

# Opal™ 4-Color and 7-Color Automation IHC Kits

For Leica Biosystems BOND RX System  
Software version 4.0

## User Manual

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# Preface

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Email: [global.techsupport@perkinelmer.com](mailto:global.techsupport@perkinelmer.com)

Internet: [www.perkinelmer.com](http://www.perkinelmer.com)

**For questions about BOND RX operation, please contact Leica Biosystem Technical support at 844-534-2262.**

Before you call, have the following information available for the technical representative:

- Reagent Lot numbers
- BOND RX Software version
- If applicable, the *error number* shown in the software or in the log file.

## Obtaining MSDSs

Material Safety Data Sheets for the reagents in the Opal 4-Color and 7-Color Automation Kits are available on the PerkinElmer website ([www.perkinelmer.com](http://www.perkinelmer.com)). MSDSs for chemicals from other manufacturers should be obtained from the chemical manufacturer.

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### NOTE











*Changes or modifications to the protocol not expressly approved by PerkinElmer could affect the results of the protocol.*

## Table of Symbols

Table 1 contains symbols that identify particularly important information and alert you to the presence of hazards. These symbols may appear in this manual and/or on the product it describes.

**Table 1. Important Symbols**

| Symbol<br>Symbole   | Description<br>Description   |
|---|--|
|    | <b>DANGER:</b> An imminently hazardous situation, which, if not avoided, will result in death or serious injury.<br><b>DANGER:</b> Situation présentant un danger imminent qui, s'il n'est pas éliminé, peut entraîner des blessures graves, voire la mort.  |
|    | <b>WARNING:</b> Caution, risk of danger. Refer to the User's documentation.<br><b>AVERTISSEMENT:</b> Attention, danger potentiel. Se reporter à la documentation de l'utilisateur.   |
|    | <b>NOTE:</b> A cautionary statement; an operating tip or maintenance suggestion; may result in instrument damage if not followed.<br><b>REMARQUE:</b> Énoncé indiquant une précaution à prendre, un conseil de fonctionnement ou une suggestion d'entretien; son non-respect peut provoquer des dommages à l'instrument. |
|  | Risk of exposure to biohazards.<br>Risque d'exposition aux risques biologiques.  |
|  | Crush hazard. Risk of body parts, hair, jewelry, or clothing getting caught in a moving part.<br>Danger d'écrasement. Faire attention que les parties corporelles, les cheveux, les bijoux ou les vêtements ne soient pas pris dans une pièce mobile.  |
|  | Risk of eye injury; wear safety glasses.<br>Risque de lésion oculaire; porter des lunettes de sécurité.  |
|  | Risk of poison.<br>Risque d'empoisonnement.  |
|  | Hazardous fumes.<br>Émanations dangereuses.  |

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# Introduction

## Product Description

Opal™ is a method for multiplex fluorescent immunohistochemistry in formalin-fixed, paraffin-embedded (FFPE) tissue. It allows use of standard unlabeled primary antibodies, including multiple antibodies raised in the same species. The basic approach was inspired by a protocol published by Zsuzsanna Tóth and Éva Mezey. The current method involves detection with Opal reactive fluorophores that covalently label the epitope. After labeling is complete, antibodies are removed in a manner that does not disrupt the Opal fluorescence signal. This allows the next target to be detected without fear of antibody cross reactivity. Opal enables development of multiplexed assays with balanced, quantitative signal for rare and abundant targets.

PerkinElmer's Opal 4-Color and 7-Color Automation IHC Kits are for Research Use Only. They are not for Use in Diagnostic Procedures.

Leica Biosystems BOND RX is for Research Use Only. It is not for Use in Diagnostic Procedures.

## Opal 4-Color and 7-Color Automation IHC Kits Reagents and Storage

Opal 4-Color or 7-Color Automation IHC Kit contains enough reagents to run 50 standard Opal 4-Color or 7-Color Assays on Leica Biosystems BOND RX System at 1:150 recommended dilution rate for all Opal fluorophores.

The reagents have a shelf life of 12 months from the shipment date when stored using indicated temperatures in Table 1.

**Table 1a. Opal 4-Color Automation IHC Kit**

| Opal 4-Color Automation IHC Kit |                         |                                |
|---------------------------------|-------------------------|--------------------------------|
| Reagent                         | Quantity                | Storage                        |
| Antibody Diluent/Block          | 100 mL                  | 4 °C                           |
| Opal Polymer HRP Ms+Rb          | 50 mL                   | 4 °C                           |
| Opal 520 Reagent                | dry material;<br>1 vial | -20 °C                         |
| Opal 570 Reagent                | dry material;<br>1 vial | -20 °C                         |
| Opal 690 Reagent                | dry material;<br>1 vial | -20 °C                         |
| Spectral DAPI                   | 1 mL                    | 4 °C                           |
| 1X Plus Amplification Diluent   | 50 mL                   | 4 °C                           |
| DMSO (Anhydrous Grade)          | 500 µL                  | Room temperature<br>(20–25 °C) |

**Table 1b. Opal 7-Color Automation IHC Kit**

| Opal 7-Color Automation IHC Kit |                         |                                |
|---------------------------------|-------------------------|--------------------------------|
| Reagent                         | Quantity                | Storage                        |
| Antibody Diluent/Block          | 100 mL                  | 4 °C                           |
| Opal Polymer HRP Ms+Rb          | 50 mL; 2 bottles        | 4 °C                           |
| Opal 520 Reagent                | dry material;<br>1 vial | -20 °C                         |
| Opal 540 Reagent                | dry material;<br>1 vial | -20 °C                         |
| Opal 570 Reagent                | dry material;<br>1 vial | -20 °C                         |
| Opal 620 Reagent                | dry material;<br>1 vial | -20 °C                         |
| Opal 650 Reagent                | dry material;<br>1 vial | -20 °C                         |
| Opal 690 Reagent                | dry material;<br>1 vial | -20 °C                         |
| Spectral DAPI                   | 1 mL                    | 4 °C                           |
| 1X Plus Amplification Diluent   | 50 mL; 2 bottles        | 4 °C                           |
| DMSO (Anhydrous Grade)          | 500 µL                  | Room temperature<br>(20–25 °C) |

## BOND RX Materials Required

The Opal 4-Color and 7-Color Automation IHC Assay require the following materials and equipment that are available only from Leica Biosystems and are listed in Table 2.

**Table 2. BOND RX Materials Required**

| Component                          | Cat. No. | Storage                        |
|------------------------------------|----------|--------------------------------|
| BOND Titration Kit                 | OPT9049  | Room temperature<br>(20–25 °C) |
| BOND Open Containers 30 mL         | OP309700 | Room temperature<br>(20–25 °C) |
| BOND Open Containers 7 mL          | OP79193  | Room temperature<br>(20–25 °C) |
| BOND Research Detection System 2   | DS9777   | Room temperature<br>(20–25 °C) |
| BOND Universal Covertiles 160 pack | S21.4611 | Room temperature<br>(20–25 °C) |
| BOND Slide Tray                    | S21.0304 | Room temperature<br>(20–25 °C) |
| BOND Reagent Tray                  | S21.1003 | Room temperature<br>(20–25 °C) |



|  |          |                                |
|--|----------|--------------------------------|
| BOND Slide Labels and Printer Ribbon       | S21.4564 | Room temperature<br>(20–25 °C) |
| BOND Apex Adhesive Slide                   | 3800040  | Room temperature<br>(20–25 °C) |
| BOND Dewax Solution – 1L (RTU)             | AR9222   | 2–26 °C                        |
| BOND Epitope Retrieval Solution 1-1L (RTU) | AR9961   | 2–8 °C                         |
| BOND Epitope Retrieval Solution 2-1L (RTU) | AR9640   | 2–8 °C                         |
| BOND Wash Solution 10X Concentrate – 1L    | AR9590   | 2–8 °C                         |
| BOND Aspirating Probe Cleaning System      | CS9100   | 2–8 °C                         |

## Equipment

| Component  | Cat. No. |
|--|----------|
| Leica Biosystems' BOND RX System - automated slide stainer   | ---      |
| PerkinElmer's Vectra <sup>®</sup> Polaris <sup>™</sup> , Vectra <sup>®</sup> 3 or Mantra <sup>™</sup> Quantitative Pathology Workstation for imaging | ---      |

## User-Supplied Materials Required

The materials below are required to run the assay and prepare the slides.

**Table 3. User-Supplied Materials Required**

| Description  | Supplier | Cat. No.    |
|--|----------|-------------|
| 200 Proof ethanol (EtOH)   | MLS      | —           |
| Xylene   | MLS      | —           |
| 10% neutral-buffered formalin (NBF)                                    | MLS      | —           |
| Paraffin wax   | MLS      | —           |
| 1X Tris-Buffered Saline (TBS)  | MLS      | —           |
| Microtome  | MLS      | —           |
| Drying oven, capable of holding temperature at 65 +/- 1 °C (optional)  | MLS      | —           |
| Slide Rack 30 - Plastic (optional)                                     | Leica    | 14047533750 |
| Water bath or incubator, capable of holding temperature at 40 +/- 1 °C | MLS      | —           |
| ProLong® Diamond Antifade Mountant                                     | MLS      | P36961      |
| Slide Rack   | MLS      | —           |
| Staining Dish  | MLS      | —           |
| Cover Glass No. 1.5  | MLS      | —           |
| Deionized Water  | MLS      | —           |
| Fume hood  | MLS      | —           |

\* MLS = Major Laboratory Supplier in North America. For other regions, please check with local vendors.

## Before You Begin

Before running either the Opal 4-Color or 7-Color Automation IHC Kit on the Leica Biosystems BOND RX:

- Read and understand the Leica Biosystems BOND RX Instructions for use.
- Read and understand this document.

## Important Guidelines

- Always use properly prepared tissue samples. See [Prepare Tissue Samples on page 11](#) for tissue sample preparation.
- Recommend running the BOND Aspirating Probe Cleaning System more often than conventional IHC.
- Always run control slides with sample slides to verify proper staining.
- Do not use consumables or reagents other than those specified.
- Changing the protocol may affect the results of the run.
- Do not let the tissue samples dry out at any time after BOND RX run is finished.
- Use good laboratory practices and follow all safety procedures. See [Safety on page 33](#) for more information.

## Prepare Tissue Samples

This section describes how to prepare and pretreat formalin-fixed, paraffin-embedded (FFPE) tissue sections for use with the Opal 4-Color or 7-Color Automation IHC Kit on the BOND RX.

### NOTE



*Using other preparation or pretreatment procedures may affect the results of the protocol.*

## Prepare the FFPE Sections

### Materials Required

- 10% neutral buffered formalin (NBF)
- 1x TBS
- Paraffin wax
- 200 Proof EtOH
- Xylene
- Microtome
- Water bath
- BOND APEX Adhesive slides
- Drying oven

### Fix the Tissue Sample

- 1 Immediately after dissection, fix tissue in 10% NBF for 16–32 hours at ROOM TEMPERATURE (RT). Fixation time will vary depending on tissue type and size.

### WARNING



*Biological Risk. Handle all samples using appropriate safety procedures.*

### NOTE



*Fixation for less than 16 hours or more than 32 hours will impair the results of the Opal 4-Color or 7-Color Automation IHC Assay.*

## Dehydrate, Embed, and Cut the Tissue Sample

### WARNING



*Do not use reagents or consumables that are past their expiration date.*

- 1 Wash the sample with 1x TBS.
- 2 Dehydrate the sample using a standard ethanol series, followed by xylene.
- 3 Embed the sample in paraffin using standard procedures. Note that paraffin-embedded samples can be stored at RT.
- 4 Trim paraffin blocks and then cut the embedded tissue into  $5 \pm 1 \mu\text{m}$  sections using a microtome.
- 5 Place the paraffin ribbon in a 35-45 °C water bath and mount sections on BOND Apex Adhesive slides or Superfrost® Plus Micro Slide.
- 6 Load slides onto the vertical 24-Slide Rack and air dry overnight.
- 7 If desired, the sectioned tissue can be stored with desiccants at RT before continuing to run the Opal 4-Color or 7-Color Automation IHC Kit.
- 8 Bake slides at 65 °C for 1 hour in drying oven before staining.

### NOTE



*Tip the slide rack to one side and place paper towels underneath to soak up the wax while drying.*

- 9 Remove and let cool.

# Opal Reagent Assay

## Workflow

The steps below represent a typical workflow for using the PerkinElmer Opal 4-Color and 7-Color Automation IHC Kits on the Leica Biosystems BOND RX.

- 1 [Optimize the Opal assay setup on page 13](#)
- 2 [Prepare the Materials on page 13](#)
- 3 [Set up the BOND RX Protocols on page 18](#)
- 4 [Run the Opal 4-Color or 7-Color Automation IHC Kit on page 25](#)

### NOTE



*PerkinElmer recommends that you run Library slides and Autofluorescence slides with the tissue samples in every run.*

## Optimize the Opal assay setup

Refer to PerkinElmer's Opal assay development guide.

## Prepare the Materials

**Table 4. Materials Required**

| Materials provided by PerkinElmer | Materials provided by Leica Biosystems | Materials provided by User           |
|-----------------------------------|--|--------------------------------------|
| • Antibody Diluent/Block          | <b>Stainer</b>                         | • 1° Antibodies                      |
| • Opal Polymer HRP Ms+Rb          | • Leica Biosystems' BOND RX System     | • 1xTBS                              |
| • 1X Plus Amplification Diluent   | <b>Bulk Reagents</b>                   | • 100% Ethanol                       |
| • Opal 520 Reagent                | • BOND Epitope Retrieval Solution 1    | • Deionized or Milli-Q Water         |
| • Opal 540 Reagent                | • BOND Epitope Retrieval Solution 2    | • ProLong® Diamond Antifade Mountant |
| • Opal 570 Reagent                | • BOND Wash Solution 10X               | • Cover Glass, No. 1.5               |
| • Opal 620 Reagent                | • BOND Dewax Solution                  | • Tissue-Tek® Vertical 24 Slide Rack |
| • Opal 650 Reagent                | <b>Consumables</b>                     | • Tissue-Tek® Staining Dish          |
| • Opal 690 Reagent                | • BOND Titration Kit                   |                                      |
| • Spectral DAPI                   | • BOND Open Containers 30 mL           |                                      |
| • DMSO                            | • BOND Research Detection System 2     |                                      |
|                                   | • BOND Universal Covertiles            |                                      |

## Prepare the Reagents

- Reconstitute each dry vial of Opal fluorophore with 75  $\mu$ L of the DMSO provided. Aliquot the Opal fluorophores if necessary. Opal fluorophores must be stored at 4 °C after reconstituted.
- Allow the reconstituted Opal fluorophore to unfreeze (if taken out of refrigerator), by allowing it to sit at room temperature for 10 min. When unfrozen, shake and spin down the vials before diluting the quantity needed for a run at 1:150 with the 1X Plus Amplification Diluent.

### NOTE



*Ensure antibodies are diluted fresh prior to starting your run.*

*Testing suggests a good signal balancing when using a dilution of 1:150 (Opal dye: Amplification Diluent). This is a good place to start; however, some antibodies/targets may require more or less concentrated Opal fluorophore.*

- Dilute all antibodies with the Antibody Diluent/Blocking (ARD1001EA) provided, using antibody concentrations obtained from previously conducted titration experiments.
- Dilute the Spectral DAPI (FP1490) at a concentration of 1 drop of DAPI to every 500  $\mu$ L of TBS.

### NOTE



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

## Prepare the BOND RX Instrument

Refer to BOND RX User Manual

## Add Opal Detection Buffer Reagent

(Optional: for those who are willing to designate a specific Research Detection System for Opal protocol)

### NOTE



*This section is made for those who do not already have a **Bond Research Detection System** made; Skip this step if you may want to use your own detection system.*

*The **Opal Detection Buffer reagent** will be used as the reagent to register with the detection system.*



- 1 In the BOND RX software, click the **Reagent Setup** icon at the top of the **System Status Screen**.
- 2 Click the **Add** button. The **Add Reagent** window opens.
- 3 Type **Opal Detection Buffer** in the **Name** text box.
- 4 Type **Buffer** in the **Abbreviated Name** text box.
- 5 Select **Ancillary** in the **Type** drop-down list.
- 6 Leave the **Supplier** text box empty for this reagent.
- 7 Click **Save**.

## Register and Prepare the Opal Detection System on the BOND RX

### NOTE



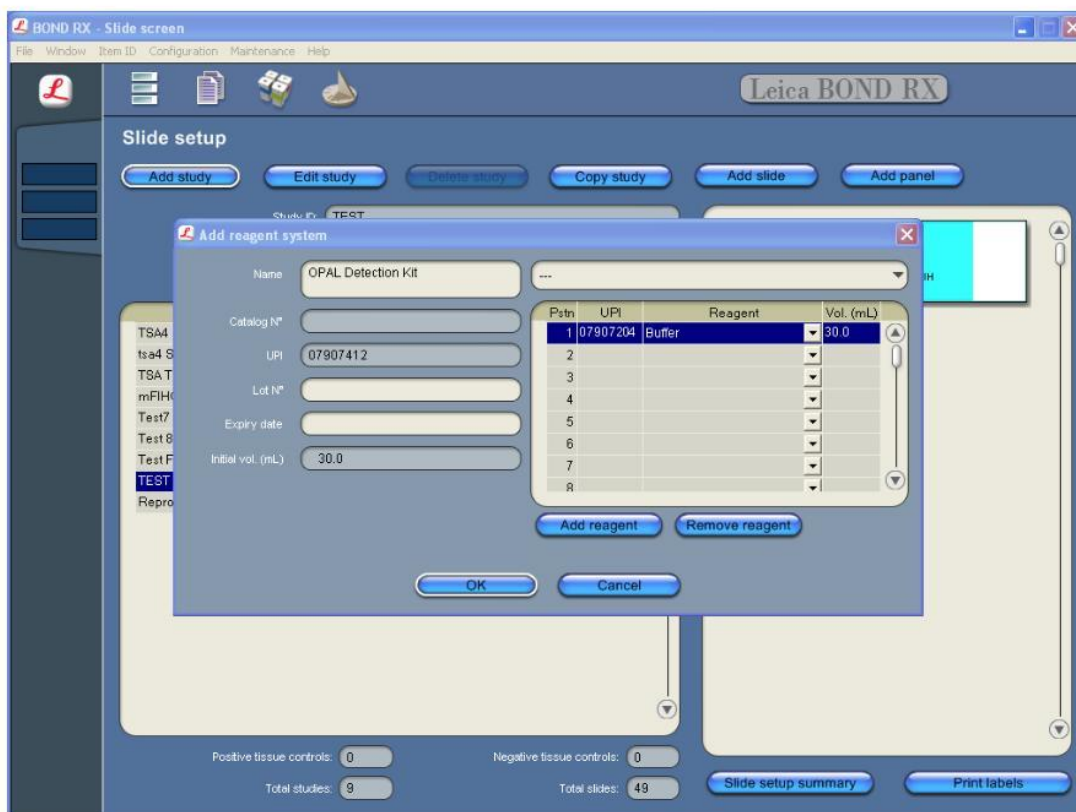
*This section is made for those who do not already have a **Bond Research Detection System** made;*

***Skip this step if you may want to use your own detection system.***

- 1 Obtain a new BOND Research Detection System.
- 2 Scan the barcode located on the front of the detection system.
- 3 Enter **OPAL Detection Kit** into the **Name** text box.
- 4 Select the first reagent slot on the right, go to the drop down and select **Opal Detection Buffer**.



- 5 Scan a new 30 mL open container and place it into the first position on the detection system.
- 6 Tape the container into place on the detection system and label it as **Opal Detection Buffer**.
- 7 Hit **OK**.



**Figure 1. Register Opal Detection System Window**

## Prepare the Instrument Reagents

### NOTE



When preparing to start a run, fill the BOND RX containers with the appropriate reagents according to Table 5. \*Open 1-6 can be modified to fit the antibody containers that the user may already have.

**Table 5. Reagent Names in BOND RX**

| Reagent to Add                              | Container Name        | Abbreviated Name | Supplier         | Container Size |
|---|-----------------------|------------------|------------------|----------------|
| TBS   | Opal Detection Buffer | Buffer           | User             | 30 mL          |
| Antibody Diluent/Block                      | *PKI Blocking Buffer  | PKIBlock         | PerkinElmer      | 30 mL          |
| Antibody 1 with Antibody Diluent            | *Open 1               | Open 1           | User/PerkinElmer | 6 mL           |
| Antibody 2 with Antibody Diluent            | *Open 2               | Open 2           | User/PerkinElmer | 6 mL           |
| Antibody 3 with Antibody Diluent            | *Open 3               | Open 3           | User/PerkinElmer | 6 mL           |
| Antibody 4 with Antibody Diluent            | *Open 4               | Open 4           | User/PerkinElmer | 6 mL           |
| Antibody 5 with Antibody Diluent            | *Open 5               | Open 5           | User/PerkinElmer | 6 mL           |
| Antibody 6 with Antibody Diluent            | *Open 6               | Open 6           | User/PerkinElmer | 6 mL           |
| Opal Polymer HRP Ms+Rb                      | *Opal Polymer HRP     | Opal HRP         | PerkinElmer      | 30 mL          |
| Opal 520 with 1X Plus Amplification Diluent | *Opal 520 Reagent     | Opal 520         | PerkinElmer      | 6 mL           |
| Opal 540 with 1X Plus Amplification Diluent | *Opal 540 Reagent     | Opal 540         | PerkinElmer      | 6 mL           |
| Opal 570 with 1X Plus Amplification Diluent | *Opal 570 Reagent     | Opal 570         | PerkinElmer      | 6 mL           |
| Opal 620 with 1X Plus Amplification Diluent | *Opal 620 Reagent     | Opal 620         | PerkinElmer      | 6 mL           |
| Opal 650 with 1X Plus Amplification Diluent | *Opal 650 Reagent     | Opal 650         | PerkinElmer      | 6 mL           |
| Opal 690 with 1X Plus Amplification Diluent | *Opal 690 Reagent     | Opal 690         | PerkinElmer      | 6 mL           |
| Spectral DAPI with TBS                      | *Spectral DAPI        | DAPI             | PerkinElmer      | 6 mL           |
| TBS   | TBS                   | TBS              | User             | 30 mL          |

### NOTE



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.

## Set up the BOND RX Protocols

Perform the following procedures to set up the BOND RX:

- [Create a Fake Probe Hybridization Protocol on page 18](#)
- [Create a Fake Probe Reagent on page 19](#)
- [Create a Staining Protocol on page 20](#)
- [Create an Autofluorescence Protocol on page 21](#)
- [Create a Library Protocol on page 21](#)
- [Create a DAPI Protocol on page 22](#)
- [Setup a Study on page 23](#)

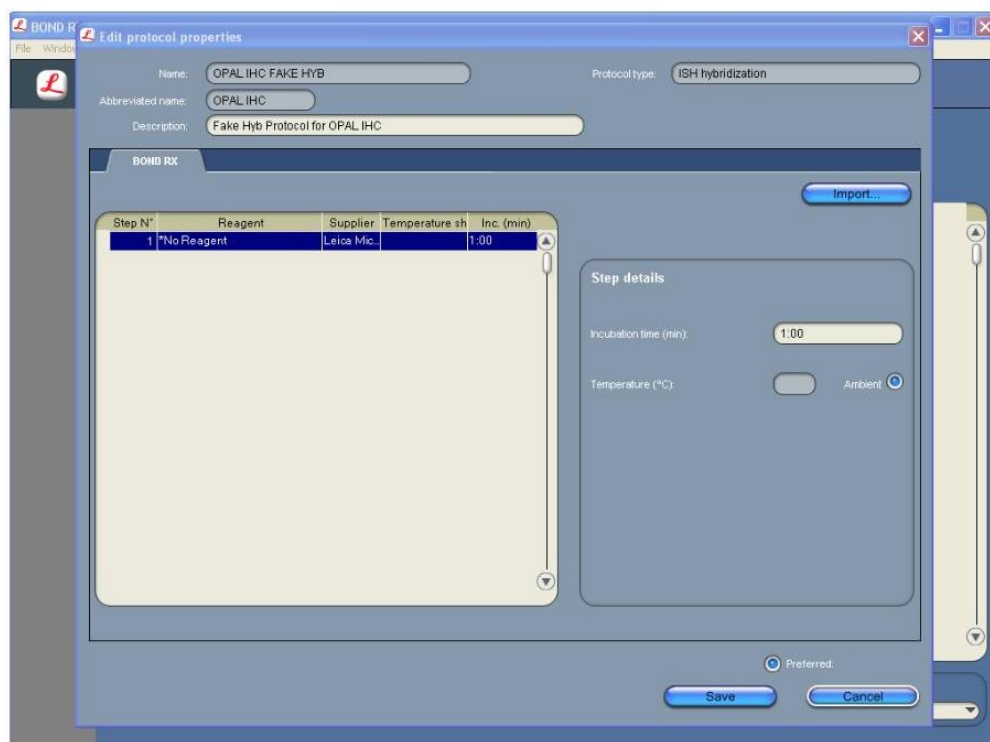
### NOTE



*Users with the **BOND RX Version 5.2** software can skip all fake probe steps.*

## Create a Fake Probe Hybridization Protocol

- 1 In the **Protocol Setup** window, select **Prestaining** under Protocol Group and **ISH Hybridization** under the Protocol Type menu.
- 2 Select the **\*ISH Hybridization (2Hr)** protocol and click **Copy**.
- 3 Change the Name to **OPAL IHC FAKE HYB**

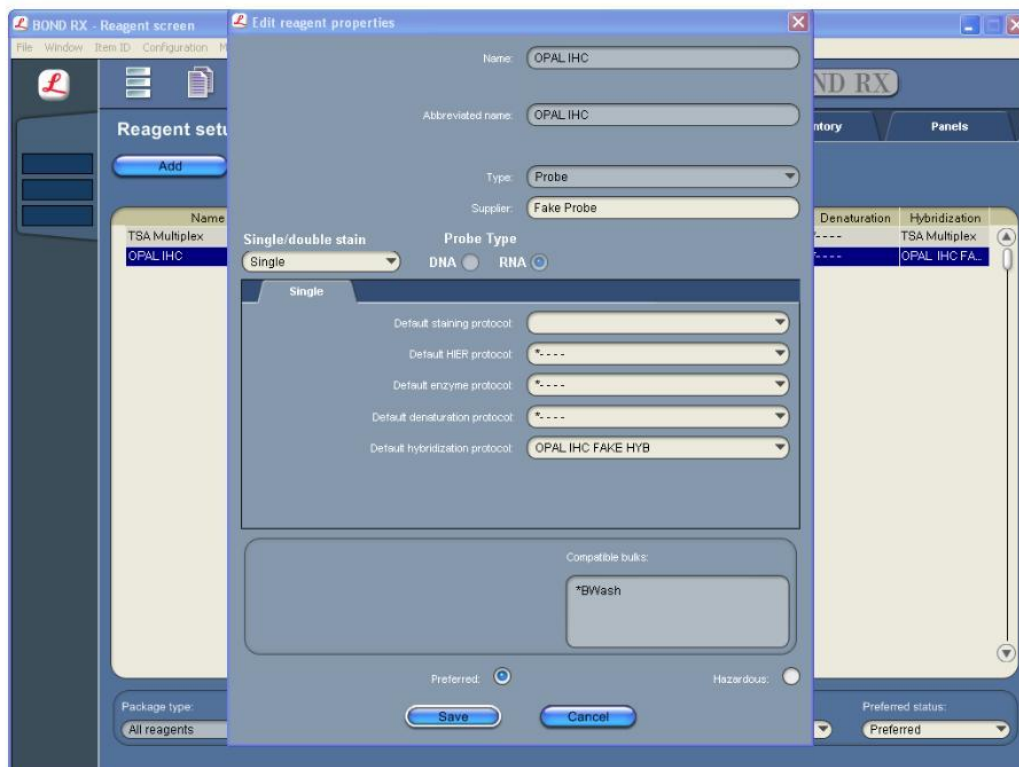


**Figure 2. Copy Protocol Window**

- 4 Change the Abbreviated Name to **OPAL IHC**.
- 5 Change the Incubation Time to **1 minute**.
- 6 Change the Temperature to **Ambient**.
- 7 Click **Save**.

## Create a Fake Probe Reagent

- 1 Click the **Reagent Setup** icon at the top of the window.
- 2 Click **Add** to open the Add Reagent window.
- 3 Type **OPAL IHC** in the Name text box.
- 4 Type **OPAL IHC** in the Abbreviated Name text box.
- 5 Select **Probe** in the Type drop-down list.
- 6 Type **Fake Probe** in the Supplier text box.
- 7 Check **RNA** as Probe Type.



**Figure 3. Add Reagent Window**

- 8 Select **OPAL IHC FAKE HYB** as the default hybridization protocol.
- 9 Click **Save**.
- 10 Register a 30 mL Open Container under the new reagent **OPAL IHC (Fake Probe)** and fill the container with TBS.

## Create a Staining Protocol

- 1 In the **Protocol Setup** window, select **Protocol Group->Staining**.
- 2 Select the **Opal 7-Color** protocol and press **Copy**.

If you are using Opal 7-Color Automation Kit, ensure the asterisk in the protocol name and abbreviated name is deleted and go to step 4.

If you are using Opal 4-Color Automation Kit, go to step 3.
- 3 For Opal 4-Color Automation Kit: Truncate the protocol for a 3-plex assay by deleting steps related to Ab2-Opal 540, Ab4-Opal 620 and Ab5-Opal 650. Name the new protocol as **Opal 4-Color** and change the abbreviated name to **PKI4c**.
- 4 Select **Opal Detection Kit** (or your preferred detection system) under the preferred detection system menu.
- 5 Replace first Bond Wash Solution step as a reagent step (figure 4 below) and input the reagent linked to your detection system for 0 minutes (**Opal Detection Buffer** for those who created their detection system with this manual). Alternatively, if you are using your own detection system and reagent, ensure the reagent linked to your detection system has a step within the 7-color or 4-color protocol.
- 6 Click **Show Wash Steps** and compare the protocol to the steps in [Appendix A](#) to ensure all steps are present and correct.
- 7 Click **Save**.

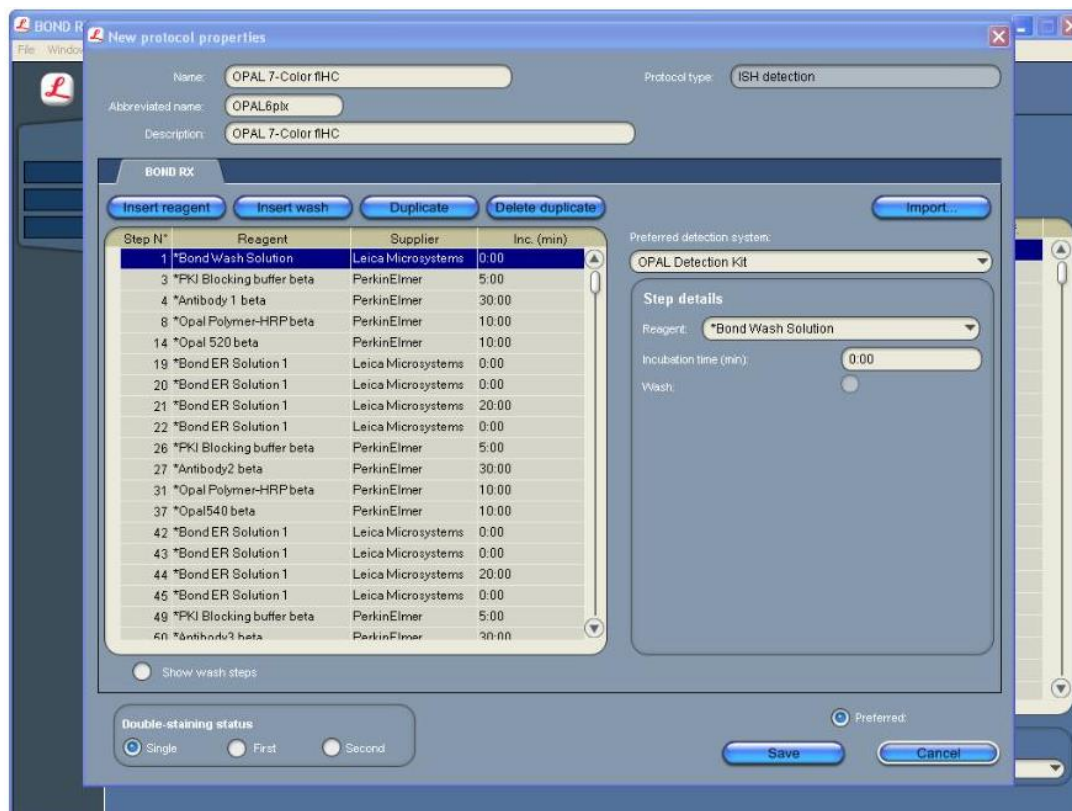


Figure 4. Add Detection System Buffer Step

## Create an Autofluorescence Protocol

- 1 In the Protocol setup window, select **Protocol Group -> Staining**.
- 2 Select either the **OPAL 4-Color** or **OPAL 7-Color** protocol created above and click **Copy**.
- 3 Change the Name to **OPAL 4-Color AF** or **OPAL 7-Color AF**.
- 4 Change the Abbreviated Name to **4plex AF** or **6plex AF**.
- 5 Select **OPAL Detection Kit** under the preferred detection system menu.
- 6 Change all 6 Opal fluorophore steps as well as DAPI to the **TBS** container, and leave all other steps the same.
- 7 Click **Save**.

## Create a Library Protocol

- 1 In the Protocol Setup window, select Protocol Group -> Staining.
- 2 Select either the **OPAL 4-Color** or **OPAL 7-Color** protocol created above and click Copy.

- 3 Change the Name to **OPAL 4-Color** or **OPAL 7-Color Ab1 Library**.
- 4 Change the Abbreviated Name to **3pxAb1Lb** or **6pxAb1Lb**.
- 5 Select **OPAL Detection Kit** under the preferred detection system menu.
- 6 Select all non-stripping reagent steps that are not involved with cycle of the library antibody, except the initial Buffer step, and change them to the **TBS container**.  
  
Example: For the library protocol of antibody 1: PKI Blocking (step 3), Antibody 1 (step 4), PKI HRP (step 8), and OPAL 520 (step 14) will remain unchanged. All other blocking, antibody, HRP, Opal fluorophore and DAPI steps will be changed to TBS.
- 7 Click **Save**.
- 8 Repeat for all other antibodies to produce 3 or 6 library slide protocols, depending on if you are using the 4-Color or 7-color protocol.

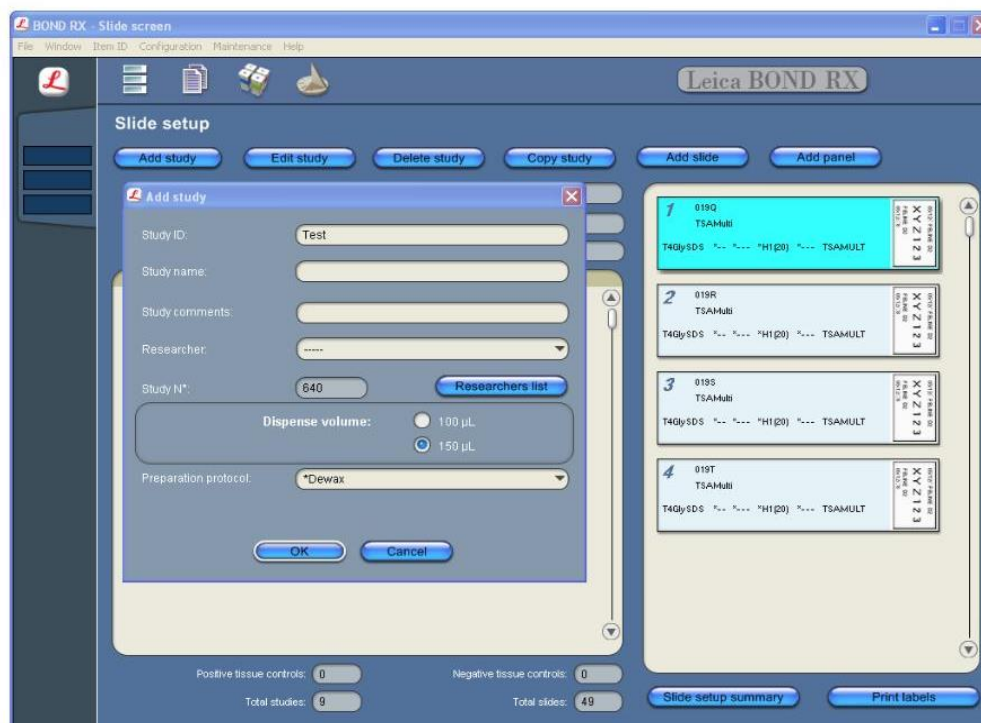
## Create a DAPI Protocol

- 1 In the Protocol Setup window, select Protocol Group -> Staining.
- 2 Select either the **OPAL 4-Color** or **OPAL 7-Color** protocol created above and click **Copy**.
- 3 Change the Name to **OPAL 4-Color DAPI** or **OPAL 7-Color DAPI**.
- 4 Change the Abbreviated Name to **3px DAPI** or **6px DAPI**.
- 5 Select **OPAL Detection Kit** under the preferred detection system menu.
- 6 Select all non-stripping reagent steps, except the initial Buffer step and DAPI, and change them to **TBS**.
- 7 Click **Save**.

## Set Up a Study

To create a study in the BOND RX software:

- 1 Click the **Slide Setup** icon on the BOND RX System Status window.
- 2 Click the **Add Study** button. The Add Study window opens (see figure 5 below).
- 3 Type a name in the **Study ID** text box.
- 4 Select **150 µL** in the **Dispense Volume** drop-down list.
- 5 Select **\*Bake and Dewax** or **\*Dewax** in the **Preparation Protocol** drop-down list, depending on whether the slides need to be baked.
- 6 Click **OK**.



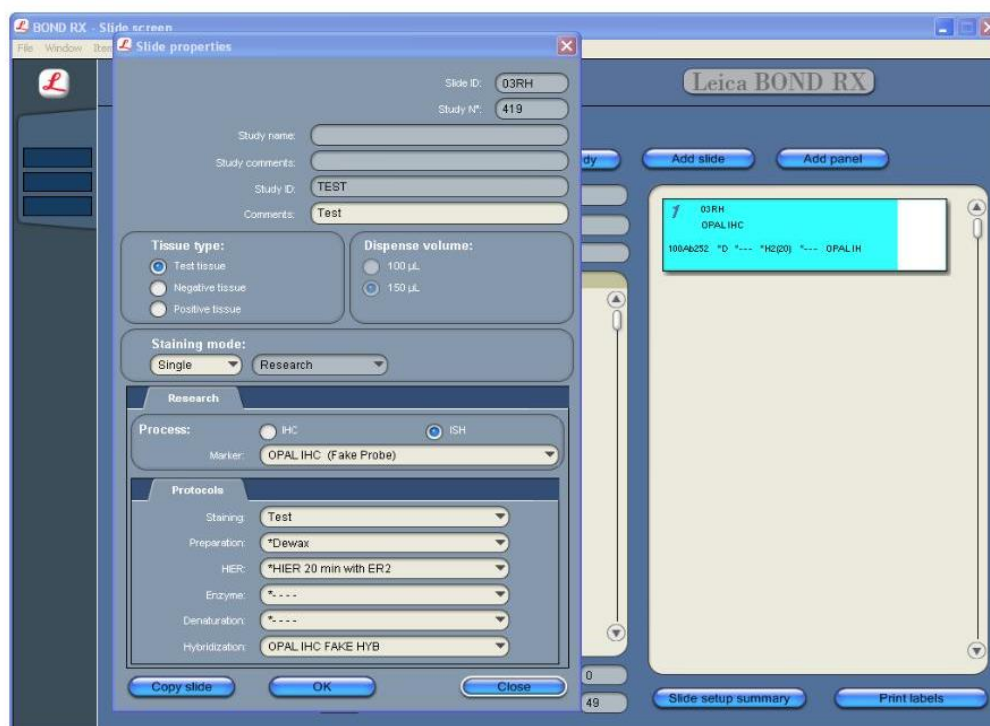
**Figure 5. Add Study Window**

- 7 Click the **Add Slide** button to assign a protocol to each slide in the study. The Add Slide window opens (see figure 6 below).
- 8 Type the name of the tissue in the **Comments** text box.
- 9 Select **ISH** as the **Process** on the **Research** tab.
- 10 Select the marker **OPAL IHC (Fake Probe)**, if using Version 4 software.
- 11 In the **Preparation Protocol** drop-down list, verify the correct



protocol, either **\*Bake and Dewax** or **\*Dewax**, is selected.

- 12 In the **Staining Protocol** drop-down list, select **OPAL 4-Color** or **OPAL 7-Color**, or other appropriate protocol.
- 13 Select the **HIER Protocol** based on the initial antibody in the 6plex. For an antibody that typically receives a pH6 epitope retrieval step, select **\*HIER 20 min with ER1**. For an antibody that typically receives a pH9 epitope retrieval step, select **\*HIER 20 min with ER2**. (For multiplex staining on version 4.0, it's recommended to start with an antibody that needs ER2 for epitope retrieval.)
- 14 Select **OPAL IHC FAKE HYB** as the **Hybridization** protocol.
- 15 Leave all other text boxes empty.
- 16 Click the **Add Slide** button to add the slide to the study.



**Figure 6. Add Slide Window**

- 17 Repeat steps 7 to 16 to add each of the slides to the protocol.
- 18 When all of the slides are added, click the **Close** button to close the Add Slide window.
- 19 Repeat the procedure above to add autofluorescent or library slides.
- 20 Click the **Print Labels** button at the bottom of the Slide Setup window to print a barcode label for each slide.

## Run the Opal 4-Color or 7-Color Automation IHC Kit

- [Materials Required on page 25](#)
- [Load the Reagents on page 25](#)
- [Start the Run on page 26](#)
- [Complete the Run on page 26](#)

### Materials Required

The following materials are required to run the assay:

- TBS
- Water (DI, MQ)
- Cover Glass, No. 1.5
- ProLong<sup>®</sup> Diamond Antifade Mountant
- Slide Rack
- Staining Dish

### Load the Reagents

- 1 Label each BOND RX container with the corresponding reagent from Table 5, using a water-resistant marker.
- 2 Use the barcode scanner to scan the barcode on the **front** of each container. A pop-up menu will appear.

#### NOTE



Do NOT scan the barcode on the top of the container.

- 3 Choose the appropriate Reagent Name, and enter the lot number and expiration date in the corresponding text boxes. Click OK.
- 4 Load the containers onto the reagent tray and slide the tray into the BOND RX.

## Start the Run

- 1 After attaching the barcode labels to the slides, add the slides to the slide tray with the labels facing up.

### NOTE



*Autofluorescent, library, and 4-Color or 7-Color protocols may be able to run on the same tray, assuming that the only differences are the reagents that are dispensed during each step, and each step across the protocols is the same amount of time.*

- 2 Add a covertile on top of each slide. The rectangular-shaped neck of the covertile should fit into the groove of the slide tray.
- 3 Place the tray in the BOND RX and press the button to load the tray onto the machine.
- 4 After the slides have been scanned, go to the **System Status Screen** and select the triangular (**PLAY**) button on the screen under the 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.

### NOTE



*Typical time for a 6plex run will take 11-13 hours, depending on the amount of slides being run. It is recommended that the delayed start is used if slides may be left on the BOND RX for more than 10 minutes beyond run completion.*

*Before leaving the instrument unattended, verify that the instrument is running properly, and that an end time has been scheduled.*

## Complete the Run

- 1 After the run is complete, press the button on the instrument to unload the slides.
- 2 Place the Slide Rack into the staining dish, and fill the dish with DI water.
- 3 Remove the covertiles and place in a separate container to clean.
- 4 Place the slides into the Slide Rack inside the staining dish.

### NOTE



*Do not leave slides on the BOND RX after the run is completed. Promptly remove and coverslip for best staining results.*

- 5 Remove the slides one at a time from the Slide Rack and wipe off excess water on the sides of the tissue and on the back of the slide.
- 6 Mount by adding 1-2 DROPS of ProLong DiamondAntifade mounting medium to each slide and carefully placing a No. 1.5 coverglass over the section. Avoid trapping air bubbles.
- 7 Air dry slides for at least 20 min.
- 8 Examine the slide results on the Vectra Automated Quantitative Pathology Workstation.

## Appendix A: Opal 7-Color Protocol

| Step No. | Reagent               | Step Type | Incubation Time | Temperature |
|----------|-----------------------|-----------|-----------------|-------------|
| 1        | Opal Detection Buffer | Reagent   | 0 min           | Ambient     |
| 2        | Bond Wash Solution    | Wash      | 0 min           | Ambient     |
| 3        | PKI Blocking Buffer   | Reagent   | 5 min           | Ambient     |
| 4        | Open 1                | Reagent   | 30 min          | Ambient     |
| 5        | Bond Wash Solution    | Wash      | 0 min           | Ambient     |
| 6        | Bond Wash Solution    | Wash      | 1 min           | Ambient     |
| 7        | Bond Wash Solution    | Wash      | 0 min           | Ambient     |
| 8        | Opal Polymer HRP      | Reagent   | 10 min          | Ambient     |
| 9        | Bond Wash Solution    | Wash      | 0 min           | Ambient     |
| 10       | Bond Wash Solution    | Wash      | 1 min           | Ambient     |
| 11       | Bond Wash Solution    | Wash      | 0 min           | Ambient     |
| 12       | Bond Wash Solution    | Wash      | 0 min           | Ambient     |
| 13       | Bond Wash Solution    | Wash      | 0 min           | Ambient     |
| 14       | Opal 520 Reagent      | Reagent   | 10 min          | Ambient     |
| 15       | Bond Wash Solution    | Wash      | 0 min           | Ambient     |
| 16       | Bond Wash Solution    | Wash      | 1 min           | Ambient     |
| 17       | Bond Wash Solution    | Wash      | 0 min           | Ambient     |
| 18       | Bond Wash Solution    | Wash      | 0 min           | Ambient     |
| 19       | Bond ER1 Solution     | Reagent   | 0 min           | Ambient     |
| 20       | Bond ER1 Solution     | Reagent   | 0 min           | 95 °C       |
| 21       | Bond ER1 Solution     | Reagent   | 20 min          | 95 °C       |
| 22       | Bond ER1 Solution     | Reagent   | 0 min           | Ambient     |
| 23       | Bond Wash Solution    | Wash      | 0 min           | Ambient     |
| 24       | Bond Wash Solution    | Wash      | 1 min           | Ambient     |
| 25       | Bond Wash Solution    | Wash      | 0 min           | Ambient     |
| 26       | PKI Blocking Buffer   | Reagent   | 5 min           | Ambient     |
| 27       | Open 2                | Reagent   | 30 min          | Ambient     |
| 28       | Bond Wash Solution    | Wash      | 0 min           | Ambient     |
| 29       | Bond Wash Solution    | Wash      | 1 min           | Ambient     |
| 30       | Bond Wash Solution    | Wash      | 0 min           | Ambient     |
| 31       | Opal Polymer HRP      | Reagent   | 10 min          | Ambient     |
| 32       | Bond Wash Solution    | Wash      | 0 min           | Ambient     |
| 33       | Bond Wash Solution    | Wash      | 1 min           | Ambient     |

| Step No. | Reagent             | Step Type | Incubation Time | Temperature |
|----------|---------------------|-----------|-----------------|-------------|
| 34       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 35       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 36       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 37       | Opal 540 Reagent    | Reagent   | 10 min          | Ambient     |
| 38       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 39       | Bond Wash Solution  | Wash      | 1 min           | Ambient     |
| 40       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 41       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 42       | Bond ER1 Solution   | Reagent   | 0 min           | Ambient     |
| 43       | Bond ER1 Solution   | Reagent   | 0 min           | 95 °C       |
| 44       | Bond ER1 Solution   | Reagent   | 20 min          | 95 °C       |
| 45       | Bond ER1 Solution   | Reagent   | 0 min           | Ambient     |
| 46       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 47       | Bond Wash Solution  | Wash      | 1 min           | Ambient     |
| 48       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 49       | PKI Blocking buffer | Reagent   | 5 min           | Ambient     |
| 50       | Open 3              | Reagent   | 30 min          | Ambient     |
| 51       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 52       | Bond Wash Solution  | Wash      | 1 min           | Ambient     |
| 53       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 54       | Opal Polymer HRP    | Reagent   | 10 min          | Ambient     |
| 55       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 56       | Bond Wash Solution  | Wash      | 1 min           | Ambient     |
| 57       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 58       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 59       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 60       | Opal 570 Reagent    | Reagent   | 10 min          | Ambient     |
| 61       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 62       | Bond Wash Solution  | Wash      | 1 min           | Ambient     |
| 63       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 64       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 65       | Bond ER1 Solution   | Reagent   | 0 min           | Ambient     |

| Step No. | Reagent             | Step Type | Incubation Time | Temperature |
|----------|---------------------|-----------|-----------------|-------------|
| 66       | Bond ER1 Solution   | Reagent   | 0 min           | 95 °C       |
| 67       | Bond ER1 Solution   | Reagent   | 20 min          | 95 °C       |
| 68       | Bond ER1 Solution   | Reagent   | 0 min           | Ambient     |
| 69       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 70       | Bond Wash Solution  | Wash      | 1 min           | Ambient     |
| 71       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 72       | PKI Blocking Buffer | Reagent   | 5 min           | Ambient     |
| 73       | Open 4              | Reagent   | 30 min          | Ambient     |
| 74       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 75       | Bond Wash Solution  | Wash      | 1 min           | Ambient     |
| 76       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 77       | Opal Polymer HRP    | Reagent   | 10 min          | Ambient     |
| 78       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 79       | Bond Wash Solution  | Wash      | 1 min           | Ambient     |
| 80       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 81       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 82       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 83       | Opal 620 Reagent    | Reagent   | 10 min          | Ambient     |
| 84       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 85       | Bond Wash Solution  | Wash      | 1 min           | Ambient     |
| 86       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 87       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 88       | Bond ER1 Solution   | Reagent   | 0 min           | Ambient     |
| 89       | Bond ER1 Solution   | Reagent   | 0 min           | 95 °C       |
| 90       | Bond ER1 Solution   | Reagent   | 20 min          | 95 °C       |
| 91       | Bond ER1 Solution   | Reagent   | 0 min           | Ambient     |
| 92       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 93       | Bond Wash Solution  | Wash      | 1 min           | Ambient     |
| 94       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 95       | PKI Blocking Buffer | Reagent   | 5 min           | Ambient     |
| 96       | Open 5              | Reagent   | 30 min          | Ambient     |

| Step No. | Reagent             | Step Type | Incubation Time | Temperature |
|----------|---------------------|-----------|-----------------|-------------|
| 97       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 98       | Bond Wash Solution  | Wash      | 1 min           | Ambient     |
| 99       | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 100      | Opal Polymer HRP    | Reagent   | 10 min          | Ambient     |
| 101      | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 102      | Bond Wash Solution  | Wash      | 1 min           | Ambient     |
| 103      | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 104      | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 105      | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 106      | Opal 650 Reagent    | Reagent   | 10 min          | Ambient     |
| 107      | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 108      | Bond Wash Solution  | Wash      | 1 min           | Ambient     |
| 109      | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 110      | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 111      | Bond ER1 Solution   | Reagent   | 0 min           | Ambient     |
| 112      | Bond ER1 Solution   | Reagent   | 0 min           | 95 °C       |
| 113      | Bond ER1 Solution   | Reagent   | 20 min          | 95 °C       |
| 114      | Bond ER1 Solution   | Reagent   | 0 min           | Ambient     |
| 115      | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 116      | Bond Wash Solution  | Wash      | 1 min           | Ambient     |
| 117      | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 118      | PKI Blocking Buffer | Reagent   | 5 min           | Ambient     |
| 119      | Open 6              | Reagent   | 30 min          | Ambient     |
| 120      | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 121      | Bond Wash Solution  | Wash      | 1 min           | Ambient     |
| 122      | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 123      | Opal Polymer HRP    | Reagent   | 10 min          | Ambient     |
| 124      | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 125      | Bond Wash Solution  | Wash      | 1 min           | Ambient     |
| 126      | Bond Wash Solution  | Wash      | 0 min           | Ambient     |
| 127      | Bond Wash Solution  | Wash      | 0 min           | Ambient     |



| Step No. | Reagent            | Step Type | Incubation Time | Temperature |
|----------|--------------------|-----------|-----------------|-------------|
| 128      | Bond Wash Solution | Wash      | 0 min           | Ambient     |
| 129      | Opal 690 Reagent   | Reagent   | 10 min          | Ambient     |
| 130      | Bond Wash Solution | Wash      | 0 min           | Ambient     |
| 131      | Bond Wash Solution | Wash      | 1 min           | Ambient     |
| 132      | Bond Wash Solution | Wash      | 0 min           | Ambient     |
| 133      | Bond Wash Solution | Wash      | 0 min           | Ambient     |
| 134      | Bond ER1 Solution  | Reagent   | 0 min           | Ambient     |
| 135      | Bond ER1 Solution  | Reagent   | 0 min           | 95 °C       |
| 136      | Bond ER1 Solution  | Reagent   | 20 min          | 95 °C       |
| 137      | Bond ER1 Solution  | Reagent   | 0 min           | Ambient     |
| 138      | Bond Wash Solution | Wash      | 0 min           | Ambient     |
| 139      | Bond Wash Solution | Wash      | 1 min           | Ambient     |
| 140      | Bond Wash Solution | Wash      | 0 min           | Ambient     |
| 141      | Spectral DAPI      | Reagent   | 5 min           | Ambient     |
| 142      | Bond Wash Solution | Wash      | 0 min           | Ambient     |
| 143      | Bond Wash Solution | Wash      | 1 min           | Ambient     |
| 144      | Bond Wash Solution | Wash      | 0 min           | Ambient     |

## Appendix B: Safety

The following safety information about the Opal 4-Color and OPAL 7-Color Automation IHC Kits is included in this documentation. Read and review all safety information before operating the Instrument or using the reagents.

- [Required Training on page 33](#)
- [Chemical and Biological Safety on page 33](#)

### Required Training

Ensure that all personnel involved with the operation of the instrument have:

- Received instruction in general safety practices for laboratories.
- Received instruction in specific safety practices for the instrument.
- Read and understood all related MSDSs.

#### WARNING



*Use this product only in the manner described in this manual. If the equipment is used in a manner not specified by the manufacturer, the protection provided by the equipment may be impaired.*

### Chemical and Biological Safety

#### WARNING



*Biohazard Risks. In some applications, chemicals or samples used with the Opal 4-Color and 7-Color Automation IHC Kit are potentially hazardous and can cause illness.*

- Read and understand the material safety data sheet (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemical or hazardous material.
- Minimize contact with and inhalation of chemicals and chemical wastes.
- Wear appropriate personal protective equipment when handling chemicals or samples (e.g., safety glasses, gloves, or clothing). For additional safety guidelines consult the MSDS.
- Handle all samples using good laboratory practices to prevent biohazards.

## Chemical and Biological Safety (Continued)

- Do not leave chemical containers open. Use only with adequate ventilation, including a fume hood, if necessary.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the chemical manufacturer's cleanup procedures as recommended on the MSDS.
- Dispose of chemical or infectious waste in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.
- After emptying waste containers, seal the waste containers appropriately.
- Comply with all local, state/provincial, or national laws and regulations related to chemical and waste storage, handling, and disposal.

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