica | 18/05/2016 | <u> </u> | |
| | *IHC Protocol J RX 40M | - IHC staining | IHC Protocol J with marker step at 40C | Leica | 18/05/2016 | v | |
| | *Opal 7-Color (v5.2 plus) | IHC staining | Perkin Elmer Opal multiplex protocol for v5.2 plus | Leica | 8/06/2017 | Г | |
| | *Top-up Dispenses Template | - | Template with multiple dispense types | Leica | 8/06/2017 | Г | |
| | | | | | | | (|
| | Protocol group: | Protocol type: | Staining status: Single | | Preferred st | atus: | _ |

- 2. Enter IHC Protocol J sequential in the Name text box, IHC Jseq in the Abbreviated name text box, and Bond Polymer Red Refine IHC Protocol for sequential IHC in the Description text box.
- 3. Select Second in the Double-staining status menu. Other buttons are optional.



4. Select Save.

RNAscope® 2.5 LS Assay- BROWN Combined with IHC



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

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

| L | 🗏 🗊 🗳 | | Lei | ca BONI | O RX | | |
|-------|----------------------------|----------------|--|-------------|--------------|----------|---|
| PENNY | Protocol setup | Open | Delete | | | | |
| | Protocol name | Protocol type | Description | Modified by | Mod. date | Pref. | |
| | *AccuCyte CTC IF Protocol | IHC staining | AccuCyte CTC IF protocol | Leica | 18/05/2016 | V | |
| EONAR | *AccuCyte CTC IHC Protocol | IHC staining | AccuCyte CTC IHC protocol | Leica | 18/05/2016 | | Ō |
| | *IF Protocol | IHC staining | IF protocol | Leica | 18/05/2016 | V | Ĭ |
| | *IHC Open Dispense Templ | . IHC staining | IHC template with Open Ancillary and Chromogen dispenses | Leica | 18/05/2016 | v | |
| | *IHC Protocol B | IHC staining | Bond Intense R IHC protocol | Leica | 26/02/2016 | Г | |
| | *IHC Protocol F | IHC staining | Bond Polymer Refine IHC protocol | Leica | 26/02/2016 | | |
| | *IHC Protocol F RX 37M | IHC staining | IHC Protocol F with marker step at 37C | Leica | 18/05/2016 | ◄ | |
| | *IHC Protocol FRX 40M | IHC staining | IHC Protocol F with marker step at 40C | Leica | 18/05/2016 | V | |
| | *IHC Protocol J | IHC staining | Bond Polymer Refine Red IHC protocol | Leica | 26/02/2016 | V | |
| | *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 | V | |
| | *Opal 7-Color (v5.2 plus) | IHC staining | Perkin Elmer Opal multiplex protocol for v5.2 plus | Leica | 8/06/2017 | Г | |
| | *Top-up Dispenses Template | e IHC staining | Template with multiple dispense types | Leica | 8/06/2017 | Г | |
| | | | | | | | (|
| | Protocol group: | Protocol type: | Staining status: Protocol orig | | Preferred st | -1 | |

- 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.

| Abbreviated name: IHC Protocol Off | | | Protocol type: (IHC staining | |
|--|--|-------------|-------------------------------|------------|
| | tefine IHC protocol for Off | line Green | | |
| Description. | tenne into protocorior on | | | |
| BOND RX | | | | |
| (Insert reagent) (Insert wash | Duplicate | Delete wash | | Import |
| | | | | |
| Step N* Reagent | Supplier | Inc. (min) | Preferred detection system: | |
| 8 *Bond Wash Solution | Leica Microsystems | 0:00 | Bond Polymer Refine Detection | |
| 9 *Post Primary | Leica Microsystems | 8:00 | Stop dotaile | |
| 10 *Bond Wash Solution 11 *Bond Wash Solution | Leica Microsystems | 2:00 2:00 | Step details | |
| 11 *Bond Wash Solution | Leica Microsystems Leica Microsystems | 2:00 | Reagent: *Deionized Water | ▼ |
| 12 "Bond Wash Solution 13 *Polymer | Leica Microsystems Leica Microsystems | 8:00 | Incubation time (min): | 0:00 |
| 13 "Polymer 14 *Bond Wash Solution | Leica Microsystems Leica Microsystems | 2:00 | | |
| 14 "Bond Wash Solution 15 *Bond Wash Solution | Leica Microsystems | 2:00 | Wash: | U |
| 16 *Deionized Water | Leica Microsystems | 0:00 | | |
| 17 *Mixed DAB Refine | Leica Microsystems | 0:00 | | |
| 18 *Mixed DAB Refine | Leica Microsystems | 10:00 | | |
| 19 *Deionized Water | Leicalviiciosystemis | 0:00 | | |
| 20 *Deionized Water | | 0:00 | | |
| 21 *Deionized Water | | 0:00 | | |
| 22 *Hernatoxylin | Leica Microsystems | 5:00 | | |
| 23 *Deionized Water | | 0:00 | | |
| 24 *Bond Wash Solution | Leica Microsystems | 0:00 | | |
| 25 *Deionized Water | | 0.00 | | |
| | | | | |
| | _ | | | |
| Show wash steps | | | | |
| | | | | |
| Double-staining status | | | | Preferred: |



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.

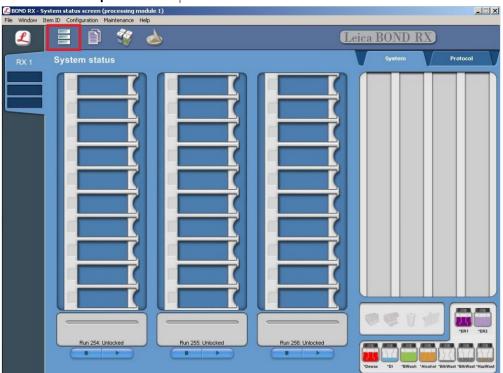
7. Select Save.

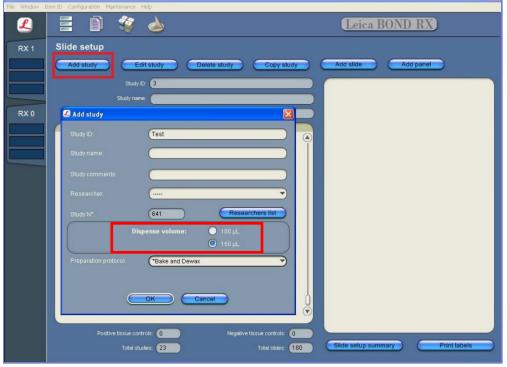


Part 4: Set up a study for sequential ISH-IHC

Build a study

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





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



4. Select **OK**.

Add an ISH and IHC protocol to each slide

1. Select Add slide.

| 尾 BOND RX - Sli | de screen | | | | | | | |
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| File Window It | em ID Configuration M | aintenance H | lelp | | | | | |
| L | | 3 | | | | Leica BC | OND RX | |
| RX 1 | Slide setup | | | | | | | |
| | Add study | Ed | it study | Delete study | Copy study | Add slide (| Add panel | |
| | | Study II | Test Run 1 | | | | | |
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| | | Researche | | | | | | |
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| | Positi | ve tissue cont | | Nacetive | tissue controls: | | | |
| | | | | Negative | | Slide setup summar | Print | abels |
| | | | | | Total slides: 0 | | | |

- 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 | | × |
|---|--|-------------|
| Study name: | Slide ID: Study N*: | 0AIC 852 |
| 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 Researce Res Researce Researce Researce Researce Researce Researce Rese | :h • | |
| Marker: | | |
| Protocols | | |
| Preparation: *Bake | 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.

| Add slide | | | | > | | |
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| Stud | dy name: 🌔 | | | | | |
| Study co | mments: (| | | | | |
| | Study ID: (| ISH-DAB/IHC-C | reen | | | |
| Co | mments: (| tissue TGFB1/0 | CD4 | | | |
| Tissue type: Image: Test tissue Image: Negative tissue Image: Positive tissue | | 0 10 | n se volume: 00 µL 50 µL | | | |
| Staining mode: Sequential D [®] (Research V | | | | | | |
| First | Second | | | | | |
| Process: | 🔘 інс | | 💽 ISH | | | |
| Marker: | (Mock Pro | ibe (ACD) | |) | | |
| Protocols | | | | | | |
| Staining: | ACD 2.5 | DAB TGFB1 no | Hematoxylin | ▼ | | |
| Preparation: | *Bake and Dewax | | | | | |
| HIER: | *ACD HIER 15 min with ER2 (95) | | | | | |
| Enzyme: | *ACD 15 min Protease | | | | | |
| Denaturation: | (* 🔻 | | | | | |
| Hybridization: | ISH Hybr | idization 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 DAB TGFB1 no Hematoxylin for the RNAscope[®] ISH Brown 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 an IHC staining under **Protocols**:
 - a. For Red IHC, select **IHC Protocol J sequential** from the Staining menu. Leave HIER and Enzyme blank.
 - b. For semi-automated Green IHC, select **IHC Protocol Offline Green** from the Staining menu. 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 | Slide properties |
|---|---|
| Slide ID: 0AIC Study Nº: (852 | Silde ID: DAHG Study N": 848 |
| Study name: Study comments: Study D: (ISH-DAB/IHC-Red Comments: Tissue ToFB1/CD4 Dispense volume: • 100 µL • 100 µL • 150 µL | Study name: Study comments: Study D: (SH-DAB/IHC-Green Comments: tissue TOFB1/CD4 Tissue type: Test tissue Postive tissue Postive tissue Postive tissue |
| Staining mode: Sequential DS First Second Process: HC Marker: *CD4 (4B12) | Staining mode: Sequential DS First Second Process: IMC Marker: *CD4 (4B12) |
| Protocols Staning: (IHC Protocol J sequential HIER: ••••• Enzyme: ••••• | Protocols Staining: (IHC Protocol Offline Green HIER: ••••• Enzyme: ••••• |
| Add slide Close | Copy slide OK 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.

IMPORTANT! For semi-automated Green IHC, follow the instructions in Part 5.



Part 5: Detect green IHC staining off the instrument

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.
- 5. 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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