USER MANUAL

# RNAscope™ LS Multiplex Fluorescent Reagent Kit for BDZ 11

For use with Leica Biosystems' BOND RX™ System

**Document Number** UM 322800

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

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**Citing RNAscope in Publications**

When describing a procedure for publication using this product, please refer to it as the RNAscope Assay and cite: Wang F, Flanagan J, Su N, Wang L-C, Bui S, Nielson A, Wu X, Vo H-T, Ma X-J and Luo Y. RNAscope: A Novel *In Situ* RNA Analysis Platform for Formalin-Fixed Paraffin-Embedded Tissues. J. Mol. Diagnostics, 2012, 14:22–29.

**Disclaimers**

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# Contents

# 1 Chapter 1. Product Information


Before using this product, read and understand the information in **Appendix F. Safety** of this document.

**IMPORTANT!** We recommend reading the entire user manual before beginning any protocols.

## About this guide

This user manual provides guidelines and protocols to use the RNAscope LS Multiplex Fluorescent Reagent Kit (Cat. No. 322800 & 323275) for use with Leica Biosystems' BOND RX Research Advanced Staining System. RNAscope LS Multiplex Fluorescent Assays are compatible with a variety of sample types.

## Product description

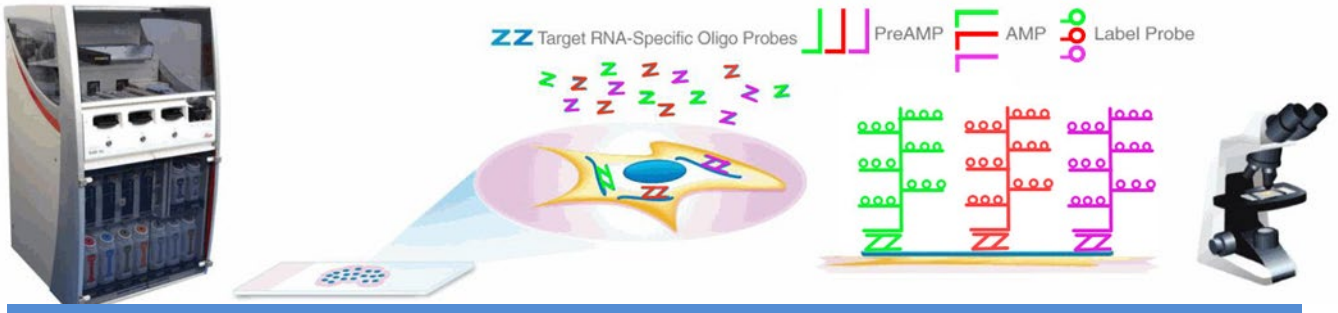
### Background

The RNAscope LS Multiplex Fluorescent Assay uses a novel and proprietary method of *in situ* hybridization (ISH) to simultaneously visualize three RNA targets in formalin-fixed, paraffin-embedded (FFPE) tissue or in fresh frozen samples mounted on slides by fluorescent detection. The assay is based on Advanced Cell Diagnostic's patented signal amplification and background suppression technology, and it can detect RNA molecules in archival samples and partially degraded specimens. The RNAscope LS Multiplex Assay allows users to automate the highly sensitive RNAscope Multiplex Assay using Leica Biosystems' BOND RX System.

### Overview

**Figure 1** on page 6 illustrates the RNAscope LS Multiplex Fluorescent Assay procedure, which can be completed on the instrument in ~14 hours. Starting with properly prepared samples, sections are first pretreated, and then RNA-specific probes are hybridized to target RNA. The RNAscope LS Multiplex Fluorescent Assay employs three independent signal amplification systems each using a different fluorophore, enabling users to investigate expression as well as positional relationship between three different genes within a cellular context. Single RNA transcripts for each target gene appear as punctate dots that are visible using a fluorescent microscope.

**Figure 1**. Procedure overview




| 1: Tissue section | 2: Hybridize to target RNA | 3: Amplify signal | 4: Image |
|---|---|---|---|
| Start with properly prepared sections and load slides onto the instrument. Pretreat tissue to allow access to target RNA. | Hybridize gene-specific probe pairs to the target mRNA. | Use three independent signal amplification systems to detect three target RNAs. Probes are hybridized to a cascade of signal amplification molecules, culminating in the binding of fluorophores. Add three fluorophores to detect RNAs. | Visualize target RNA using a fluorescent microscope. |

# Kit contents and storage

The RNAscope LS Multiplex Fluorescent Assay requires the RNAscope 2.5 LS Probes and the RNAscope LS Multiplex Reagents, available from Advanced Cell Diagnostics.

## RNAscope 2.5 LS Probes

The RNAscope 2.5 LS Probes consist of the user-specified Target Probe and the Positive and Negative Control Probes. Visit **https://acdbio.com/products** to find a gene-specific target probe or appropriate control probes. Each target probe contains a mixture of short oligonucleotides designed to bind to a specific target RNA and detectable in one of three probe channels, C1, C2, or C3.

Channel C1 target probes are Ready-To-Use (RTU), while channels C2 and C3 probes are shipped as a 50X concentrated stock. To independently detect three target RNAs in a multiplex assay, each target probe must be in a different channel. If you are using only the C2 and C3 probes, you can use Probe Diluent (Cat. No. 300048).

Each probe is sufficient for staining ~30 standard slides. The probes have a shelf life of two years from the date of manufacturing when stored as indicated in the following tables:

| Target Probes | | | | | |
|---|---|---|---|---|---|
| ☑ | Reagent | Cat. No. | Content | Quantity | Storage |
| | RNAscope LS Multiplex Target Probe – *[species]* – *[gene]* | Various | Ready-To-Use (RTU) probe for channel C1 | 16 mL x 1 bottle | 2–8°C |
| | RNAscope LS Multiplex Target Probe – *[species]* – *[gene]* – C2 | Various | 50X probe for channel C2 | 320 µL x 1 tube | 2–8°C |
| | RNAscope LS Multiplex Target Probe – *[species]* – *[gene]* – C3 | Various | 50X probe for channel C3 | 320 µL x 1 tube | 2–8°C |

| Control Probes | | | | | |
|---|---|---|---|---|---|
| ☑ | Reagent | Cat. No. | Content | Quantity | Storage |
| | RNAscope 2.5 LS 3-plex Positive Control Probe-Hs | 320868 | RNAscope 2.5 LS Multiplex Positive Control Probe for the RNAscope LS Multiplex Fluorescent Assay - *POLR2A* (C1 channel), *PPIB* (C2 channel), *UBC* (C3 channel). *UBC* has the highest relative expression levels, followed by *PPIB* and *POLR2A* in that order. | 16 mL x 1 bottle | 2–8°C |
| | RNAscope 2.5 LS 3-plex Positive Control Probe-Mm | 320888 | RNAscope 2.5 LS Multiplex Mouse Positive Control Probe for the RNAscope LS Multiplex Fluorescent Assay - *Polr2A* (C1 channel), *Ppib* (C2 channel), *Ubc* (C3 channel). *Ubc* has the highest relative expression levels, followed by *Ppib* and *Polr2A* in that order. | 16 mL x 1 bottle | 2–8°C |
| | RNAscope 2.5 LS Multiplex Positive Control Probe-HeLa Cells | 320838 | RNAscope 2.5 LS Multiplex Positive Control Probe for the RNAscope LS Multiplex Fluorescent Assay - *TBP* (C1 channel), *PPIB* (C2 channel), *POLR2A* (C3 channel), specific for HeLa cells. | 16 mL x 1 bottle | 2–8°C |
| | RNAscope 2.5 LS 3-plex Negative Control Probe | 320878 | RNAscope 2.5 LS Multiplex Negative Control Probe for the RNAscope LS Multiplex Fluorescent Assay - *dapB* (*Bacillus subtilis* strain). | 16 mL x 1 bottle | 2–8°C |

Different fluorophores are assigned to the C1, C2, and C3 channels depending on the TSA Vivid™, Opal™ or TSA® plus fluorophore selected for that channel.

**Note:** Tyramide linked fluorophores are assigned to the C1, C2, and C3 channels, depending on the particular RNAscope Assay. The fluorophore channels recommended for the RNAscope LS Multiplex Fluorescent Assay are shown in the following table:

| Probe Channel ID | Fluorophore Labels | | | |
|---|---|---|---|---|
| | Enzyme | Recommended | Compatible | Compatible |
| C1* | HRP | TSA Vivid Fluorophore 520 | Opal 520 | Opal 570 |
| C2 | HRP | TSA Vivid Fluorophore 570 | Opal 570 | Opal 690 |
| C3 | HRP | TSA Vivid Fluorophore 650 | Opal 690 | Opal Polaris 780 |

* Default channel

**Note:** If you assign Opal 690 to the C1 or C2 channel, you may need to increase the concentration of Opal 690. If you use Opal Polaris 780, develop it last.

### RNAscope LS Multiplex Reagents

The RNAscope LS Multiplex Fluorescent Reagent Kit (Cat. No. 322800) contains all the reagents needed to run the RNAscope LS Multiplex Fluorescent Assay on Leica Biosystems' BOND RX System, except for the RNA-specific probes, Opal dyes or TSA Plus fluorophores, and mounting medium. The kits provide enough reagents to stain ~60 standard slides.

The reagents are Ready-To-Use (RTU) except for the TSA buffer and are stored as indicated in the following tables:

| RNAscope LS Multiplex Reagent Kit (Cat. No. 322800) | | | |
|---|---|---|---|
| ☑ | **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 Rinse | 29 mL x 2 bottles | 2–8°C |
| | RNAscope LS Multiplex AMP 1 | 21 mL x 1 bottle | 2–8°C |
| | RNAscope LS Multiplex AMP 2 | 21 mL x 1 bottle | 2–8°C |
| | RNAscope LS Multiplex AMP 3 | 21 mL x 1 bottle | 2–8°C |
| | RNAscope LS Multiplex HRP C1 | 21 mL x 1 bottle | 2–8°C |
| | RNAscope LS Multiplex HRP C2 | 21 mL x 1 bottle | 2–8°C |
| | RNAscope LS Multiplex HRP C3 | 21 mL x 1 bottle | 2–8°C |
| | RNAscope LS Multiplex TSA Buffer | 29 mL x 3 bottles | 2–8°C |
| | RNAscope LS Multiplex HRP Blocker | 29 mL x 2 bottles | 2–8°C |
| | RNAscope LS Multiplex DAPI | 21 mL x 1 bottle | 2–8°C |

### RNAscope LS Multiplex Reagents with TSA Vivid Dyes

The RNAscope LS Multiplex Fluorescent Reagent Kit with TSA Vivid Dyes (Cat No. 323275) contains all the reagents listed above with the addition of the TSA Vivid fluorophores in the 520, 570 and 650 channels. RNA-specific probes and the mounting medium are required to run the assay. The kits provide enough reagents to stain ~60 standard slides. To perform a 3-plex assay, use TSA Vivid 520, 570, and 650.

| TSA Vivid Fluorophores* (supplied with Cat. No. 323275) | | | | |
|---|---|---|---|---|
| ☑ | **Fluorophores** | **Part number** | **Recommended dilution range** | **Reagent registration name** |
| | TSA Vivid Fluorophore 520 | 323271 | 1:750-1:3000 | ACD Multiplex TSA-F1 |
| | TSA Vivid Fluorophore 570 | 323272 | 1:750-1:3000 | ACD Multiplex TSA-F2 |
| | TSA Vivid Fluorophore 650 | 323273 | 1:750-1:3000 | ACD Multiplex TSA-F3 |

* 1. Reconstitute the TSA Vivid reagent with 100 µL Dimethylsulfoxide (DMSO). 2. Recommended working dilution range: 1:750 - 1:3000. We recommend starting with a dilution of 1:1500 and adjusting based on signal intensity.

# Additional materials and equipment

## Opal dyes or TSA Plus fluorophores – required if using the RNAscope LS Multiplex Fluorescent Reagent Kit (Cat. No. 322800)

The RNAscope LS Multiplex Fluorescent Reagent Kit (Cat. No. 322800) requires purchase of Opal dyes or TSA Plus fluorophores from Akoya Biosciences. Dilute the fluorophores in TSA buffer provided in the RNAscope LS Multiplex Fluorescent Reagent Kit. The Opal Polaris 780 Reagent Pack contains two reagents: Opal TSA-DIG and Opal Polaris 780. We recommend diluting Polaris TSA-DIG in TSA buffer, and diluting Opal Polaris 780 in either Primary Antibody Diluent BOND from Leica (PN: AR9352) or Antibody Diluent/Block from Akoya Biosciences (PN: ARD1001EA). Choose a dilution factor for each fluorophore based on recommendations from ACD and your specific experimental conditions such as target expression levels, tissue quality, or microscope setting. Materials are qualified with 1:1500 dilution for all three fluorophores. We cannot guarantee assay results if you use other fluorescent dyes.

To perform a 3-plex assay, use Opal 520, 570, and 690. When autofluorescence in the FITC channel is a concern and a Cy7 filter is available, use Opal Polaris 780 instead of Opal 520.

| Fluorophores* | Part number (Akoya Biosciences) | Recommended dilution range | Reagent registration name |
|---|---|---|---|
| Opal 520 | FP1487001KT | 1:750-1:3000 | ACD Multiplex TSA-F1 |
| Opal 570 | FP1488001KT | 1:750-1:3000 | ACD Multiplex TSA-F2 |
| Opal 690 | FP1497001KT | 1:750-1:3000 | ACD Multiplex TSA-F3 |
| Opal 780 Polaris Reagent Pack | FP1501001KT | Opal TSA-DIG: 1:750–1:3000 | TSA-DIG |
| | | Opal Polaris 780: 1:187.5–1:750 | Polaris 780 |

* Reconstitute all Opal dyes (except Opal Polaris 780) with 75 µL Dimethylsulfoxide (DMSO). Reconstitute Opal Polaris 780 with 300 µL double distilled water (ddH2O).

You may replace an Opal dye with the compatible TSA Plus fluorophore. If you are performing a 4-plex assay, refer to the *RNAscope LS 4-plex Fluorescent Assay Technical Note* (Doc. No. 322380-TN) available at **https://acdbio.com/technical-support/user-manuals.**

## Required materials and equipment from Leica BOND RX

The RNAscope LS Multiplex Fluorescent Assay requires specific materials and equipment available *only* from Leica Biosystems.

| ☑ | Component | Cat. No. | Storage |
|---|---|---|---|
| | Leica Biosystems' BOND RX System — automated slide stainer | — | — |
| | BOND 30 mL Open containers | OP309700 | Room temp (20–25°C) |
| | BOND 7 mL Open containers* | OP79193 | Room temp (20–25°C) |
| | BOND Research Detection System | DS9455 | Room temp (20–25°C) |
| | BOND Universal Covertile | S21.4611 | Room temp (20–25°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) |

* (Optional) Recommended for use with Opal dyes.

## Other materials

**IMPORTANT!** Do not substitute other materials for the SuperFrost® Plus Slides listed in the following table.

| ☑ | Description | Supplier | Cat. No. |
|---|---|---|---|
| | SuperFrost Plus Slides (required) | Fisher Scientific | 12-550-15 |
| | 95% Ethanol (EtOH) | American Master Tech Scientific/MLS* | ALREACS |
| | Xylene | Fisher Scientific/MLS | X3P-1GAL |
| | 10% neutral-buffered formalin (NBF) | MLS | — |
| | Paraffin wax | MLS | — |
| | 1X PBS | MLS | — |
| | Microtome | MLS | — |
| | Drying oven, capable of holding temperature at 60 +/– 1°C (optional) | MLS | — |
| | Water bath or incubator, capable of holding temperature at 40 +/– 1°C | MLS | — |
| | Opal dyes or TSA Plus fluorophores | Akoya Biosciences | — |
| | Either Primary Antibody Diluent BOND or<br>Antibody Diluent/Block (if Opal Polaris 780 is used) | Leica Biosystems<br>Akoya Biosciences | AR9352<br>ARD1001EA |
| | Prolong Gold Antifade | Thermo Fisher | P36930; P10144; P36934 |
| | Tissue-Tek® Vertical 24 Slide Rack | American Master Tech Scientific/MLS | LWSRA24 |
| | Tissue-Tek Staining Dish (4 required) | American Master Tech Scientific/MLS | LWT4457EA |
| | Tissue-Tek Clearing Agent Dish, xylene resistant (2 required) | American Master Tech Scientific/MLS | LWT4456EA |
| | Cover Glass 24 x 50 mm | Fisher Scientific/MLS | 12-545-F |
| | Distilled water | MLS | — |
| | Fume hood | MLS | — |

* Major Laboratory Supplier in North America. For other regions, please check Catalog Numbers with your local lab supplier.

# 2 Chapter 2. Before You Begin

Prior to running the RNAscope LS Multiplex Fluorescent Assay on your samples for the first time, we recommend that you:

- Become familiar with Leica Biosystems' BOND RX Research Advanced Staining System. Refer to the *Leica Biosystems' BOND RX System Instructions For Use*.
- Run the assay on RNAscope Control Slides (Cat. No. 310045 for Human HeLa Cell Pellet, and Cat. No. 310023 for Mouse 3T3 Cell Pellet) using the RNAscope 2.5 LS Positive and Negative Multiplex Control Probes.

## Important procedural guidelines

- Start with properly fixed and prepared sections. Refer **to Chapter 3. Prepare and Pretreat Samples** for preparation of FFPE slides. For preparation of other sample types, contact **support.acd@bio-techne.com**.
- Regularly maintain and clean your automated staining instrument.
- Always run positive and negative control probes on your sample to assess sample RNA quality and optimal permeabilization.
- Do not substitute required materials. Assay has been validated with these materials only.
- Follow the protocol exactly for best results.
- Do not let your sections dry out during the procedure.
- Use good laboratory practices and follow all necessary safety procedures. Refer to **Appendix F. Safety** for more information.

# 3 Chapter 3. Prepare and Pretreat Samples

The following protocols describe formalin-fixed, paraffin-embedded (FFPE) sample preparation and pretreatment.

**IMPORTANT!** We highly recommend following these guidelines. We cannot guarantee assay results with other preparation methods.

## Prepare FFPE sections

### Materials required

- 10% neutral buffered formalin (NBF)
- 1X PBS
- Paraffin wax
- 95% Ethanol (EtOH)
- Xylene
- Microtome
- Water bath
- SuperFrost Plus slides

### Fix the sample

1. Immediately following dissection cut the tissue into blocks of 3–4 mm in thickness.
2. Place the tissue blocks into fixative within **1 HR** of biopsy.
3. Fix the tissue in 10% NBF for **16–32 HRS** at **ROOM TEMPERATURE (RT)**. Fixation time will vary depending on tissue type and size.

⚠ CAUTION! Handle biological specimens appropriately.

**IMPORTANT!** Fixation for **<16 HRS** or **>32 HRS** will impair the performance of the RNAscope LS Multiplex Fluorescent Assay.

### Dehydrate, embed, and cut the sample

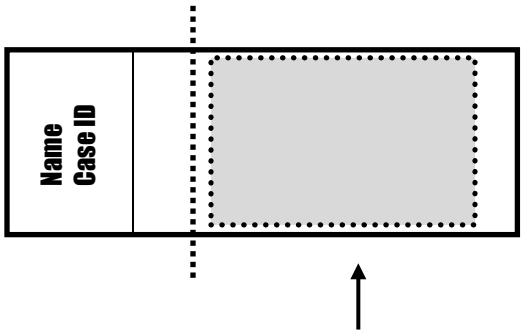
**IMPORTANT!** Use fresh reagents.

4. Wash sample with 1X PBS.
5. Dehydrate sample using a standard ethanol series, followed by xylene.
6. Embed sample in paraffin using standard procedures.

**Note:** Store embedded samples at room temperature with desiccation. To better preserve RNA quality over a long period (>1 yr), we recommend storing at 2–8°C with desiccation.

7. Trim paraffin blocks as needed and cut embedded tissue into 5 +/– 1 µm sections using a microtome.

8. Place the paraffin ribbon in a **40–45°C** water bath, and mount the sections on **SUPERFROST PLUS SLIDES.** Place tissue as shown for optimal staining:




**Tissue section location**

**IMPORTANT!** Do not mount more than one section per slide. Place each section in the center of the slide.

9. Air dry the slides **OVERNIGHT** at **RT**.

OPTIONAL STOPPING POINT. Use sectioned tissue within three months. Store sections with desiccants at room temperature.

# 4 Chapter 4. Set Up the BDZ 11 Software

**IMPORTANT!** We strongly recommend you run the RNAscope Control Slides (Cat. No. 310045 or Cat. No. 310023) using the RNAscope 2.5 LS positive and negative control probes along with your samples in every run.

## Workflow

**Register the reagents**

⬇

**Create and register the BOND Research Detection System**

⬇

**Create a one-minute probe hybridization protocol (ONE TIME ONLY)**

⬇

**Create a staining protocol (ONCE FOR EACH PROBE)**

⬇

**Register the mock probe (ONE TIME ONLY)**

⬇

**Dilute TSA Vivid/Opal fluorophores in the TSA buffer**

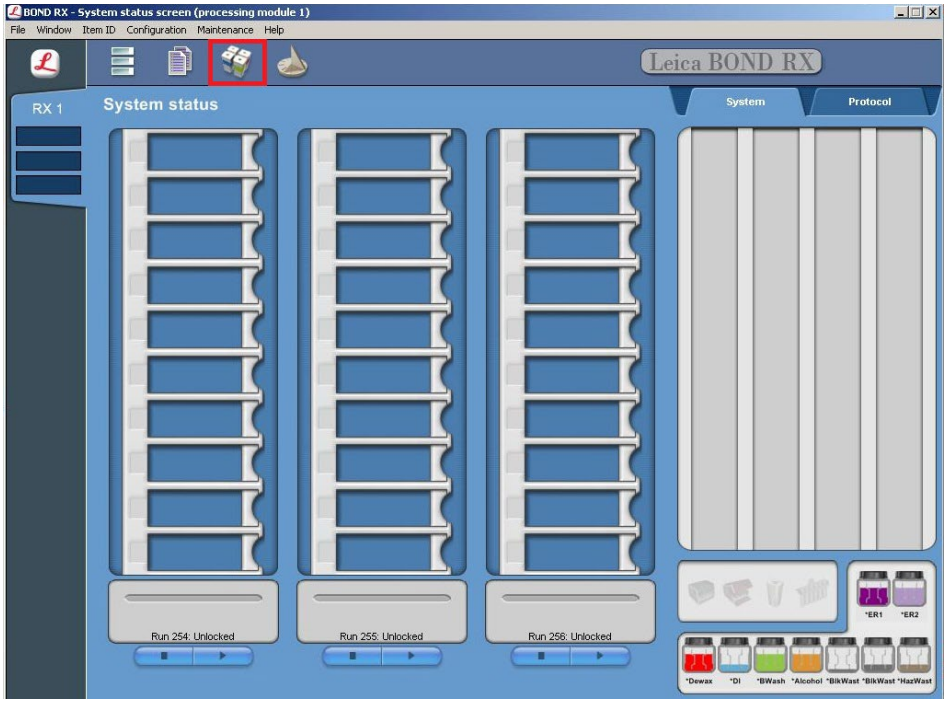
⬇

**Set up a study for the multiplex assay**

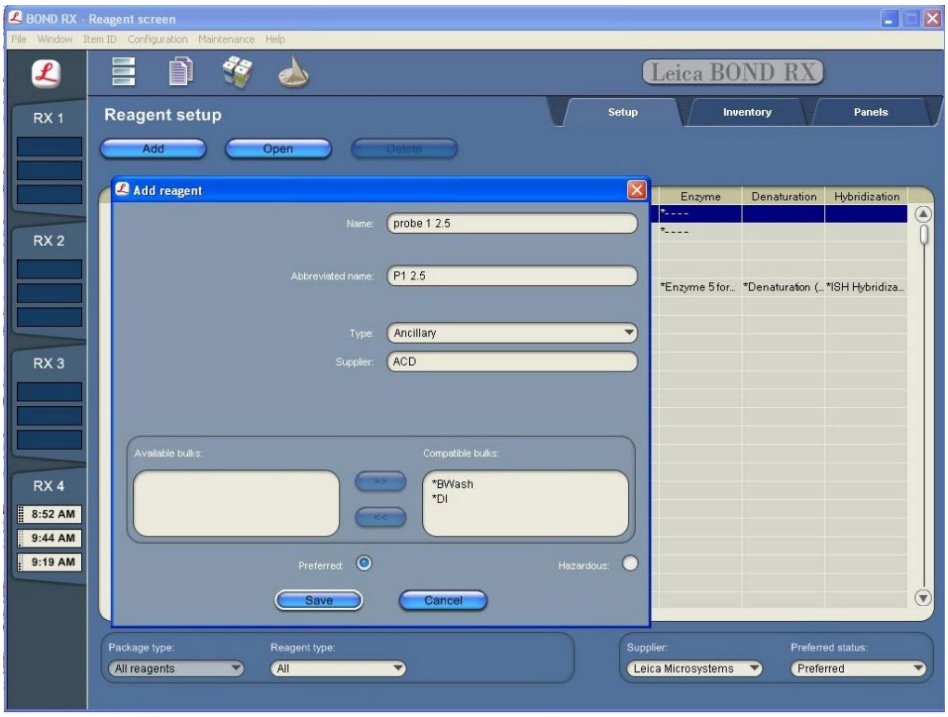
# Register the reagents

This step is a "workaround" to the existing BDZ 11 software to accommodate the RNAscope LS Multiplex Fluorescent Assay. Your ACD Field Application Specialist (FAS) should implement this procedure. In summary, a probe is created as an ancillary reagent and added to the staining protocol.

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




2. Select **Add** to enter reagent information.
3. To create probe, enter a name in the Name text box.




4. Enter **P1 2.5** (for example) in the Abbreviated name text box.
5. Select **Ancillary** in the Type drop-down menu.
6. Enter **ACD** in the Supplier text box.
7. Check both the Preferred and Hazardous boxes (for probes only).

**IMPORTANT!** Only probes are marked hazardous. RNAscope Amp reagents do not require that designation.

8. Select **Save**.
9. Repeat Steps 2–7 to register the rest of the reagents using the container names in the following table:

| Reagents | Container Name |
|---|---|
| RNAscope LS Multiplex AMP 1 | ACD Multiplex Amp 1 |
| RNAscope LS Multiplex AMP 2 | ACD Multiplex Amp 2 |
| RNAscope LS Multiplex AMP 3 | ACD Multiplex Amp 3 |
| RNAscope LS Multiplex HRP C1 | ACD Multiplex HRP-C1 |
| RNAscope LS Multiplex HRP C2 | ACD Multiplex HRP-C2 |
| RNAscope LS Multiplex HRP C3 | ACD Multiplex HRP-C3 |
| RNAscope LS Multiplex HRP Blocker | ACD Multiplex HRP blocker |
| TSA Vivid/Opal-fluorophore 1 (user to dilute in TSA buffer) | ACD Multiplex TSA-F1 |
| TSA Vivid/ Opal-fluorophore 2 (user to dilute in TSA buffer) | ACD Multiplex TSA-F2 |
| TSA Vivid/ Opal-fluorophore 3 (user to dilute in TSA buffer) | ACD Multiplex TSA-F3 |
| Opal TSA-DIG (if Opal Polaris 780 is used) | TSA-DIG |

| Reagents | Container Name |
|---|---|
| Opal Polaris 780 (if Opal Polaris 780 is used) | Polaris 780 |
| RNAscope LS Multiplex DAPI | DAPI |
| BOND Wash | Bond Wash |

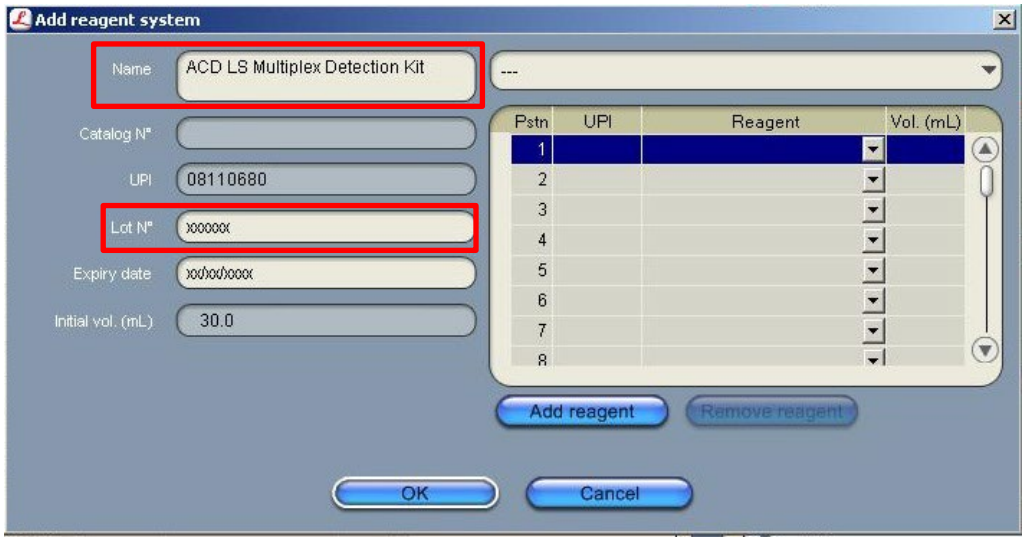
* Indicates step is hard-coded in the software by Leica Biosystems.

## Create and register the BOND Research Detection System

A BOND Research Detection System from Leica is required to setup the RNAscope LS Multiplex Fluorescent Assay. Your ACD Field Application Specialist (FAS) should implement this procedure. Each detection system barcode is valid for up to 40 mL of use (equivalent to ~260 slides or four RNAscope LS Multiplex Fluorescent Reagent Kits).

1. Scan the barcode on the tray of a new BOND Research Detection System.
2. To setup a new detection system for the assay, enter **ACD LS Multiplex Detection Kit** in the Name text box and enter the lot number of the RNAscope LS Multiplex Fluorescent Reagent Kit.

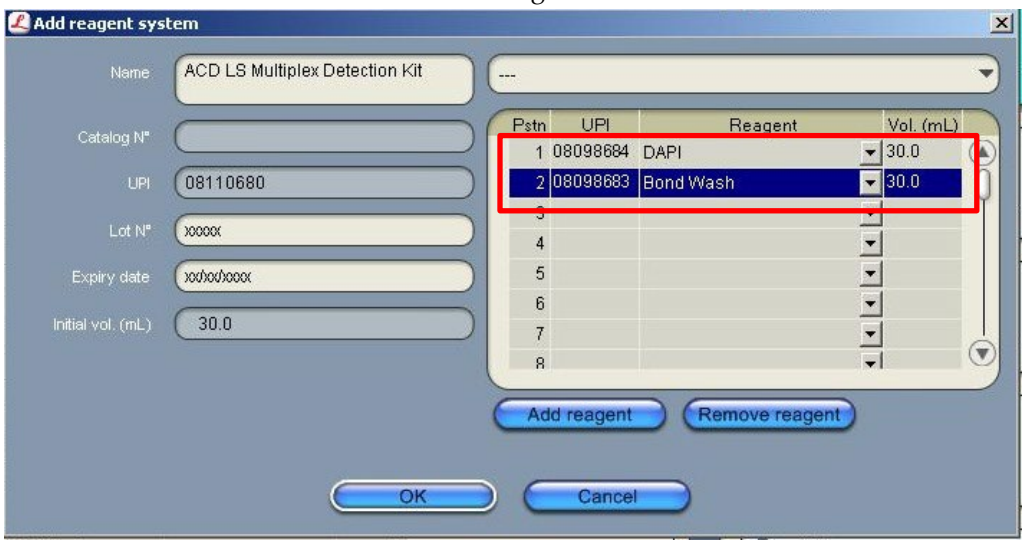
**Note:** Creating the detection system needs to be performed only once on each BOND RX controller.



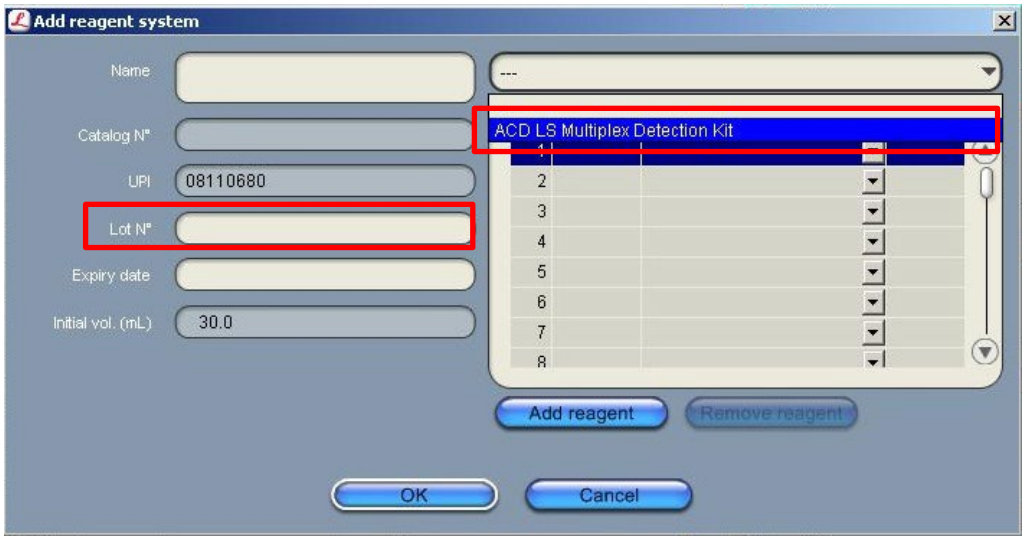

3. Place two new BOND 30 mL Open containers on the Research Detection System rack.
4. Scan the first container and select the registration name **DAPI**. You can mix different lots of DAPI in the same container.

**Note:** If you prefer not to use DAPI on the instrument or want to perform immunohistochemistry (IHC) steps after the assay, you may use BOND Wash in place of DAPI in the protocol.

5. Scan the second container and select the registration name **Bond Wash**.

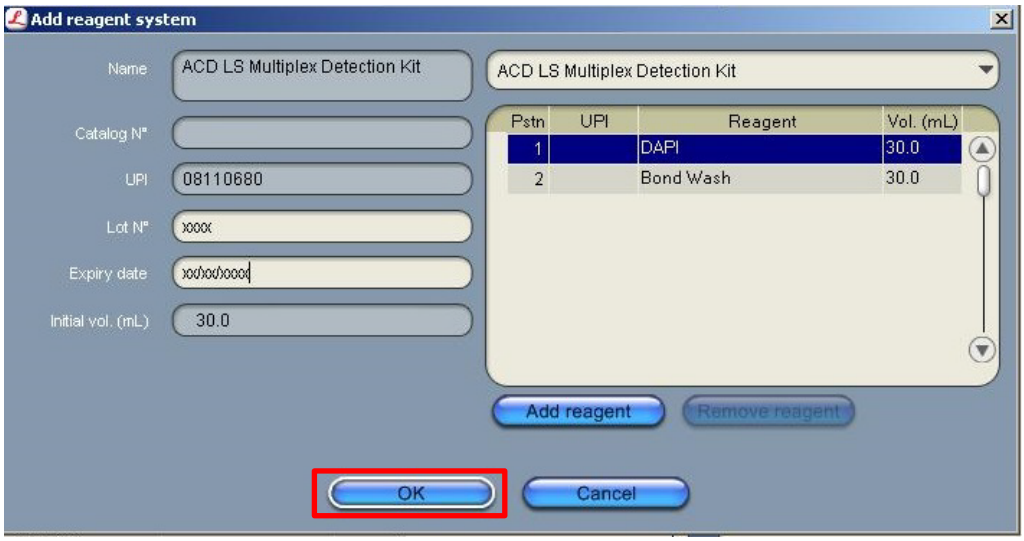



6. When one Research Detection System is finished (up to 40 mL), register a new detection system by scanning the barcode on the tray and select **ACD LS Multiplex Detection Kit** from the drop down menu on the right. Enter the new lot number.




7. Register two new 30 mL containers for DAPI and BOND Wash by first selecting the reagent name and then scanning the barcode on the container.

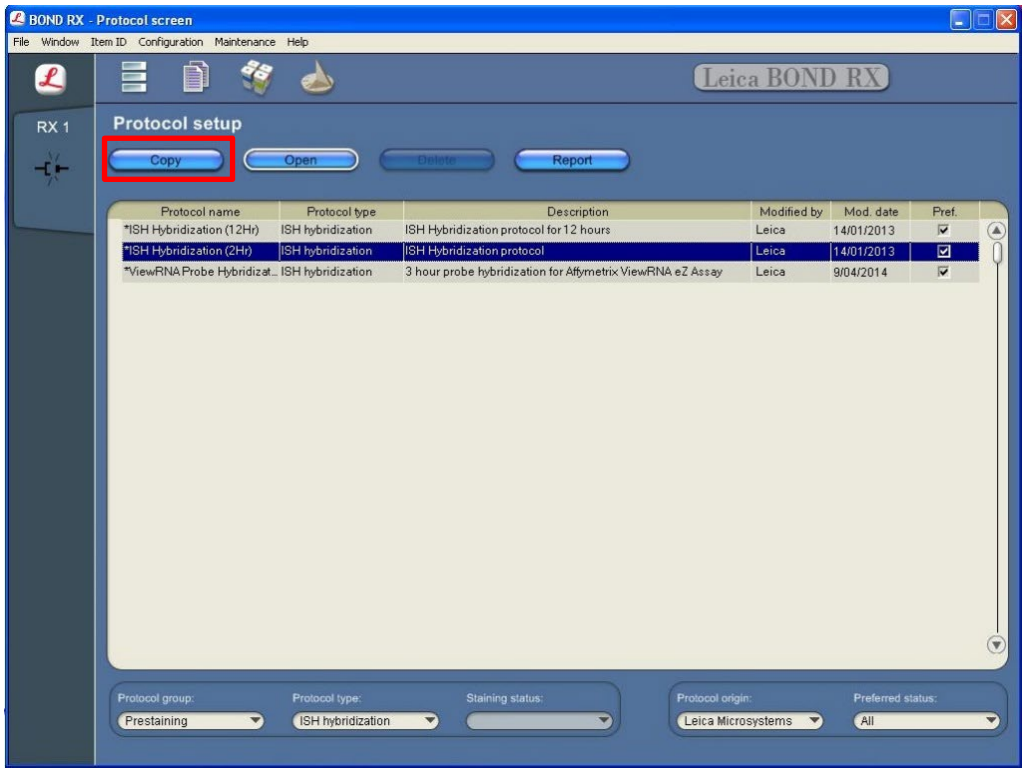
8. Select **OK**.



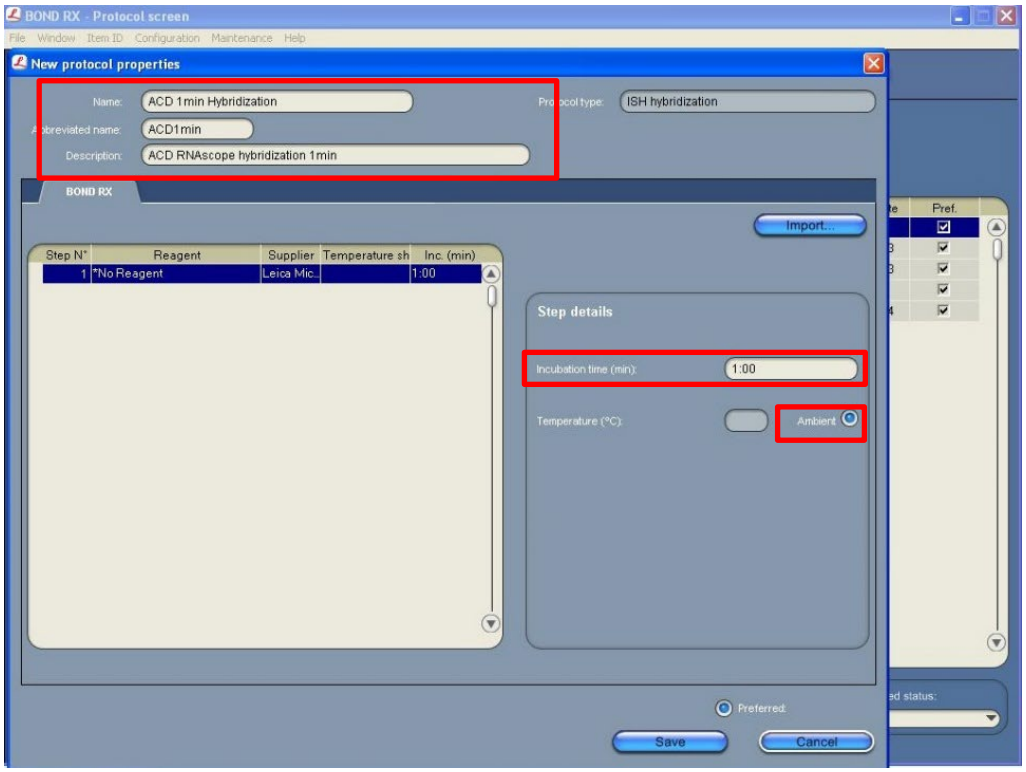

# Create a one-minute probe hybridization protocol

A mock probe hybridization step must be created as part of BDZ 11 software workaround for the RNAscope LS Multiplex Fluorescent Assay. The following example copies the existing two hour hybridization protocol and changes the incubation time to one minute.

1. In the Protocol setup screen, select **ISH hybridization** under the Protocol type menu.
2. Highlight the ***ISH Hybridization (2Hr)** protocol. Select **Copy.**




3. Change the Name to **ACD 1min Hybridization**, the Abbreviated Name to **ACD1min**, and the Description to **ACD RNAscope hybridization 1min**.

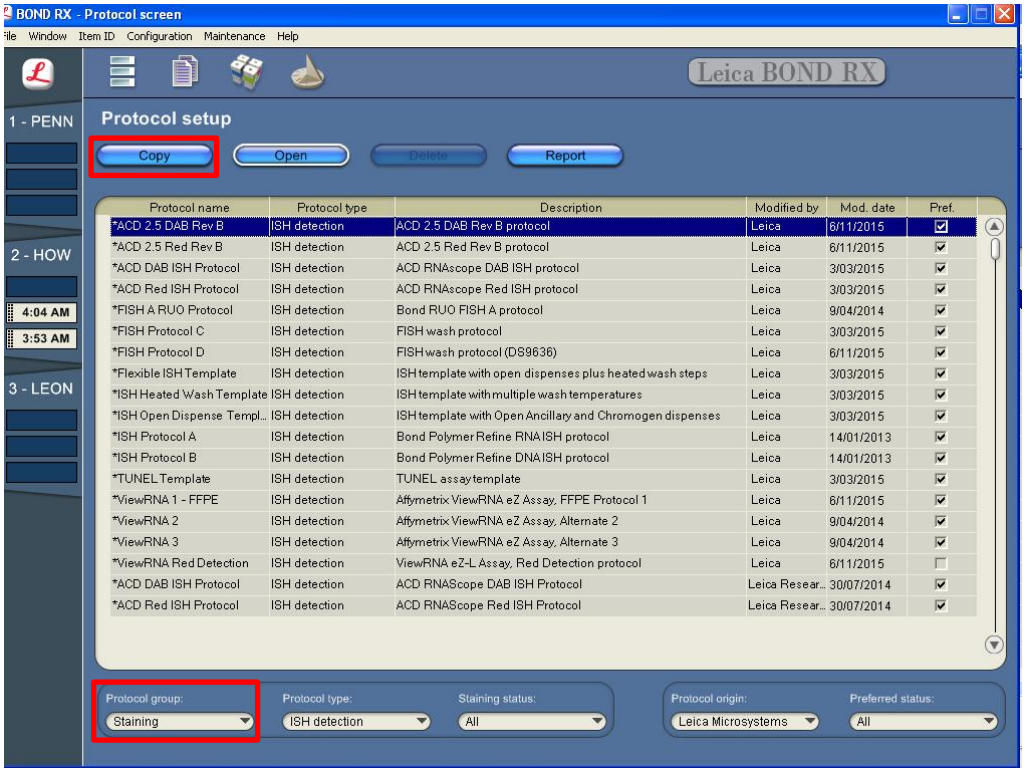



4. Highlight the *No Reagent step.
5. Change the incubation time to **1 MIN** and select **Ambient** as Temperature (°C).
6. Select **Save**.

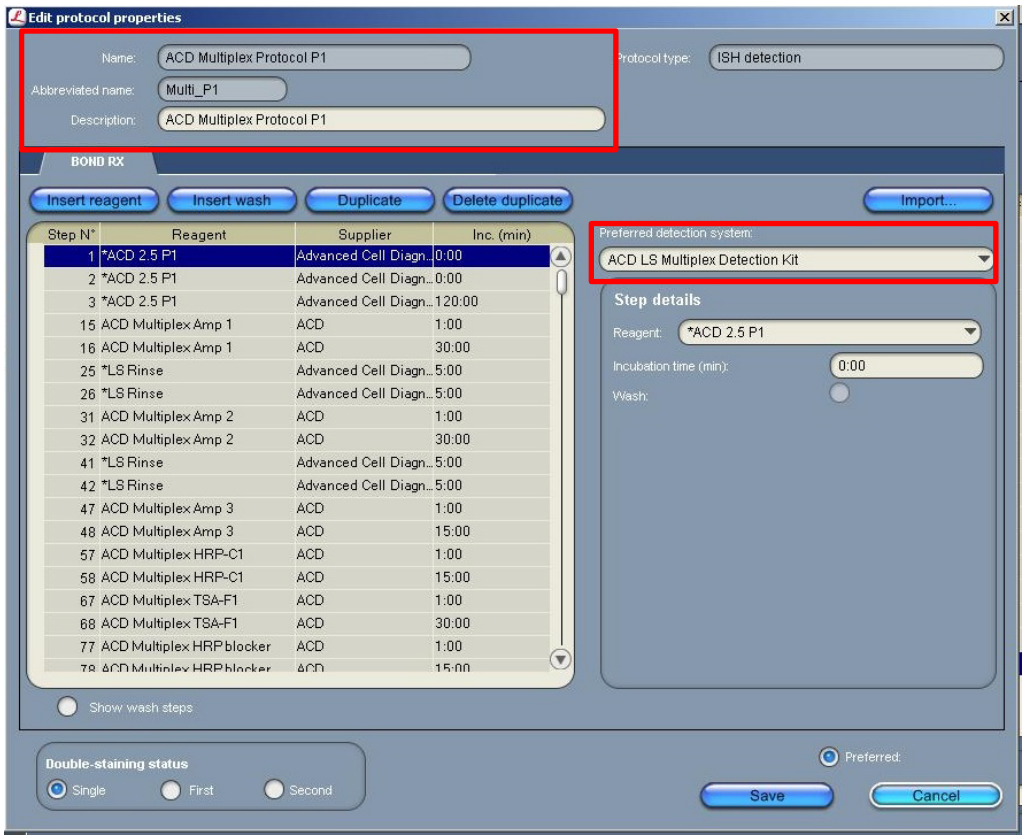
# Create a staining protocol

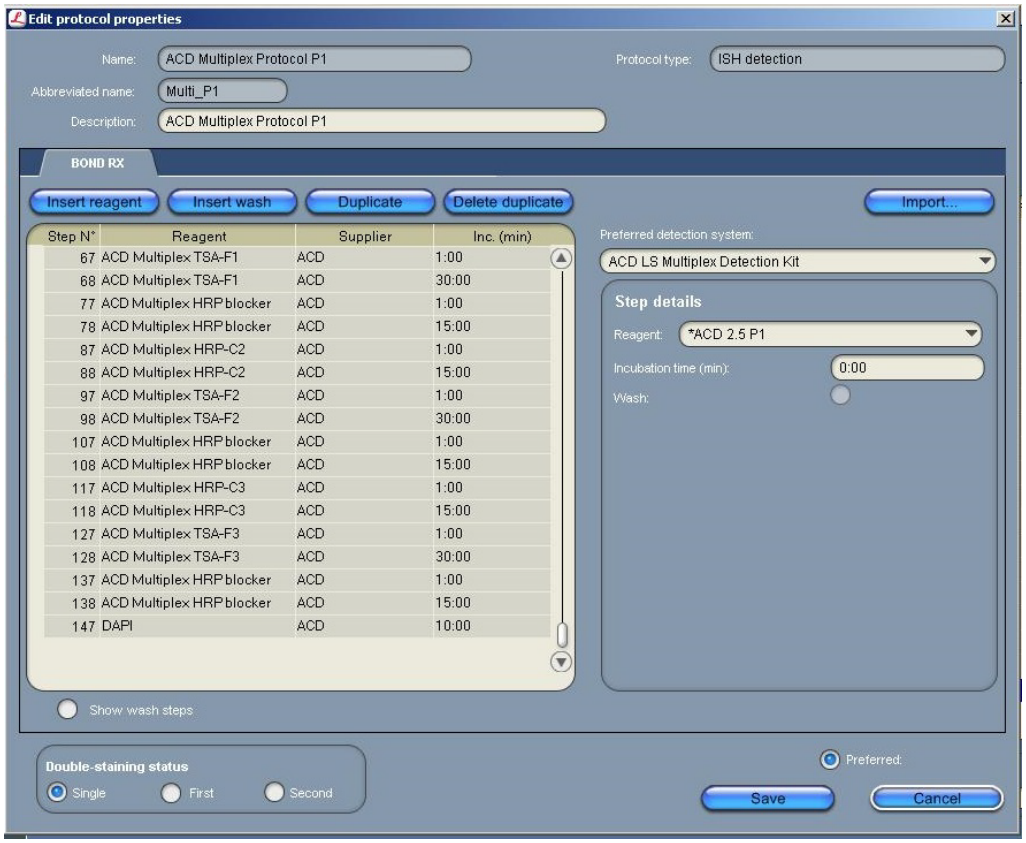
Due to the BDZ 11 software workaround for the RNAscope LS Multiplex Fluorescent Assay, unique staining protocols *must be created for each probe*. Your ACD Field Application Specialist (FAS) should implement this procedure.

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.**




3. For the following steps, refer to the next figure:
   a. Change the protocol name for your first probe to **ACD Multiplex Protocol P1** in the Name text box, **Multi_P1** in the Abbreviated name text box, and **ACD Multiplex Protocol P1** in the Description text box.
   b. Select **ACD LS Multiplex Detection Kit** from the Preferred detection system menu.



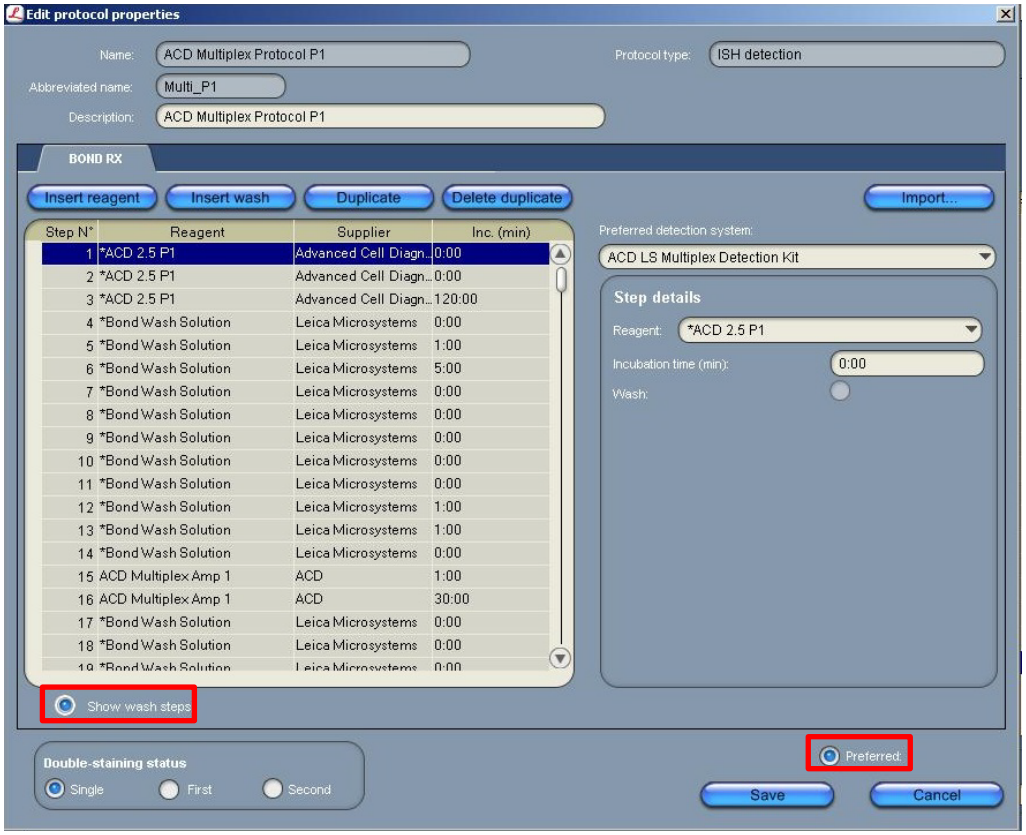




**Note:** The preceding two figures display all reagent steps.

4. Highlight and select each step to edit. See **Appendix A. Multiplex Protocol** for the full protocol.

**IMPORTANT!** You can only change the incubation times, not the temperatures, of these steps.

5. Click Show wash steps to also view the wash steps. Insert BOND Washes to match each of the protocol steps shown.
6. Compare and confirm the on-screen protocol with the protocol listed in **Appendix A. Multiplex Protocol**. If you use Opal Polaris 780, refer to **Appendix B. Multiplex Protocol Using Opal Polaris 780** instead.
7. Select **Preferred** in the bottom right corner of the window.



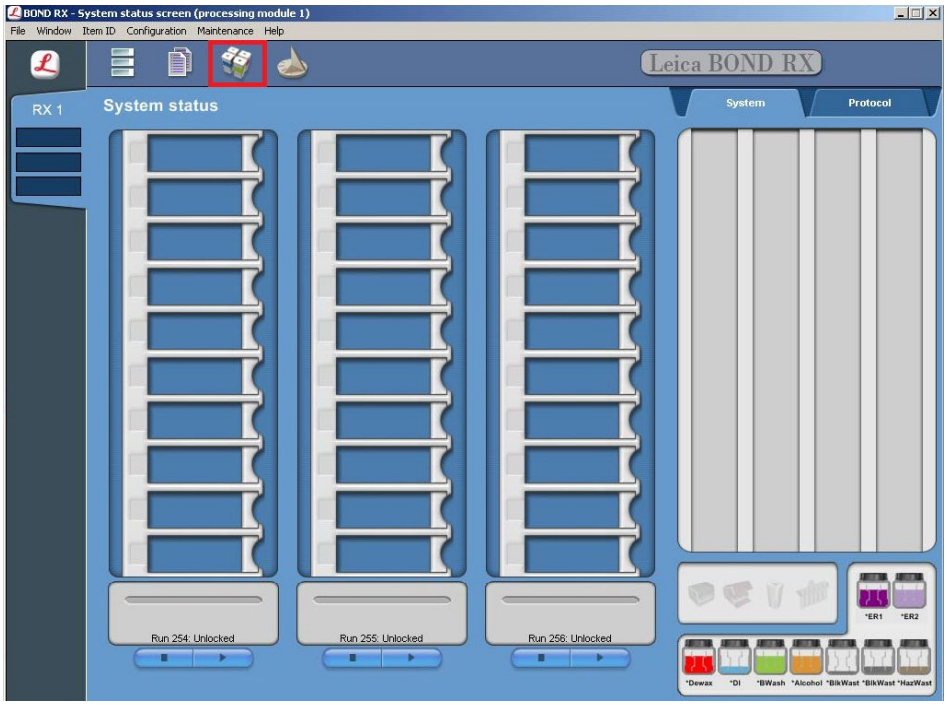

8. Select **Save**.
9. Click **Next** to proceed. Ignore any pop-ups that may appear on the screen.
10. Create a new probe protocol.

**Note:** You must create a new protocol for each new probe you use.

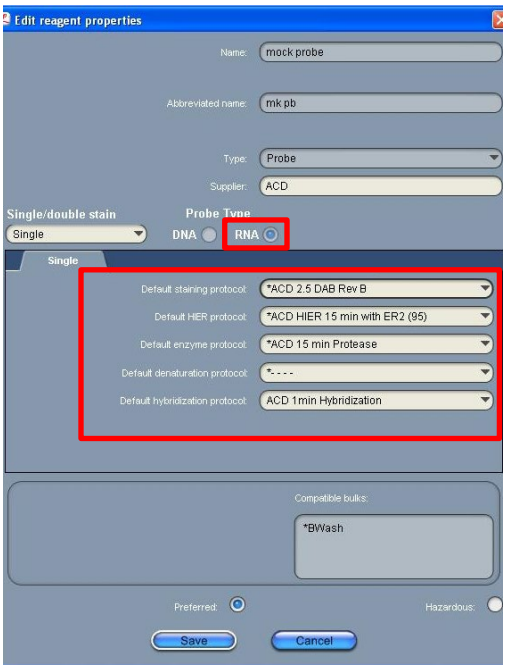
# Register the mock probe

Create a mock probe in the reagent set up.

1. Click the **Reagent setup** icon.



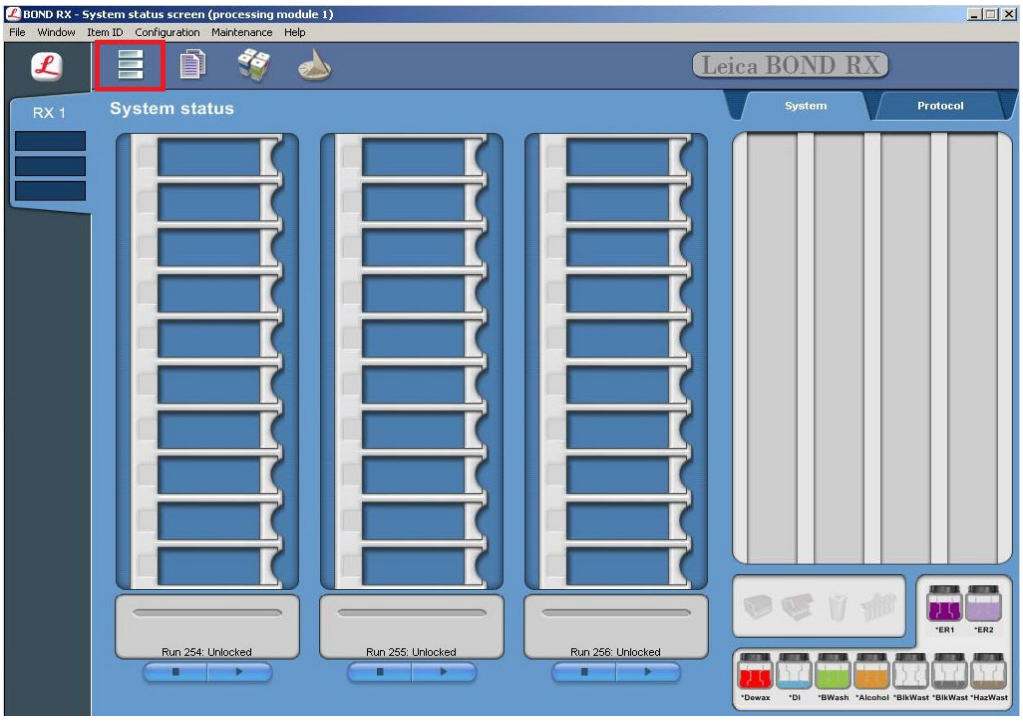

2. Select **Add**.
3. Enter the mock probe in the Name and Abbreviated name text boxes.
4. Select **Probe** in the Type drop-down menu. Enter **ACD** in the Supplier text box.
5. Check **RNA** for Probe Type.
6. Select ***ACD 2.5 DAB RevB** (or your most frequently used protocol) as the Default staining protocol.
7. Select ***ACD HIER 15min with ER2 (95)** as the Default HIER protocol.
8. Select ***ACD 15min Protease** as the Default enzyme protocol.
9. Leave the Default denaturation protocol blank.
10. Select **ACD 1 min Hybridization** as the Default hybridization protocol.
11. Select **Save**.



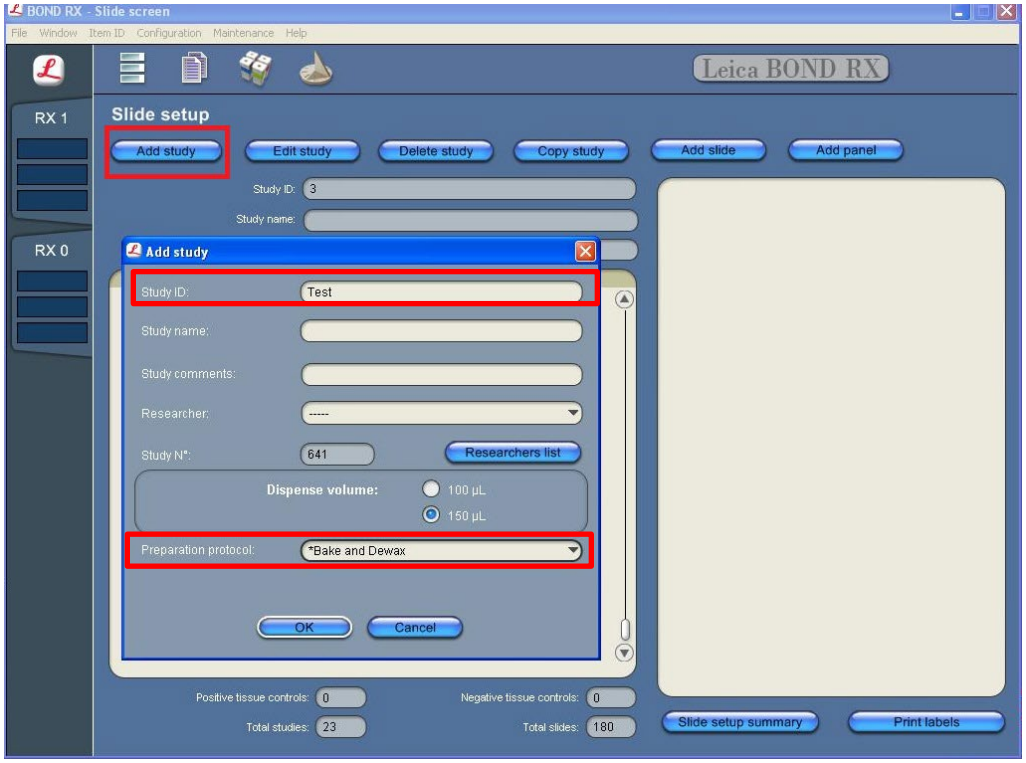

## Set up a study for the multiplex assay

**IMPORTANT!** To be able to use all three trays for the multiplex assay (three channels), strictly follow the protocol steps listed in **Appendix A. Multiplex Protocol**. Adding any additional steps will prevent the system from running all three trays.

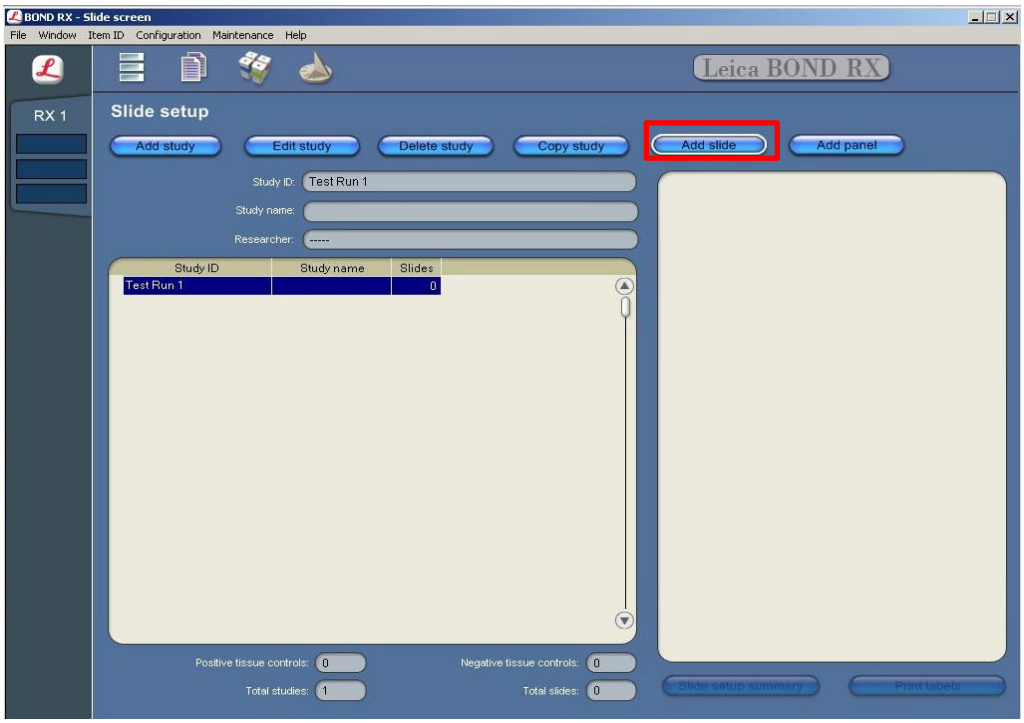
1. To build a study for the LS Multiplex Fluorescent Assay, select the **Slide setup** icon at the top of the screen.



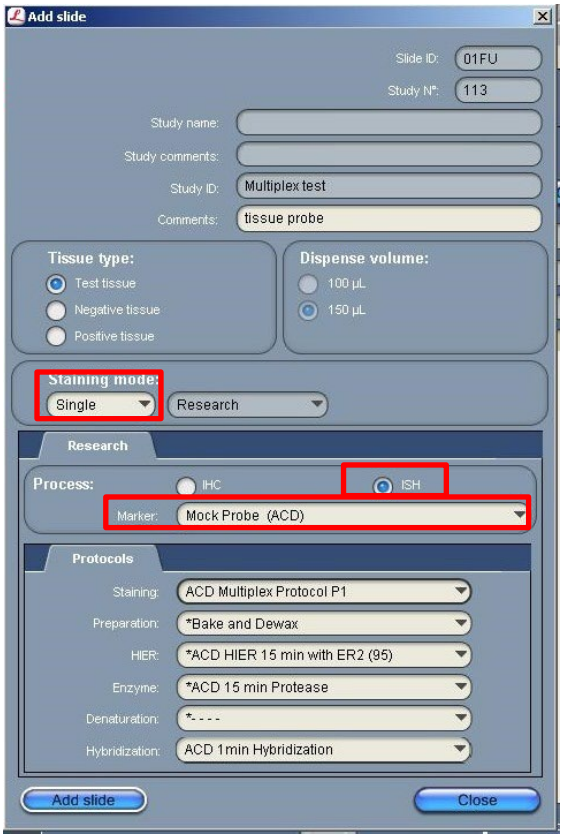
2. Select **Add study** and enter a name in the Study ID field (keep the Dispense volume at 150 µl as shown).




3. For FFPE tissues, select ***Bake and Dewax** as the Preparation protocol. For fresh frozen tissues, select ***----** instead of *Bake and Dewax.
4. Select **OK**.
5. Select **Add slide** to assign a protocol to each slide.



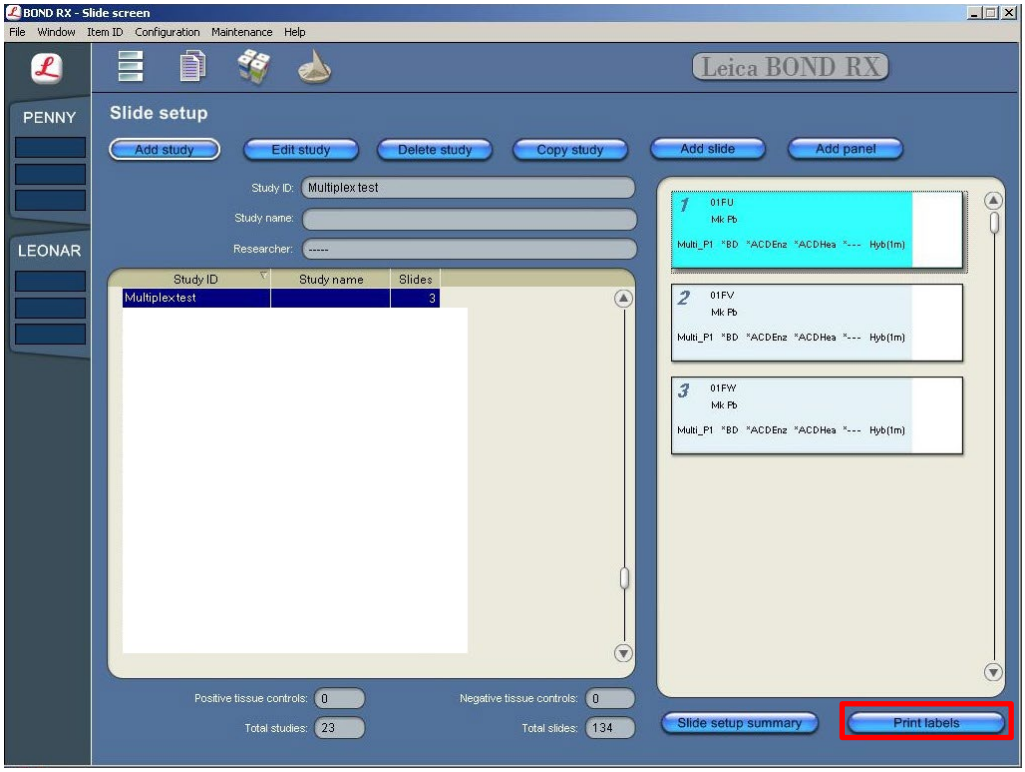

6. Enter the tissue type and probe name under the Comments field.
7. Keep **Single** as default from the Staining mode drop down menu.
8. Select **ISH** under Process and **mock probe (ACD)** from the Marker drop down menu.




9. Under the Protocols tab, do the following:

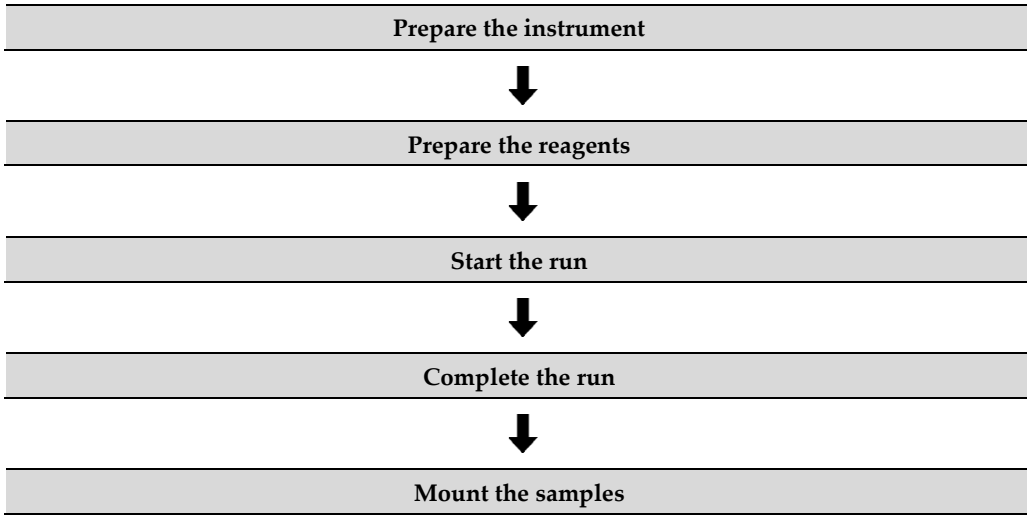
a. For each distinct probe, select a different protocol from the Staining drop down menu (for example, ACD Multiplex Protocol P1).
b. For standard FFPE tissues, select the protocol ***Bake and Dewax** from the Preparation drop down menu. For fresh frozen tissues, select ***----** instead of *Bake and Dewax.
c. Select ***ACD HIER 15 min with ER2 (95)** as the HIER protocol or the appropriate HIER protocol for your tissue.
d. Select ***ACD 15 min Protease** for Enzyme, or the appropriate enzyme protocol for your tissue.
e. Select **ACD 1 min Hybridization** for Hybridization.

10. Select **Add slide** for each target probe and for each of the slides used in the run.
11. After adding all the slides to the study, select **Close** to return to the Slide setup screen.
12. Select **Print labels** to print barcodes to attach to the slides.



# 5 Chapter 5. Run the RNAscope LS Multiplex Fluorescent Assay

## Workflow



# Materials required

| Materials provided by Advanced Cell Diagnostics | Materials provided by Leica Biosystems | Other materials and equipment |
|---|---|---|
| • RNAscope 2.5 LS C1 Target Probe<br>• RNAscope 2.5 LS C2 Target Probe (50X)<br>• RNAscope 2.5 LS C3 Target Probe (50X)<br>• RNAscope LS Multiplex Positive Control Probe<br>• RNAscope LS Multiplex Negative Control Probe<br>• RNAscope 2.5 LS Hydrogen Peroxide<br>• RNAscope 2.5 LS Protease III<br>• RNAscope LS Multiplex AMP 1<br>• RNAscope LS Multiplex AMP 2<br>• RNAscope LS Multiplex AMP 3<br>• RNAscope LS Multiplex HRP-C1<br>• RNAscope LS Multiplex HRP-C2<br>• RNAscope LS Multiplex HRP-C3<br>• RNAscope LS Multiplex HRP Blocker<br>• TSA Vivid (diluted in RNAscope LS Multiplex TSA Buffer)<br>• TSA Vivid (diluted in RNAscope LS Multiplex TSA Buffer)<br>• TSA Vivid (diluted in RNAscope LS Multiplex TSA Buffer)<br>• RNAscope 2.5 LS Rinse<br>• RNAscope LS Multiplex DAPI | • Leica Biosystems' BOND RX System<br>• BOND 30 mL Open containers<br>• BOND 7 mL Open containers<br>• BOND Research Detection System<br>• BOND Universal Covertiles<br>• BOND Aspirating Probe Cleaning System<br>• BOND Mixing Stations<br><br>LS Bulk Reagents<br>• BOND Wash Solution, 10X<br>• BOND Dewax Solution<br>• BOND Epitope Retrieval Solution 1<br>• BOND Epitope Retrieval Solution 2 | • Prolong Gold Antifade Mountant<br>• Opal or TSA Plus fluorophores<br>• Either Leica Primary Antibody Diluent BOND or Akoya Antibody Diluent/Block<br>• Cover Glass |

# Prepare the instrument

1. Fill the large containers located in the bottom of the instrument with the Leica BOND RX bulk reagents.
2. Dilute BOND Wash Solution 1:10.

**Note:** Insufficient bulk reagent volumes may lead to run failure.

**IMPORTANT!** Do not introduce bubbles into the solutions by shaking the containers. To mix reagents, gently invert the containers several times. If bubbles are present, leave the containers out at room temperature until the bubbles dissipate.

3. Use clean, dry covertiles for every run. Follow Leica instructions to clean used covertiles with water, bleach, and ethanol. Air dry before reuse.
4. Before starting a run, empty bulk waste containers. Discard waste according to all local, state/provincial and/or national regulations.

## Prepare the instrument reagents

1. Obtain one empty BOND Open container and label it **Mock Probe**.
2. Fill the Mock Probe container with Leica Biosystems' 1X BOND Wash.
3. Carefully transfer all the RNAscope LS reagents except for the TSA buffer into empty 30 mL BOND Open containers.

**Note:** Before each run, make sure you have enough of each reagent. See the table following Step 8 on page 32 for the reagent volume required per slide.

4. Fill the DAPI container with DAPI and the Bond Wash container with Leica Biosystems' 1X BOND Wash. Use 150 µL DAPI or Bond Wash per slide.

**Note:** You may use your own DAPI, or other counterstain, in place of the DAPI provided in the kit.

5. Prepare the LS Multiplex probe mix:
   a. Determine the volume of multiplex probe needed (volume needed for the total number of slides plus container dead-volume). Make sure to add enough dead-volume to your calculation depending on the container type used:
      - 2.5 mL dead-volume when using a BOND 30 mL Open container.
      - 1 mL dead-volume when using a BOND 7 mL Open container.
      - 600 µL dead-volume when using a BOND Titration container (6 mL).
   b. Dilute the 50X C2 and C3 probe stocks 1:50 into the Ready-To-Use C1 probe. For example, add 320 µL 50X C2 probe and 320 µL 50X C3 probe to a tube, then add enough C1 probe to bring the final volume to 16 mL.
   c. Transfer the LS Multiplex probe mix into the appropriate Bond container.

**Note:** The LS Multiplex probe mix is stable for one year at 2–8°C.

6. Prepare the TSA Vivid/Opal fluorophore dilutions:
   a. Determine the volume of TSA Vivid/Opal fluorophore needed (see **Appendix E. Dilute the TSA Vivid/Opal Fluorophore**), and make sure to include dead volume per container (0.6–2.5 mL).
   b. Dilute the TSA Vivid/Opal fluorophore stock using the TSA buffer provided in the reagent kit.
   c. Add the diluted fluorophores to the appropriate Leica containers.

| Reagents | Recommended dilution range | Reagent registration name |
|---|---|---|
| TSA Vivid 520/Opal 520 | 1:750–1:3000 | ACD Multiplex TSA-F1 |
| TSA Vivid 570/Opal 570 | 1:750–1:3000 | ACD Multiplex TSA-F2 |
| TSA Vivid 650/Opal 690 | 1:750–1:3000 | ACD Multiplex TSA-F3 |
| TSA-DIG (if using Opal 780) | 1:750–1:3000 | TSA-DIG |
| Opal Polaris 780 (if using Opal 780) | 1:187.5–1:750 | Polaris 780 |

**Note:** TSA Vivid/ Opal fluorophore diluted in TSA buffer is stable for one month at 2–8°C in the dark.

7. Using the Barcode Scanner, scan the barcode located on the front of the BOND Open container. A window will appear.
8. From the drop-down menu, select the corresponding name of the reagent as shown in the following table under **Container name**:

| Reagents | Container name | Volume required per slide |
|---|---|---|
| RNAscope 2.5 LS Hydrogen Peroxide | *Open 0 Haz | 150 µl |
| RNAscope 2.5 LS Protease III | *ACD Enzyme | 200 µl |
| RNAscope 2.5 LS Rinse | *LS Rinse | 600 µl |
| RNAscope LS Multiplex AMP 1 | ACD Multiplex Amp 1 | 300 µl |
| RNAscope LS Multiplex AMP 2 | ACD Multiplex Amp 2 | 300 µl |
| RNAscope LS Multiplex AMP 3 | ACD Multiplex Amp 3 | 300 µl |
| RNAscope LS Multiplex HRP C1 | ACD Multiplex HRP-C1 | 300 µl |
| RNAscope LS Multiplex HRP C2 | ACD Multiplex HRP-C2 | 300 µl |
| RNAscope LS Multiplex HRP C3 | ACD Multiplex HRP-C3 | 300 µl |
| RNAscope LS Multiplex HRP Blocker | ACD Multiplex HRP blocker | 900 µl |
| TSA Vivid/Opal-fluorophore 1 (diluted in TSA buffer) | ACD Multiplex TSA-F1 | 300 µl |
| TSA Vivid/Opal-fluorophore 2 (diluted in TSA buffer) | ACD Multiplex TSA-F2 | 300 µl |
| TSA Vivid/ Opal-fluorophore 3 (diluted in TSA buffer) | ACD Multiplex TSA-F3 | 300 µl |
| Opal TSA-DIG (diluted in TSA buffer)† | TSA-DIG | 300 µl |
| Opal Polaris 780 (diluted in Leica Primary Antibody Diluent BOND or Akoya Antibody Diluent/Block)† | Polaris 780 | 300 µl |
| 1X BOND Wash | Mock probe | 220 µl |
| LS Multiplex probe mix | Variable | 450 µl |

†These reagents are only needed when Opal Polaris 780 is used in the assay.

9. Enter the RNAscope LS Multiplex Reagent Kit lot number and the expiration date in their respective fields. Select **OK**.

**IMPORTANT!** Do not introduce bubbles into the solutions by shaking the containers. To mix reagents, gently invert the containers several times. If bubbles are present, leave the containers out at room temperature until the bubbles dissipate.

## Start the run

1. Attach the barcodes to the slides, and add the slides to the slide tray with the label sides facing up.
2. Add a covertile on top of each slide, and verify placement and seating of each covertile.

**Note:** The rectangular-shaped neck of the covertile should fit into the groove of the slide tray.

3. Place the tray in the Leica BOND RX, and press the button to load the tray onto the machine.
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 scanned label images, and select Delayed Start to start the run at a future time.

**Note:** The total run time for LS Multiplex Fluorescent Assay is 13–14 hrs depending on the number of slides.

**IMPORTANT!** Before leaving the instrument unattended, ensure that the instrument is running successfully. Refer to **Troubleshooting** on page 36 if any issues occur.

## Complete the run and mount the samples

1. After the run is complete, press the button on the front of the instrument to unload the slides.
2. Remove the covertiles.
3. Add a drop of Prolong Gold Antifade mounting media to each slide. Avoid introducing bubbles.
4. Carefully place a glass coverslip on the slides, and dry overnight in the dark.
5. Store the slides at **4˚C** in the dark for up to two weeks.

# 6 Chapter 6. Evaluate the Results

## Fluorescent Imaging Recommendations

| Microscope | Optics | Image Capture |
|---|---|---|
| • Leica DM series or equivalent<br>• Zeiss Axio Imager or equivalent<br>• Inverted microscope, if optics and condenser meet requirements<br>• Required excitation/emission filter cube: DAPI/FITC/Cy3/Cy5/Cy7 (if Opal Polaris 780 is used) | • 20X (N.A 0.75) air<br>• 40X (N.A. 0.8) air (recommended)<br>• 40X (N.A. 1.3) oil<br>• 63X (N.A. 1.3) oil – use for low expression targets, if needed<br>• Use 20X and 40X to visualize high expression genes and low expression genes, respectively | • Microscope with camera for fluorescence capturing. Multispectrum microscope/camera system (eg. Nuance® EX, Mantra, Vectra and Polaris) recommended for imaging tissue with high autofluorescence.<br>• For optimal fluorescence detection, we recommend using a high resolution and high sensitivity cooled CCD camera that is 64 µm pixel size or smaller with > 65% peak quantum efficiency. Common models include: Orca-Flash 4.0 (Hamamatsu) and Nuance® EX (Perkin Elmer). |

## Evaluate the samples

Examine tissue sections under a standard fluorescent microscope at 20–40X magnification. You may also use a confocal microscope.

- Assess tissue and cell morphology.
- Assess the negative control background first. One dot to every 10 cells displaying background staining per 20X microscope field is acceptable. Set the light source and exposure time of image acquisition to acceptable background levels.
- Assess positive control signal strength. Positive control signal should be visible as punctate dots within the cell at 20–40X magnification.

# Control examples

If the assay is successful, the staining should look like the following image:

**Figure 2**. RNAscope LS Multiplex Fluorescent Assay detection of TBP (green), PPIB (orange), and POLR2A (white) mRNA in HeLa FFPE tissue at 40X magnification.

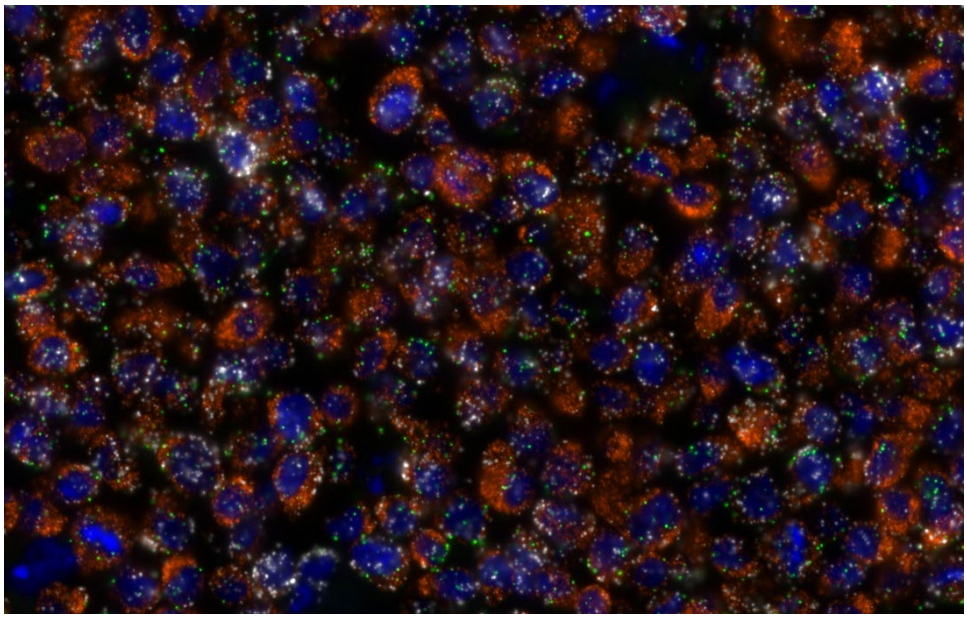
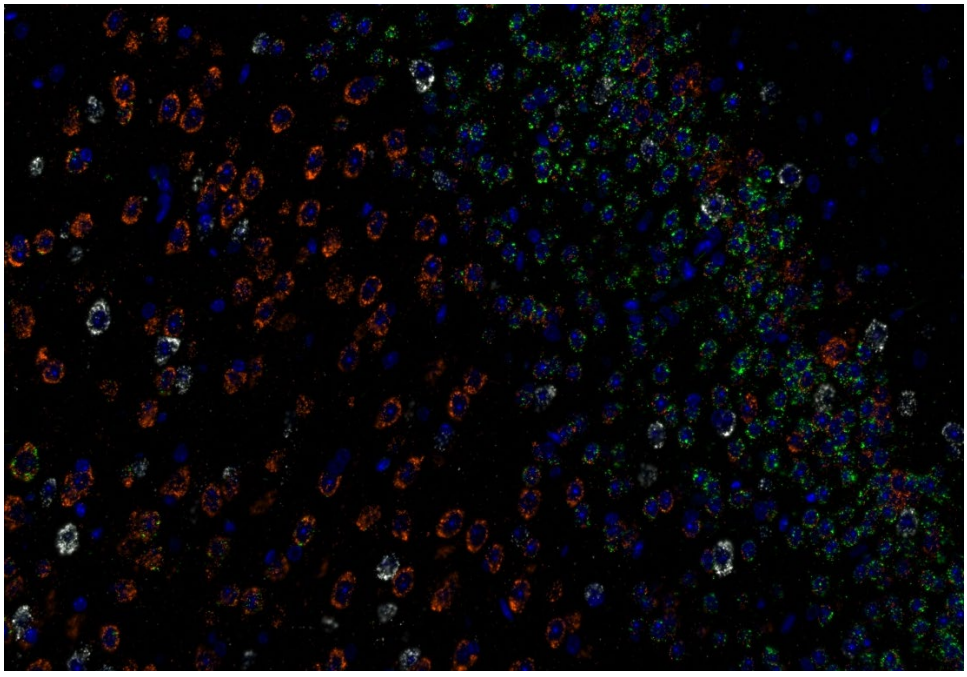


**Figure 3**. RNAscope LS Multiplex Assay detection of Vglut2 (green), Vglut1 (orange), and Gad1 (white) mRNA in mouse brain FFPE tissue at 20X magnification.



# Troubleshooting

If you obtain less than satisfactory results, troubleshoot your assay by following these simple guidelines:

- Always use optimal fluorescent filter settings and imaging tools.
- If signal intensity is too low for your imaging tools, increase the TSA Vivid/Opal fluorophore concentration.
- Use optimized fluorescence filter sets to reduce signal bleed-through. If you observe fluorescence bleed-through, reduce the TSA Vivid/Opal fluorophore concentration of the bleeding-through channel and/or reduce the exposure time during image acquisition to avoid over-exposure.
- If your RNA ISH signal cannot be distinguished from autofluorescence in tissues with high autofluorescence, increase the TSA Vivid/Opal fluorophore concentration.
- If you observe the presence of background staining, limit the sensitivity of image acquisition or reduce the corresponding TSA Vivid/Opal fluorophore concentration. Always acquire images using the setting in which background is under-detected.
- If the signal-to-noise ratio is low due to high background, increase the Enzyme time (protease) and/or Epitope Retrieval 2 (ER2) time in increments of five minutes (see Appendix B and Appendix C on pages 47 and 50). We recommend increasing the Enzyme time first.
- Use the above process for over-fixed tissues.
- The RNAscope LS Multiplex Fluorescence Assay uses only the Leica Biosystem BOND Research Detection System. Do not use BOND Polymer Refine DAB/Red Detection kits or any other chromogen kits.
- Do not shake the contents in the dispensers as this will form bubbles and may lead to weak or no staining. If bubbles are present, leave the containers out at room temperature until the bubbles dissipate.

For troubleshooting information, please contact technical support at **support.acd@bio-techne.com**.

# A Appendix A. Multiplex Protocol

The following table displays the full software protocol.

**Note:** Heated bond washes 4–6 come from the bulk reagents and are heated by the instrument. You cannot delete these steps. You may delete other wash steps.

**IMPORTANT!** To be able to use all three trays for the multiplex assay (three channels), follow the protocol steps listed in the following table. Adding any additional steps will prevent the system from running all three trays.

| Step No. | Reagent | Step Type | Incubation Time | Temperature† |
|---|---|---|---|---|
| 1 | *ACD 2.5 P1 | Reagent | 0 MIN | Ambient |
| 2 | *ACD 2.5 P1 | Reagent | 0 MIN | Ambient |
| 3 | *ACD 2.5 P1 | Reagent | 120 MIN | 42°C |
| 4 | *Bond Wash Solution | Wash | 0 MIN | 42°C |
| 5 | *Bond Wash Solution | Wash | 1 MIN | 42°C |
| 6 | *Bond Wash Solution | Wash | 5 MIN | 42°C |
| 7 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 8 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 9 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 10 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 11 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 12 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 13 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 14 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 15 | ACD Multiplex Amp 1 | Reagent | 1 MIN | 42°C |
| 16 | ACD Multiplex Amp 1 | Reagent | 30 MIN | 42°C |
| 17 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 18 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 19 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 20 | *Bond Wash Solution | Wash | 3 MIN | Ambient |
| 21 | *Bond Wash Solution | Wash | 3 MIN | Ambient |
| 22 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 23 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 24 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 25 | *LS Rinse | Reagent | 5 MIN | Ambient |

| Step No. | Reagent | Step Type | Incubation Time | Temperature† |
|---|---|---|---|---|
| 26 | *LS Rinse | Reagent | 5 MIN | Ambient |
| 27 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 28 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 29 | *Bond Wash Solution | Open Wash | 0 MIN | Ambient |
| 30 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 31 | ACD Multiplex Amp 2 | Reagent | 1 MIN | 42°C |
| 32 | ACD Multiplex Amp 2 | Reagent | 30 MIN | 42°C |
| 33 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 34 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 35 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 36 | *Bond Wash Solution | Wash | 3 MIN | Ambient |
| 37 | *Bond Wash Solution | Wash | 3 MIN | Ambient |
| 38 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 39 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 40 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 41 | *LS Rinse | Reagent | 5 MIN | Ambient |
| 42 | *LS Rinse | Reagent | 5 MIN | Ambient |
| 43 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 44 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 45 | *Bond Wash Solution | Open Wash | 1 MIN | Ambient |
| 46 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 47 | ACD Multiplex Amp 3 | Reagent | 1 MIN | 42°C |
| 48 | ACD Multiplex Amp 3 | Reagent | 15 MIN | 42°C |
| 49 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 50 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 51 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 52 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 53 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 54 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 55 | *Bond Wash Solution | Open Wash | 1 MIN | Ambient |
| 56 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 57 | ACD Multiplex HRP-C1 | Reagent | 1 MIN | 42°C |
| 58 | ACD Multiplex HRP-C1 | Reagent | 15 MIN | 42°C |
| 59 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 60 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 61 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 62 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 63 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 64 | *Bond Wash Solution | Wash | 1 MIN | Ambient |

| Step No. | Reagent | Step Type | Incubation Time | Temperature† |
|---|---|---|---|---|
| 65 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 66 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 67 | ACD Multiplex TSA-F1 | Reagent | 1 MIN | Ambient |
| 68 | ACD Multiplex TSA-F1 | Reagent | 30 MIN | Ambient |
| 69 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 70 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 71 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 72 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 73 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 74 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 75 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 76 | ACD Multiplex HRP blocker | Reagent | 1 MIN | 42°C |
| 77 | ACD Multiplex HRP blocker | Reagent | 15 MIN | 42°C |
| 78 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 79 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 80 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 81 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 82 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 83 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 84 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 85 | ACD Multiplex HRP-C2 | Reagent | 1 MIN | 42°C |
| 86 | ACD Multiplex HRP-C2 | Reagent | 15 MIN | 42°C |
| 87 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 88 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 89 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 90 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 91 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 92 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 93 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 94 | ACD Multiplex TSA-F2 | Reagent | 1 MIN | Ambient |
| 95 | ACD Multiplex TSA-F2 | Reagent | 30 MIN | Ambient |
| 96 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 97 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 98 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 99 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 100 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 101 | *Bond Wash Solution | Wash | 1 MIN | Ambient |

| Step No. | Reagent | Step Type | Incubation Time | Temperature† |
|---|---|---|---|---|
| 102 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 103 | ACD Multiplex HRP blocker | Reagent | 1 MIN | 42°C |
| 104 | ACD Multiplex HRP blocker | Reagent | 15 MIN | 42°C |
| 105 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 106 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 107 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 108 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 109 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 110 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 111 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 112 | ACD Multiplex HRP-C3 | Reagent | 1 MIN | 42°C |
| 113 | ACD Multiplex HRP-C3 | Reagent | 15 MIN | 42°C |
| 114 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 115 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 116 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 117 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 118 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 119 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 120 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 121 | ACD Multiplex TSA-F3 | Reagent | 1 MIN | Ambient |
| 122 | ACD Multiplex TSA-F3 | Reagent | 30 MIN | Ambient |
| 123 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 124 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 125 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 126 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 127 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 128 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 129 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 130 | ACD Multiplex HRP blocker | Reagent | 1 MIN | 42°C |
| 131 | ACD Multiplex HRP blocker | Reagent | 15 MIN | 42°C |
| 132 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 133 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 134 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 135 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 136 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 137 | DAPI/Bond Wash‡ | Reagent | 10 MIN | Ambient |

| Step No. | Reagent | Step Type | Incubation Time | Temperature† |
|---|---|---|---|---|
| 138 | *De-ionized Water | Wash | 0 MIN | Ambient |
| 139 | *De-ionized Water | Wash | 0 MIN | Ambient |
| 140 | *De-ionized Water | Wash | 0 MIN | Ambient |

* Indicates reagent is hard–coded in the software by Leica Biosystems.

† Temperatures cannot be changed in 4.0 software.

‡ The standard protocol uses DAPI. Use BOND Wash instead of DAPI, if you are using DAPI offline or performing IHC steps afterwards on your samples.

# B Appendix B. Multiplex Protocol Using Opal Polaris 780

The following table displays the full software protocol.

**Note:** Heated bond washes 4–6 come from the bulk reagents and are heated by the instrument. You cannot delete these steps. You may delete other wash steps.

**IMPORTANT!** To be able to use all three trays for the multiplex assay (three channels), follow the protocol steps listed in the following table. Adding any additional steps will prevent the system from running all three trays.

| Step No. | Reagent | Step Type | Incubation Time | Temperature† |
|---|---|---|---|---|
| 1 | *ACD 2.5 P1 | Reagent | 0 MIN | Ambient |
| 2 | *ACD 2.5 P1 | Reagent | 0 MIN | Ambient |
| 3 | *ACD 2.5 P1 | Reagent | 120 MIN | 42°C |
| 4 | *Bond Wash Solution | Wash | 0 MIN | 42°C |
| 5 | *Bond Wash Solution | Wash | 1 MIN | 42°C |
| 6 | *Bond Wash Solution | Wash | 5 MIN | 42°C |
| 7 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 8 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 9 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 10 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 11 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 12 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 13 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 14 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 15 | ACD Multiplex Amp 1 | Reagent | 1 MIN | 42°C |
| 16 | ACD Multiplex Amp 1 | Reagent | 30 MIN | 42°C |
| 17 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 18 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 19 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 20 | *Bond Wash Solution | Wash | 3 MIN | Ambient |
| 21 | *Bond Wash Solution | Wash | 3 MIN | Ambient |
| 22 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 23 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 24 | *Bond Wash Solution | Wash | 0 MIN | Ambient |

| Step No. | Reagent | Step Type | Incubation Time | Temperature† |
|---|---|---|---|---|
| 25 | *LS Rinse | Reagent | 5 MIN | Ambient |
| 26 | *LS Rinse | Reagent | 5 MIN | Ambient |
| 27 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 28 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 29 | *Bond Wash Solution | Open Wash | 0 MIN | Ambient |
| 30 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 31 | ACD Multiplex Amp 2 | Reagent | 1 MIN | 42°C |
| 32 | ACD Multiplex Amp 2 | Reagent | 30 MIN | 42°C |
| 33 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 34 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 35 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 36 | *Bond Wash Solution | Wash | 3 MIN | Ambient |
| 37 | *Bond Wash Solution | Wash | 3 MIN | Ambient |
| 38 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 39 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 40 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 41 | *LS Rinse | Reagent | 5 MIN | Ambient |
| 42 | *LS Rinse | Reagent | 5 MIN | Ambient |
| 43 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 44 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 45 | *Bond Wash Solution | Open Wash | 1 MIN | Ambient |
| 46 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 47 | ACD Multiplex Amp 3 | Reagent | 1 MIN | 42°C |
| 48 | ACD Multiplex Amp 3 | Reagent | 15 MIN | 42°C |
| 49 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 50 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 51 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 52 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 53 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 54 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 55 | *Bond Wash Solution | Open Wash | 1 MIN | Ambient |
| 56 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 57 | ACD Multiplex HRP-C1 | Reagent | 1 MIN | 42°C |
| 58 | ACD Multiplex HRP-C1 | Reagent | 15 MIN | 42°C |
| 59 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 60 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 61 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 62 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 63 | *Bond Wash Solution | Wash | 1 MIN | Ambient |

| Step No. | Reagent | Step Type | Incubation Time | Temperature† |
|---|---|---|---|---|
| 64 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 65 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 66 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 67 | ACD Multiplex TSA-F1 | Reagent | 1 MIN | Ambient |
| 68 | ACD Multiplex TSA-F1 | Reagent | 30 MIN | Ambient |
| 69 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 70 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 71 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 72 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 73 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 74 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 75 | ACD Multiplex HRP blocker | Reagent | 1 MIN | 42°C |
| 76 | ACD Multiplex HRP blocker | Reagent | 15 MIN | 42°C |
| 77 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 78 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 79 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 80 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 81 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 82 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 83 | ACD Multiplex HRP-C2 | Reagent | 1 MIN | 42°C |
| 84 | ACD Multiplex HRP-C2 | Reagent | 15 MIN | 42°C |
| 85 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 86 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 87 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 88 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 89 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 90 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 91 | ACD Multiplex TSA-F2 | Reagent | 1 MIN | Ambient |
| 92 | ACD Multiplex TSA-F2 | Reagent | 30 MIN | Ambient |
| 93 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 94 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 95 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 96 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 97 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 98 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 99 | ACD Multiplex HRP blocker | Reagent | 1 MIN | 42°C |

| Step No. | Reagent | Step Type | Incubation Time | Temperature† |
|---|---|---|---|---|
| 100 | ACD Multiplex HRP blocker | Reagent | 15 MIN | 42°C |
| 102 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 103 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 104 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 105 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 106 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 107 | ACD Multiplex HRP-C3 | Reagent | 1 MIN | 42°C |
| 108 | ACD Multiplex HRP-C3 | Reagent | 15 MIN | 42°C |
| 109 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 110 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 111 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 112 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 113 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 114 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 115 | TSA-DIG | Reagent | 1 MIN | Ambient |
| 116 | TSA-DIG | Reagent | 30 MIN | Ambient |
| 117 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 118 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 119 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 120 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 121 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 122 | ACD Multiplex HRP blocker | Reagent | 1 MIN | 42°C |
| 123 | ACD Multiplex HRP blocker | Reagent | 15 MIN | 42°C |
| 124 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 125 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 126 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 127 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 128 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 129 | Polaris 780 | Reagent | 1 MIN | Ambient |
| 130 | Polaris 780 | Reagent | 30 MIN | Ambient |
| 131 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 132 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 133 | *Bond Wash Solution | Wash | 0 MIN | Ambient |
| 134 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 135 | *Bond Wash Solution | Wash | 1 MIN | Ambient |
| 136 | DAPI/Bond Wash‡ | Reagent | 10 MIN | Ambient |

| Step No. | Reagent | Step Type | Incubation Time | Temperature† |
|---|---|---|---|---|
| 137 | *De-ionized Water | Wash | 0 MIN | Ambient |
| 138 | *De-ionized Water | Wash | 0 MIN | Ambient |
| 139 | *De-ionized Water | Wash | 0 MIN | Ambient |

* Indicates reagent is hard–coded in the software by Leica Biosystems.

† Temperatures cannot be changed in 4.0 software.

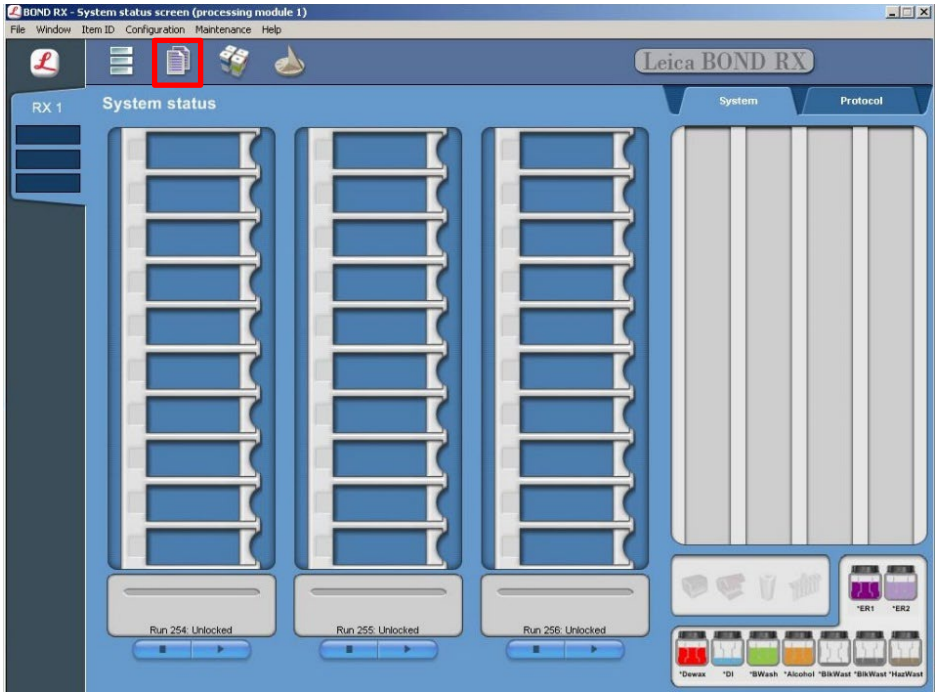
‡ The standard protocol uses DAPI. If you are using DAPI offline or performing IHC steps afterwards on your samples, use BOND Wash instead of DAPI.

# Appendix C. Edit the Epitope Retrieval Protocol

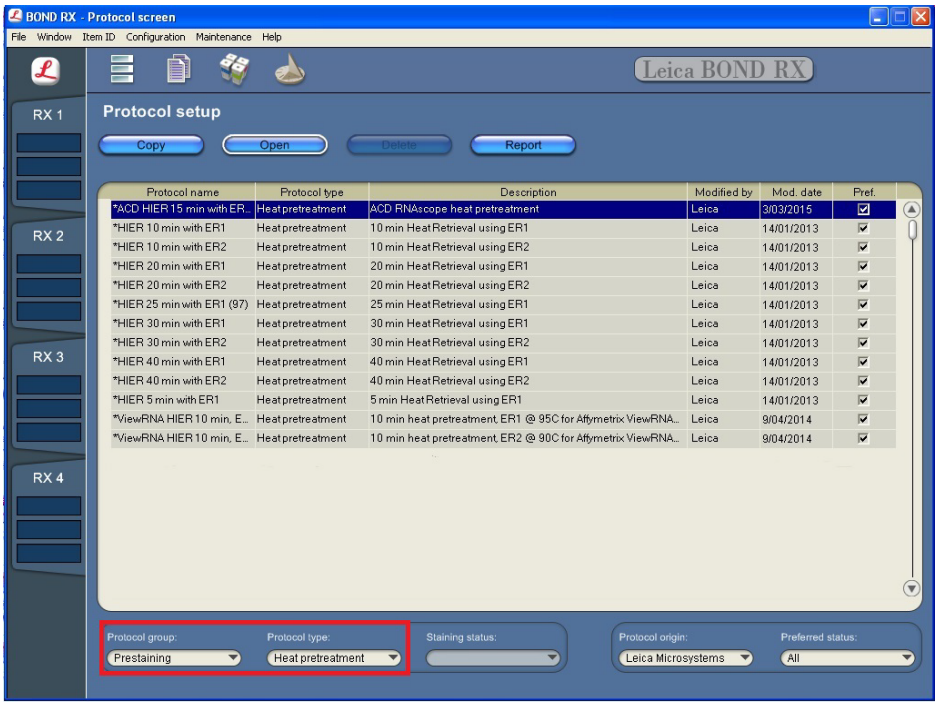
The following example shows how to edit the Epitope Retrieval procedure from within the software. The same pretreatment conditions can be used for LS single-plex, duplex, and multiplex assays.

## Create a prestaining protocol

1. Open the Leica BOND software and click on the Protocol setup icon as shown.



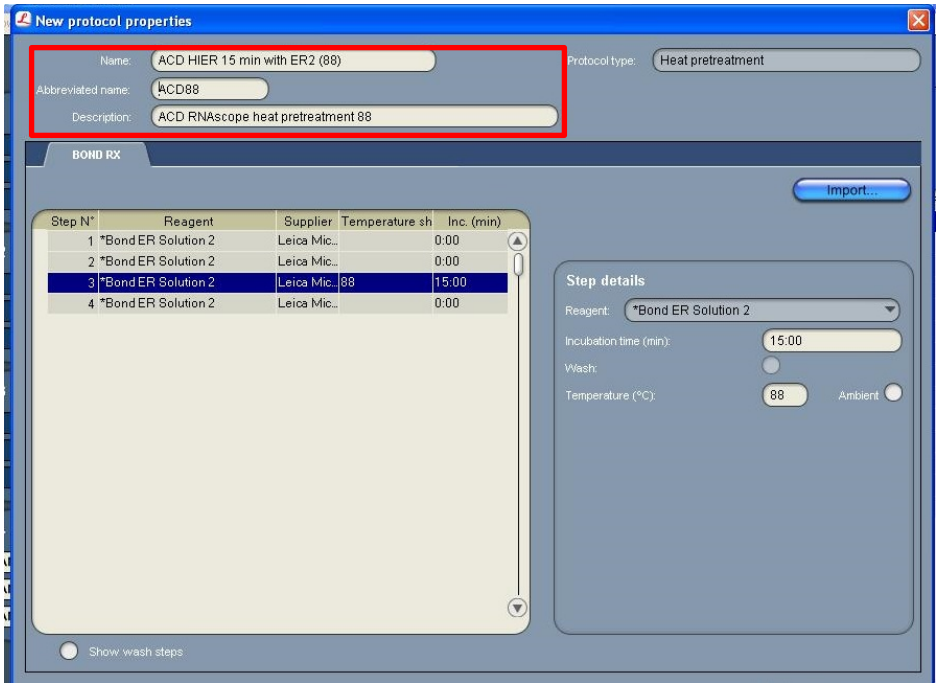

2. Select **Prestaining** under the Protocol group menu and **Heat pretreatment** under the Protocol type menu to access the heat pretreatment protocols.




3. Highlight the ***ACD HIER 15 min with ER2 (95)** protocol**.** Select **Copy.**

**Note:** ER2 = Epitope Retrieval 2.

4. Rename the protocol as **ACD HIER 15 min with ER2 (88)**.
5. Rename the Abbreviated name as **ACD88**.
6. Rename the Description to **ACD RNAscope heat pretreatment 88**.




7. Highlight the third ***Bond ER Solution 2** step. Depending on the tissue type used, change the temperature and time as shown in the following table:

| Tissue Type | ER2 Incubation Time | Temperature |
|---|---|---|
| Brain and spinal cord | 15 MIN | 95°C |
| Breast cancer | 15 MIN | 95°C |
| Cell pellet | 15 MIN | 88°C |
| Colon | 15 MIN | 95°C |
| GI tract | 15 MIN | 95°C |
| Head and neck cancer | 15 MIN | 95°C |
| Heart | 15 MIN | 95°C |
| Kidney | 15 MIN | 95°C |
| Liver | 15 MIN | 95°C |
| Lung | 15 MIN | 95°C |
| Lymphoma | 15 MIN | 95°C |
| Placenta | 15 MIN | 95°C |
| Prostate | 15 MIN | 95°C |
| Skin | 15 MIN | 95°C |
| Stomach | 15 MIN | 95°C |
| Thymus | 15 MIN | 88°C or 95°C |
| Tonsil | 15 MIN | 88°C or 95°C |
| Xenograft | 15 MIN | 88°C |
| Mouse tissue | 15 MIN | 88°C or 95°C |
| Fresh Frozen tissue* | No | No |

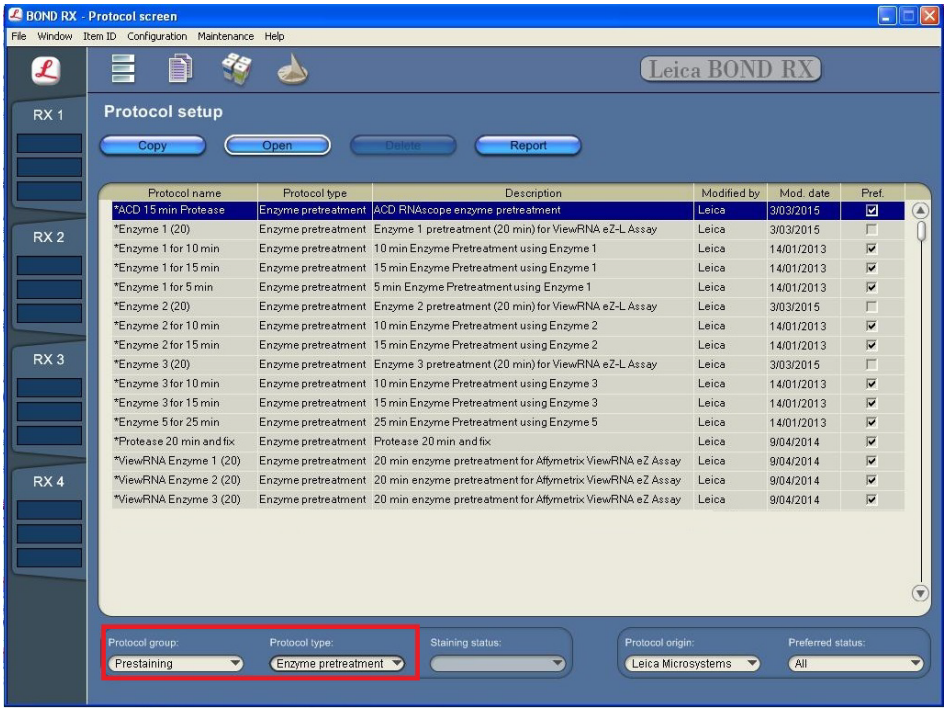
* For fresh frozen tissue, the HIER step is unnecessary. Select "*----"for the HIER protocol.

8. Select **Save** to create a protocol for ER2 pretreatment at 88°C.
9. If needed, repeat Steps 1–8 to create a new heating protocol (for example, **ACD 25min ER2**).

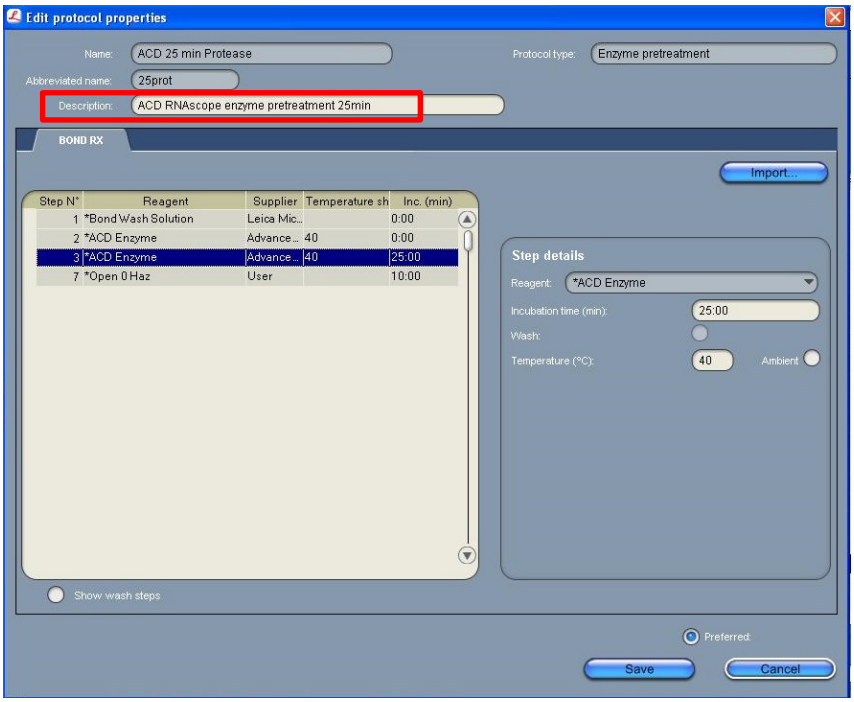
# D Appendix D. Edit the Protease Protocol

The following example shows how to edit the Protease procedure from within the software.

1. Select Enzyme Pretreatment under the Protocol type menu (bottom left).
2. Highlight the ***ACD 15min Protease** protocol. Select **Copy**.




3. Rename the protocol to **ACD 25min Protease**.
4. Rename the Abbreviated name to **25prot**.
5. Rename the Description to **ACD RNAscope enzyme pretreatment 25min**.




6. Highlight the second ***ACD Enzyme** step. Keep the temperature at **40°C** and set the enzyme incubation time to **25 MIN**.
7. Select **Save**.
8. If needed, repeat Steps 1–7 to create a new protease protocol for different sample types.
9. For the following FFPE tissues, we recommend a treatment of LS Protease III for **15 MIN** at **40°C**:

| Tissue Type | |
|---|---|
| Brain and spinal cord | Lung |
| Breast cancer | Lymphoma |
| Cell pellet | Placenta |
| Colon | Prostate |
| GI tract | Skin |
| Head and neck cancer | Stomach |
| Heart | Thymus |
| Kidney | Tonsil |
| Liver | Xenograft |

10. For fresh frozen tissue, we recommend a treatment of Protease IV for **30 MIN** at ambient temperature. Contact ACD support for availability of Protease IV for use on the Bond RX system.

# Appendix E. Dilute the TSA Vivid/Opal Fluorophore

The following guide shows how to dilute the TSA Vivid/Opal fluorophore with the TSA buffer provided.

1. Reconstitute the TSA Vivid reagent with 100 µL DMSO. Reconstitute lyophilized Opal fluorophore stock according to manufacturer's instruction.
2. Determine the number of slides needed for the run.
3. Use the following table to determine the volumes needed for the run (volume needed for the total number of slides plus container dead-volume). Make sure to add enough dead-volume to your calculation depending on the container type used. Use 6 mL BOND Titration containers for up to 18 slides; Use 7 mL BOND Titration containers for up to 22 slides.
   - 2.5 mL dead-volume when using a BOND 30 mL Open container
   - 1 mL dead-volume when using a BOND 7 mL Open container
   - 600 µL dead-volume when using a BOND Titration container (6mL)
4. Dilute all three fluorophores.
5. Add the diluted fluorophores to the appropriate containers.

| Number of slides | TSA Vivid/Opal-fluorophore solution per channel after dilution (µL) | TSA Vivid/Opal-fluorophore stock needed if using 1:750 dilution (µL) | TSA Vivid/Opal-fluorophore stock needed if using 1:1500 dilution (µL) | Opal-fluorophore stock needed if using 1:3000 dilution (µL) |
|---|---|---|---|---|
| 5 | 1500 | 2.0 | 1.0 | 0.5 |
| 6 | 1800 | 2.4 | 1.2 | 0.6 |
| 7 | 2100 | 2.8 | 1.4 | 0.7 |
| 8 | 2400 | 3.2 | 1.6 | 0.8 |
| 9 | 2700 | 3.6 | 1.8 | 0.9 |
| 10 | 3000 | 4.0 | 2.0 | 1.0 |
| 11 | 3300 | 4.4 | 2.2 | 1.1 |
| 12 | 3600 | 4.8 | 2.4 | 1.2 |
| 13 | 3900 | 5.2 | 2.6 | 1.3 |
| 14 | 4200 | 5.6 | 2.8 | 1.4 |
| 15 | 4500 | 6.0 | 3.0 | 1.5 |
| 16 | 4800 | 6.4 | 3.2 | 1.6 |
| 17 | 5100 | 6.8 | 3.4 | 1.7 |
| 18 | 5400 | 7.2 | 3.6 | 1.8 |
| 19 | 5700 | 7.6 | 3.8 | 1.9 |
| 20 | 6000 | 8.0 | 4.0 | 2.0 |
| 21 | 6300 | 8.4 | 4.2 | 2.1 |
| 22 | 6600 | 8.8 | 4.4 | 2.2 |
| 23 | 6900 | 9.2 | 4.6 | 2.3 |

| Number of slides | TSA Vivid/Opal-fluorophore solution per channel after dilution (µL) | TSA Vivid/Opal-fluorophore stock needed if using 1:750 dilution (µL) | TSA Vivid/Opal-fluorophore stock needed if using 1:1500 dilution (µL) | Opal-fluorophore stock needed if using 1:3000 dilution (µL) |
|---|---|---|---|---|
| 24 | 7200 | 9.6 | 4.8 | 2.4 |
| 25 | 7500 | 10.0 | 5.0 | 2.5 |
| 26 | 7800 | 10.4 | 5.2 | 2.6 |
| 27 | 8100 | 10.8 | 5.4 | 2.7 |
| 28 | 8400 | 11.2 | 5.6 | 2.8 |
| 29 | 8700 | 11.6 | 5.8 | 2.9 |
| 30 | 9000 | 12.0 | 6.0 | 3.0 |

**Note:** Diluted TSA Vivid/Opal fluorophores are stable in TSA buffer at 2–8˚C for up to one month in the dark.

When running a small number of slides, you may use the TSA buffer to make a small amount of 1:10 stock. Use the 1:10 stock to make the final dilution.

# F Appendix F. Safety

## Chemical safety

**WARNING! GENERAL CHEMICAL HANDLING.** To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, visit http://www.acdbio.com/technical-support/user-manuals.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- IMPORTANT! Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

## Biological hazard safety

**WARNING! BIOHAZARD.** Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

### In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at www.cdc.gov/biosafety
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030)
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials
- Additional information about biohazard guidelines is available at www.cdc.gov/

### In the EU:

- Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition
- Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)

# Documentation and Support

## Obtaining SDSs

Safety Data Sheets (SDSs) are available at: **https://acdbio.com/technical-support/user-manuals**. For the SDSs of chemicals not distributed by Advanced Cell Diagnostics, contact the chemical manufacturer.

## Obtaining support

For the latest services and support information, go to: **https://acdbio.com/technical-support/support-overview.**

At the website, you can:

- Access telephone and fax numbers to contact Technical Support and Sales facilities.
- Search through frequently asked questions (FAQs).
- Submit a question directly to Technical Support.
- Search for user documents, SDSs, application notes, citations, training videos, and other product support documents.
- Find out information about customer training events.

## Contact information

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Newark, CA 94560
Toll Free: 1-877-576-3636
Direct: 1-510-576-8800
Fax: 1-510-576-8801
Information: **info.acd@bio-techne.com**
Orders: **orders.acd@bio-techne.com**
Support Email: **support.acd@bio-techne.com**

## Limited product warranty

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. If you have any questions, please contact Advanced Cell Diagnostics at: https://acdbio.com/about/contact.

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For support, email **support.acd@bio-techne.com**
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