RNAscope® LS 4-plex Fluorescent Assay

Introduction

This Technical Note provides guidelines for performing automated 4-plex *in situ* hybridization (ISH) on the Leica BOND RX System. The procedure is based on the standard 3-plex automated fluorescent assay using the RNAscope® LS Multiplex Fluorescent Reagent Kit (Cat. No. 322800), and requires the RNAscope® LS 4-plex Ancillary Kit (Cat. No. 322830) to detect four different mRNA species. Before starting the procedure, create a protocol for the RNAscope® 4-plex assay on the RX controller with the help of your ACD FAS. For every chemical, read the Material Safety Data Sheet (MSDS) and follow handling instructions. Wear appropriate protective eyewear, clothing, and gloves. For the latest service and support information, go to **www.acdbio.com/support**.

Materials Required

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

Target Probes					
☑	Reagent	Cat. No.	Content	Quantity	Storage
	RNAscope® LS Multiplex Target Probe – *[species]*– *[gene]*	Various	Ready-To-Use (RTU) probe for color channel C1	18 mL x 1 bottle	2–8°C
	RNAscope® LS Multiplex Target Probe – *[species]*– *[gene]*– C2	Various	50X probe for color channel C2	320 µL x 1 tube	2–8°C
	RNAscope® LS Multiplex Target Probe – *[species]*– *[gene]*– C3	Various	50X probe for color channel C3	320 µL x 1 tube	2–8°C
	RNAscope® LS Multiplex Target Probe – *[species]*– *[gene]*– C4	Various	50X probe for color channel C4	320 µL x 1 tube	2–8°C
Control Probes					
☑	Reagent	Cat. No.	Content	Quantity	Storage
	RNAscope® 2.5 LS Multiplex Positive Control Probe- Hs	321808	RNAscope® 2.5 LS Human Positive Control Probe for the RNAscope® LS 2.5 Multiplex Fluorescent Assay - *POLR2A* (C1 channel), *PPIB* (C2 channel), *UBC* (C3 channel), and HPRT-1 (C4 channel).	18 mL x 1 bottle	2–8°C
	RNAscope® 2.5 LS Multiplex Positive Control Probe- Mm	321818	RNAscope® 2.5 LS Mouse Positive Control Probe for the RNAscope® LS 2.5 Multiplex Fluorescent Assay - *Polr2A* (C1 channel), *Ppib* (C2 channel), *Ubc* (C3 channel), and *Hprt-1* (C4 channel).	18 mL x 1 bottle	2–8°C

Control Probes					
☑	Reagent	Cat. No.	Content	Quantity	Storage
	RNAscope® 2.5 LS Multiplex Negative Control Probe	321838	RNAscope® 4-plex Multiplex Negative Control Probe for the RNAscope® 2.5 LS Multiplex Fluorescent Assay - *DapB* (*Bacillus subtilis* strain).	18 mL x 1 bottle	2–8°C

RNAscope® LS Multiplex Fluorescent Reagent Kit

The kit provides 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 table:

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® Multiplex TSA buffer	29 mL x 3 bottle	2–8°C
	RNAscope® LS Multiplex HRP Blocker	29 mL x 2 bottle	2–8°C
	RNAscope® LS Multiplex DAPI	21 mL x 1 bottle	2–8°C

RNAscope® LS 4-plex Ancillary Kit

The kit provides enough reagents to stain ~60 standard slides in combination with RNAscope® LS Multiplex Fluorescent Reagent Kit. The reagents are Ready-To-Use (RTU) except for the Multiplex TSA buffer, and are stored as indicated in the following table:

RNAscope® LS 4-plex Ancillary Reagent Kit (Cat. No. 322830)			
☑	Reagent	Quantity	Storage
	RNAscope® LS Multiplex HRP C4	21 mL x 1 bottle	2–8°C
	RNAscope® Multiplex TSA Buffer	29 mL x 1 bottle	2–8°C
	RNAscope® LS Multiplex HRP blocker	29 mL x 1 bottle	2–8°C

Materials from Leica BOND RX

The RNAscope® LS 4-plex Fluorescent Assay requires specific materials and equipment available *only* from Leica Biosystems.

☑	Component	Cat. No.	Storage
	BOND 30 mL Open containers	OP309700	Room temp (20–25°C)
	BOND 7 mL Open containers*	OP79193	Room temp (20–25°C)
	Bond Titration Kit	OPT9049	Room temp (20–25°C)
	BOND Research Detection System	DS9455	Room temp (20–25°C)
	BOND Universal Covertiles 100 pack	S21.2001	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™ fluorophores.

Opal™ fluorophores

The assay requires Opal™fluorophores from PerkinElmer (see the following table). Dilute the fluorophores in TSA® buffer provided by the RNAscope® LS Multiplex Reagent Kit and RNAscope® LS 4-plex ancillary kit. Choose a dilution factor for each fluorophore based on recommendations from ACD and your needs (for example, tissue quality or microscope setting). Materials are qualified using a 1:1500 dilution for all fluorophores. We cannot guarantee assay results if you use other fluorescent dyes.

Fluorophore Kit	PerkinElmer Part Number	Recommended dilution range
Opal 520 Reagent Pack*	FP1487001KT	1:750–1:3000
Opal 570 Reagent Pack*	FP1488001KT	1:750–1:3000
Opal 620 Reagent Pack*	FP1495001KT	1:750–1:3000
Opal 690 Reagent Pack*	FP1497001KT	1:750–1:3000

* Depending on the dilution factor used, this stock size (150 µl) is sufficient to run the assay on 375–1500 slides.

Recommended fluorophore combinations

To perform 4-plex RNAscope® *in situ* hybridization (ISH) assay, see the following table for examples below of four fluorophore combinations. You will assign each Opal™ fluorophore to one channel from C1, C2, C3 and C4. Do not use the same Opal™ fluorophore for different channels.

Reagent registration name	Corresponding RNAscope® channel	Option 1 (recommended)	Option 2	Option 3	Option 4
ACD Multiplex TSA-F1	C1	Opal 520	Opal 570	Opal 620	Opal 620
ACD Multiplex TSA-F2	C2	Opal 570	Opal 620	Opal 570	Opal 520
ACD Multiplex TSA-F3	C3	Opal 620	Opal 520	Opal 520	Opal 570

Reagent registration name	Corresponding RNAscope® channel	Option 1 (recommended)	Option 2	Option 3	Option 4
ACD Multiplex TSA-F4	C4	Opal 690	Opal 690	Opal 690	Opal 690

IMPORTANT! Use a multiplexed biomarker imaging system, such as the Nuance® EX, Mantra™ or Vectra® System, to successfully analyze your multiplex fluorescent staining.

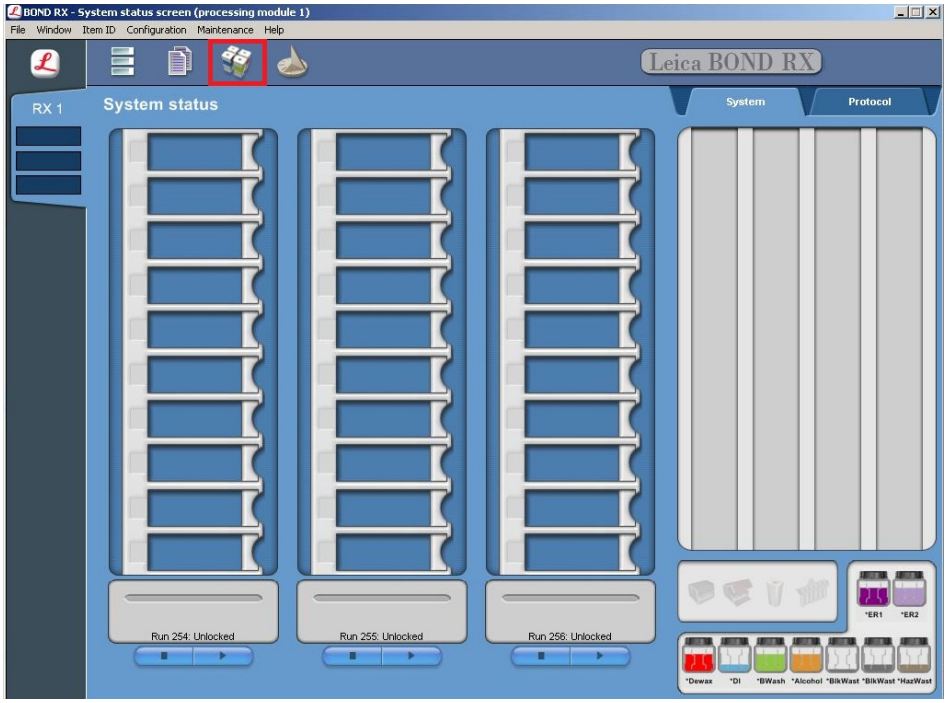
Workflow

Create software protocols to perform 4-Plex in situ hybridization (ISH)

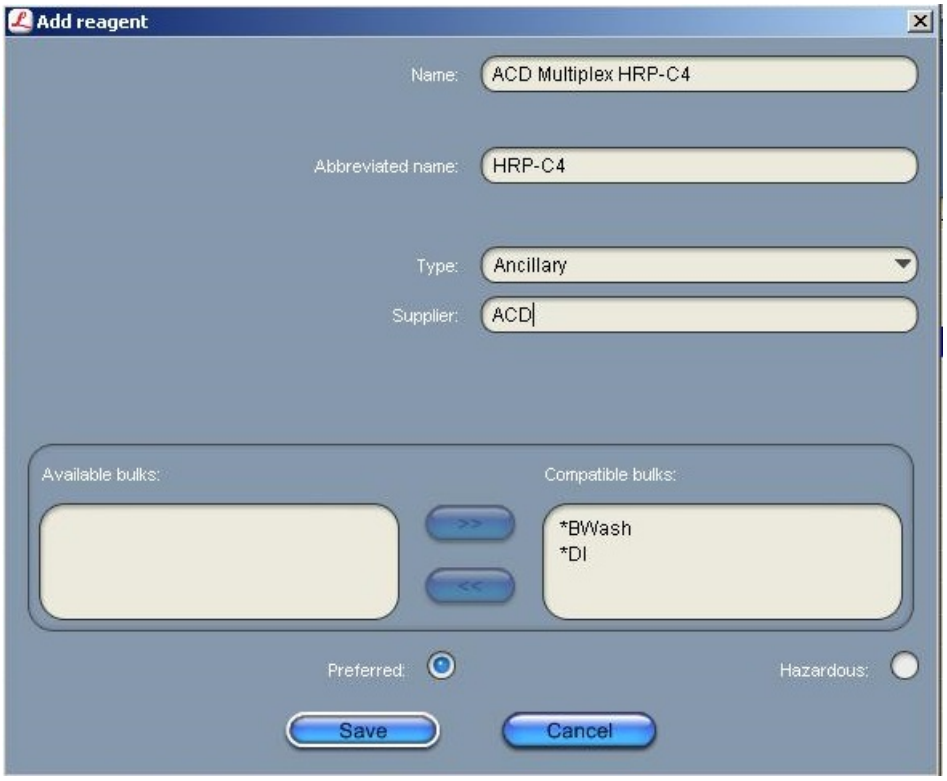
This section provides instructions for creating 4-plex *in situ* hybridization (ISH) software protocols on the Leica BOND RX System based on ACD LS Multiplex protocol.

Register the reagents

1. To add the HRP-C4 to the assay, select the **Reagent Setup** icon at the top of the screen.
2. Select **Add** to enter reagent information.

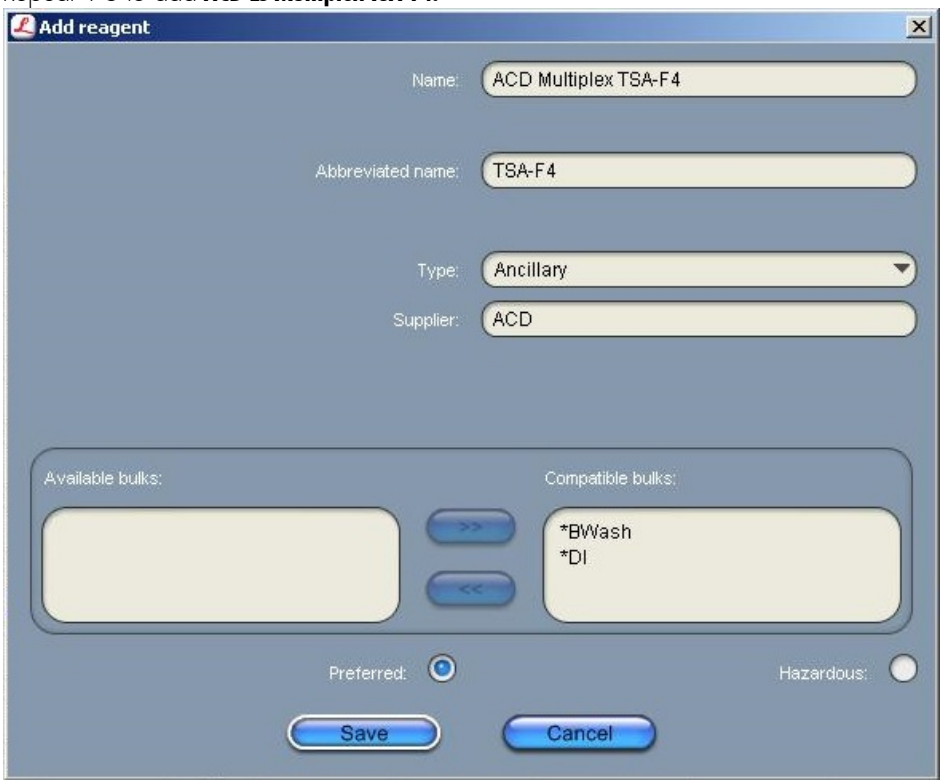


3. Enter the name **ACD LS Multiple HRP-C4** in the Name text box.
4. Enter **HRP-C4** in the Abbreviated name text box.
5. Select **Ancillary** in the Type drop-down menu.



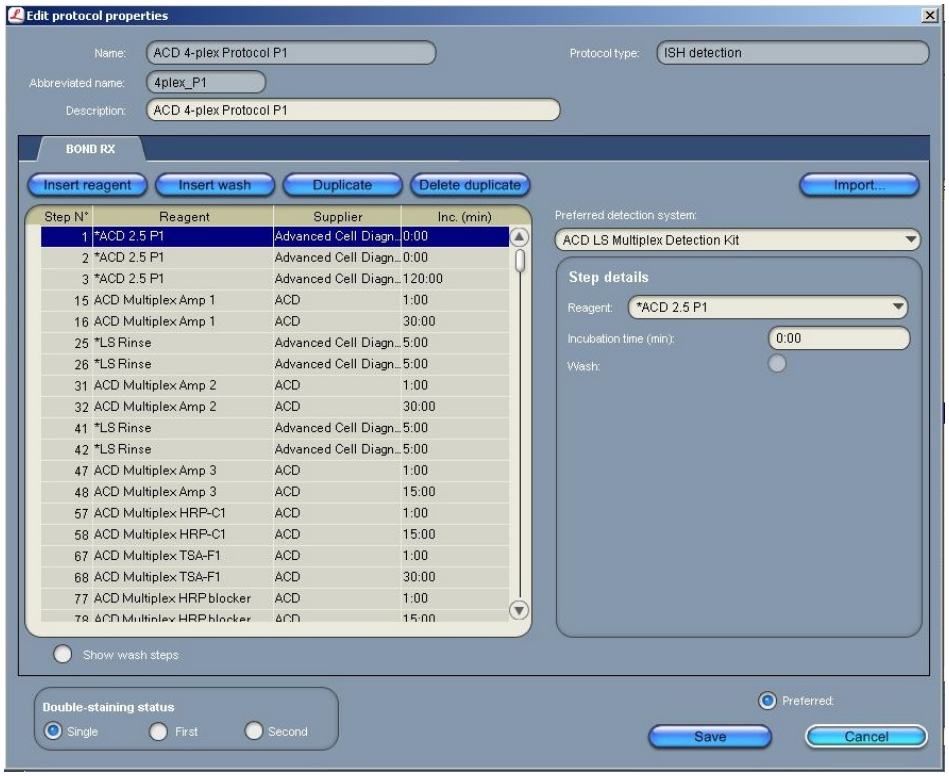
Note: You may leave the Supplier text box empty.

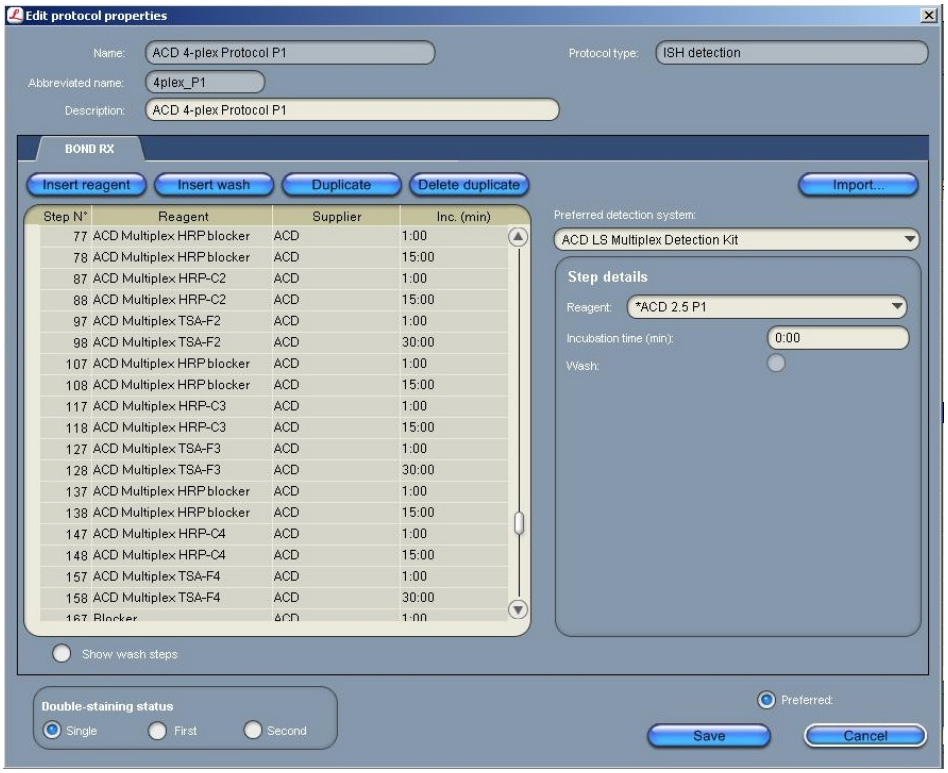
6. Select **Save**.
7. Repeat 1-6 to add **ACD LS Multiplex TSA-F4.**

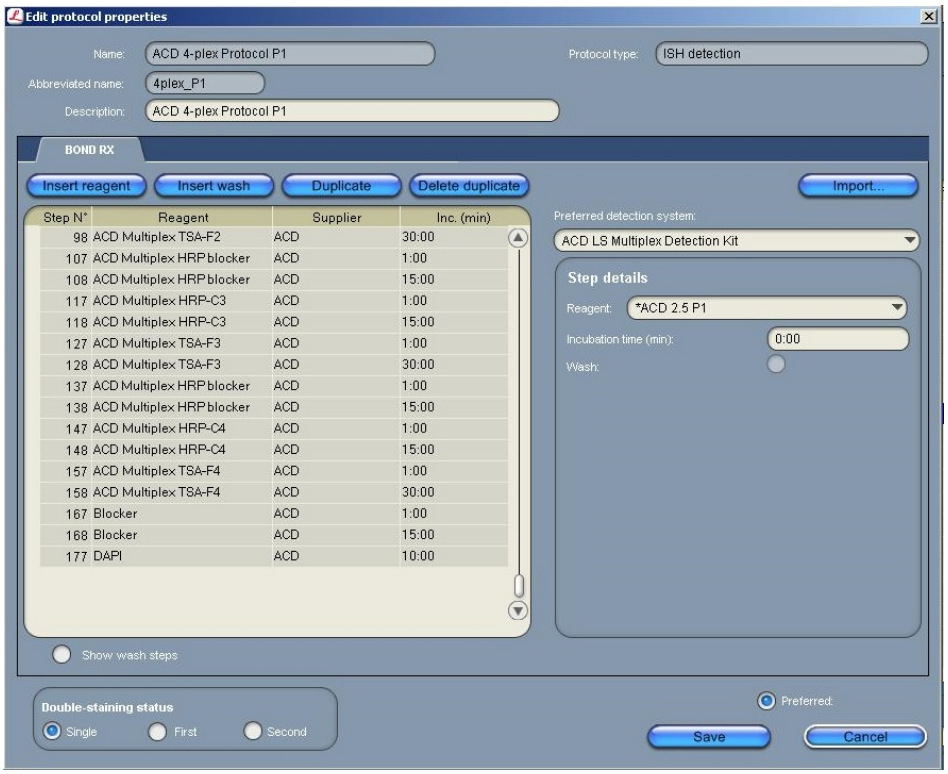


Create a 4-Plex Protocol

1. In the Protocol setup screen, select **Staining** under the Protocol group menu.
2. Highlight the protocol for the standard RNAscope® LS Multiplex Fluorescent Assay set up by your ACD FAS (for example, **ACD Multiplex Protocol P1**). Select **Copy**.
3. Change the protocol name for your first probe to **ACD 4-Plex Protocol P1** in the Name text box, **4Plex_P1** in the Abbreviated name text box, and **ACD 4-Plex Protocol P1** in the Description text box.
4. Follow Appendix A and modify the protocol to include the fourth channel for ISH.
5. Click **Show wash steps** to display the wash steps.
6. Select **Insert wash** to add BOND Washes. Match each of the protocol steps shown in Appendix A.
7. Select **Save**.
8. Click **Next** to proceed. Ignore any pop ups that may appear on the screen.
9. A new protocol needs to be created for each additional probe.







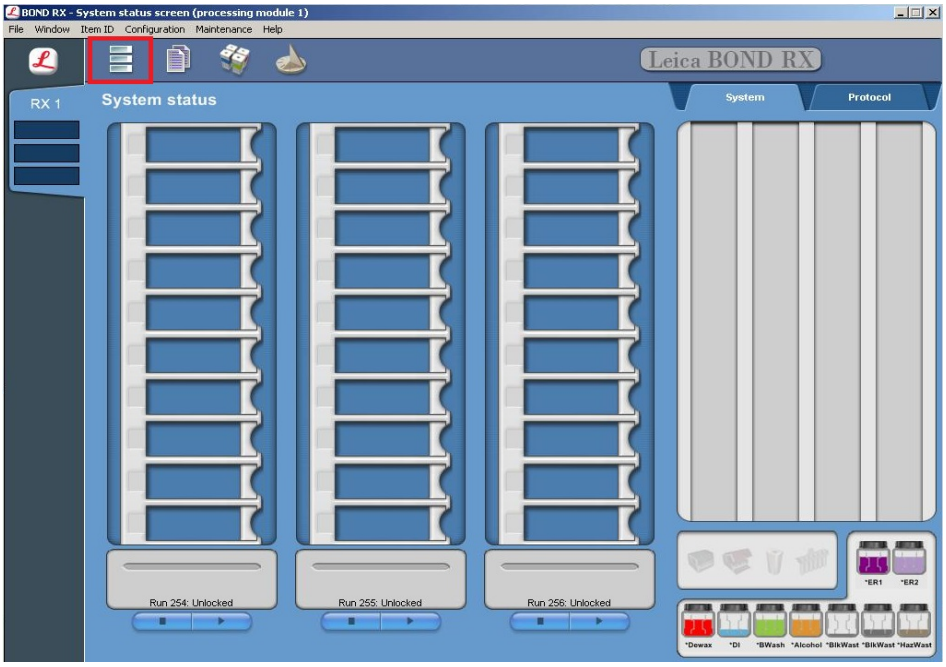
Note: The preceding three figures display all reagent steps.

Part 3: Set up a 4-Plex ISH Study

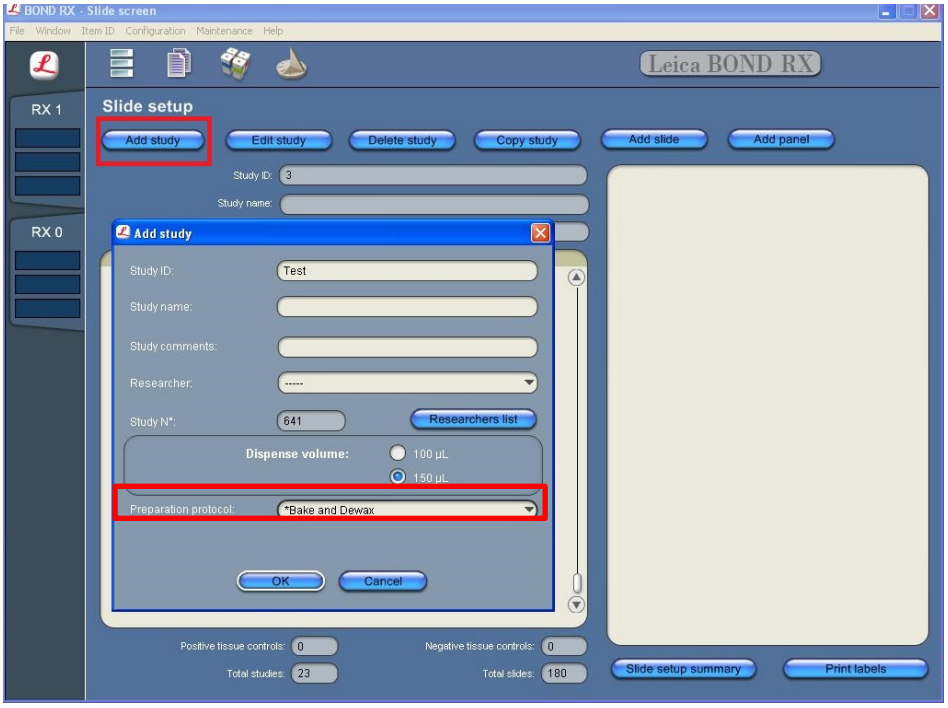
IMPORTANT! Only run a maximum of two trays. Running three trays will result in significant instrument errors including loss of dispensers.

Build a study

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



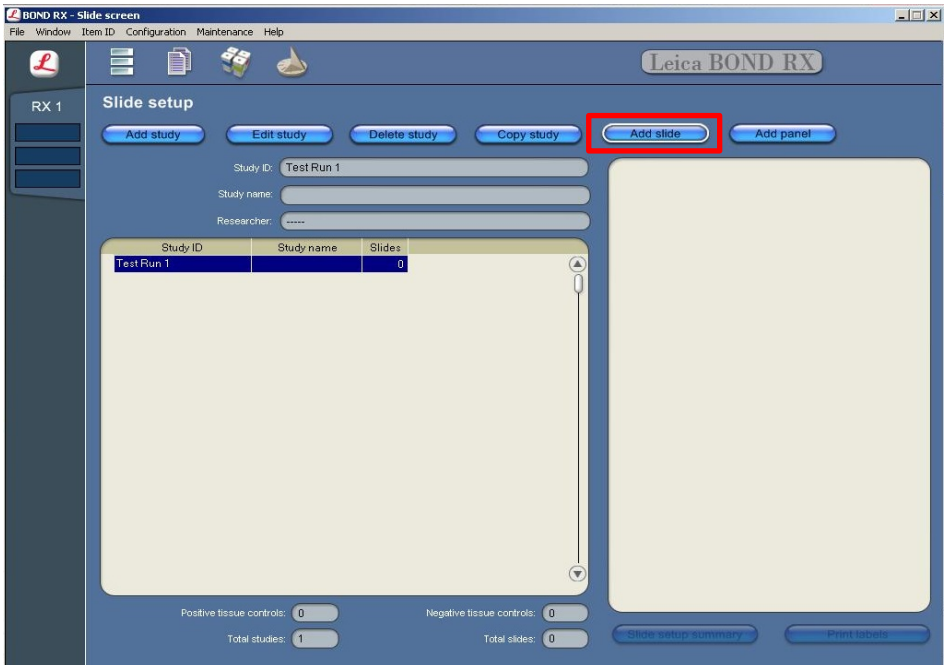
2. Select **Add study** and enter a name in the Study ID field (keep the Dispense volume at **150** µL as shown).



3. For FFPE tissues, select ***Bake and Dewax** as the Preparation protocol (otherwise, leave blank).
4. Select **OK**.

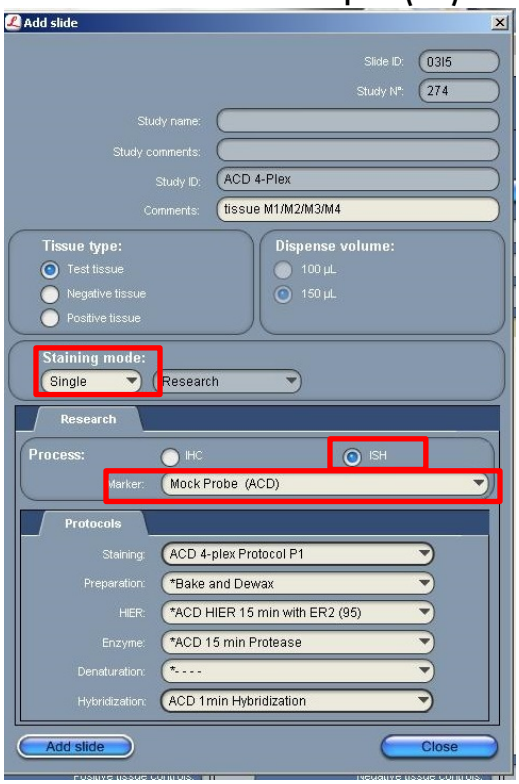
Add a 4-Plex ISH protocol to each slide

1. Select **Add slide**.



2. Enter the tissue type and probe name under the Comments field.
3. Keep **Single** as default from the Staining mode drop down menu.

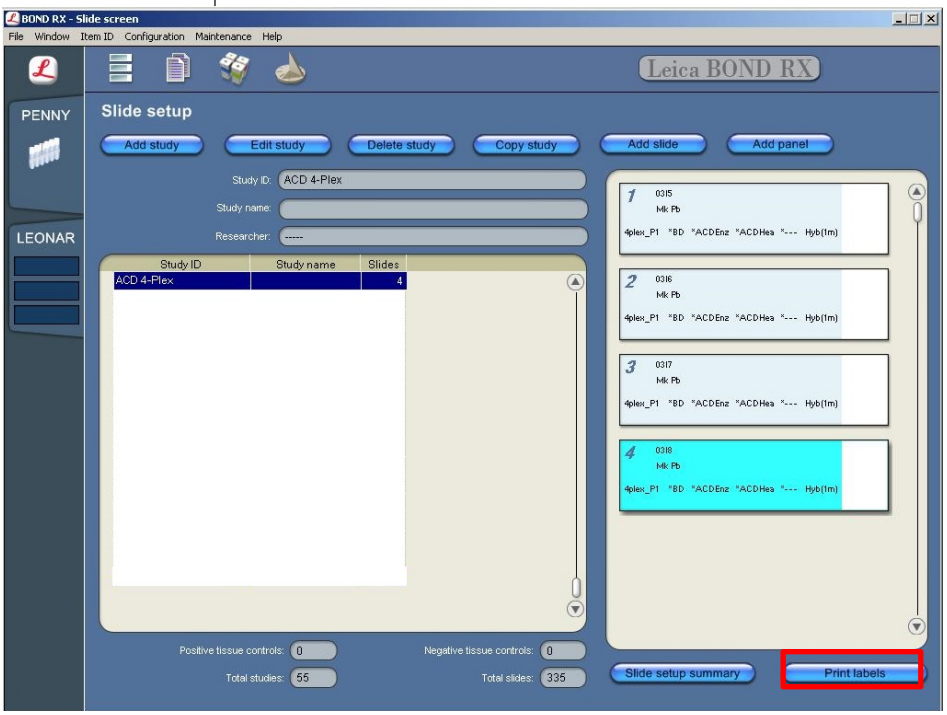
4. Select **ISH** under Process and **mock probe (ACD)** from the Marker drop down menu.



5. For the RNAscope® LS 4-Plex Fluorescent Assay, under the **Protocols** tab:
   a. For each distinct probe, select a different protocol from the Staining drop down menu (for example, ACD 4-Plex 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.
6. Select **Add slide** for each target probe, and for each of the slides used in the run.

Complete the study

1. After adding all the slides to the study, select **Close** to return to the Slide setup screen.
2. Select **Print labels** to print barcodes to attach to the slides.



Part 4: Imaging

To image fourplex fluorescent staining, use a multiplex biomarker imaging system, such as the Nuance® EX, Mantra™, or Vectra® System. Please refer to Perkin Elmer's guideline for imaging. The following table lists the corresponding filter setting for each dye:

Opal™ system	Filter setting
Opal 520	FITC
Opal 570	Cy3
Opal 620	Texas Red
Opal 690	Cy5

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.

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
26	*LS Rinse	Reagent	5 MIN	Ambient
27	*Bond Wash Solution	Wash	0 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature†
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
65	*Bond Wash Solution	Wash	1 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature†
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	*Bond Wash Solution	Wash	1 MIN	Ambient
77	ACD Multiplex HRP blocker	Reagent	1 MIN	42°C
78	ACD Multiplex HRP blocker	Reagent	15 MIN	42°C
79	*Bond Wash Solution	Wash	0 MIN	Ambient
80	*Bond Wash Solution	Wash	0 MIN	Ambient
81	*Bond Wash Solution	Wash	0 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	*Bond Wash Solution	Wash	1 MIN	Ambient
86	*Bond Wash Solution	Wash	1 MIN	Ambient
87	ACD Multiplex HRP-C2	Reagent	1 MIN	42°C
88	ACD Multiplex HRP-C2	Reagent	15 MIN	42°C
89	*Bond Wash Solution	Wash	0 MIN	Ambient
90	*Bond Wash Solution	Wash	0 MIN	Ambient
91	*Bond Wash Solution	Wash	0 MIN	Ambient
92	*Bond Wash Solution	Wash	1 MIN	Ambient
93	*Bond Wash Solution	Wash	1 MIN	Ambient
94	*Bond Wash Solution	Wash	1 MIN	Ambient
95	*Bond Wash Solution	Wash	1 MIN	Ambient
96	*Bond Wash Solution	Wash	1 MIN	Ambient
97	ACD Multiplex TSA-F2	Reagent	1 MIN	Ambient
98	ACD Multiplex TSA-F2	Reagent	30 MIN	Ambient
99	*Bond Wash Solution	Wash	0 MIN	Ambient
100	*Bond Wash Solution	Wash	0 MIN	Ambient
101	*Bond Wash Solution	Wash	0 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature†
102	*Bond Wash Solution	Wash	1 MIN	Ambient
103	*Bond Wash Solution	Wash	1 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 blocker	Reagent	1 MIN	42°C
108	ACD Multiplex HRP blocker	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	*Bond Wash Solution	Wash	1 MIN	Ambient
116	*Bond Wash Solution	Wash	1 MIN	Ambient
117	ACD Multiplex HRP-C3	Reagent	1 MIN	42°C
118	ACD Multiplex HRP-C3	Reagent	15 MIN	42°C
119	*Bond Wash Solution	Wash	0 MIN	Ambient
120	*Bond Wash Solution	Wash	0 MIN	Ambient
121	*Bond Wash Solution	Wash	0 MIN	Ambient
122	*Bond Wash Solution	Wash	1 MIN	Ambient
123	*Bond Wash Solution	Wash	1 MIN	Ambient
124	*Bond Wash Solution	Wash	1 MIN	Ambient
125	*Bond Wash Solution	Wash	1 MIN	Ambient
126	*Bond Wash Solution	Wash	1 MIN	Ambient
127	ACD Multiplex TSA-F3	Reagent	1 MIN	Ambient
128	ACD Multiplex TSA-F3	Reagent	30 MIN	Ambient
129	*Bond Wash Solution	Wash	0 MIN	Ambient
130	*Bond Wash Solution	Wash	0 MIN	Ambient
131	*Bond Wash Solution	Wash	0 MIN	Ambient
132	*Bond Wash Solution	Wash	1 MIN	Ambient
133	*Bond Wash Solution	Wash	1 MIN	Ambient
134	*Bond Wash Solution	Wash	1 MIN	Ambient
135	*Bond Wash Solution	Wash	1 MIN	Ambient
136	*Bond Wash Solution	Wash	1 MIN	Ambient
137	ACD Multiplex HRP blocker	Reagent	1 MIN	42°C
138	ACD Multiplex HRP blocker	Reagent	15 MIN	42°C
139	*Bond Wash Solution	Wash	0 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature†
140	*Bond Wash Solution	Wash	0 MIN	Ambient
141	*Bond Wash Solution	Wash	0 MIN	Ambient
142	*Bond Wash Solution	Wash	1 MIN	Ambient
143	*Bond Wash Solution	Wash	1 MIN	Ambient
144	*Bond Wash Solution	Wash	1 MIN	Ambient
145	*Bond Wash Solution	Wash	1 MIN	Ambient
146	*Bond Wash Solution	Wash	1 MIN	Ambient
147	ACD Multiplex HRP-C4	Reagent	1 MIN	42°C
148	ACD Multiplex HRP-C4	Reagent	15 MIN	42°C
149	*Bond Wash Solution	Wash	0 MIN	Ambient
150	*Bond Wash Solution	Wash	0 MIN	Ambient
151	*Bond Wash Solution	Wash	0 MIN	Ambient
152	*Bond Wash Solution	Wash	1 MIN	Ambient
153	*Bond Wash Solution	Wash	1 MIN	Ambient
154	*Bond Wash Solution	Wash	1 MIN	Ambient
155	*Bond Wash Solution	Wash	1 MIN	Ambient
156	*Bond Wash Solution	Wash	1 MIN	Ambient
157	ACD Multiplex TSA-F4	Reagent	1 MIN	Ambient
158	ACD Multiplex TSA-F4	Reagent	30 MIN	Ambient
159	*Bond Wash Solution	Wash	0 MIN	Ambient
160	*Bond Wash Solution	Wash	0 MIN	Ambient
161	*Bond Wash Solution	Wash	0 MIN	Ambient
162	*Bond Wash Solution	Wash	1 MIN	Ambient
163	*Bond Wash Solution	Wash	1 MIN	Ambient
164	*Bond Wash Solution	Wash	1 MIN	Ambient
165	*Bond Wash Solution	Wash	1 MIN	Ambient
166	*Bond Wash Solution	Wash	1 MIN	Ambient
167	ACD Multiplex HRP blocker	Reagent	1 MIN	42°C
168	ACD Multiplex HRP blocker	Reagent	15 MIN	42°C
169	*Bond Wash Solution	Wash	0 MIN	Ambient
170	*Bond Wash Solution	Wash	0 MIN	Ambient
171	*Bond Wash Solution	Wash	0 MIN	Ambient
172	*Bond Wash Solution	Wash	1 MIN	Ambient
173	*Bond Wash Solution	Wash	1 MIN	Ambient
174	*Bond Wash Solution	Wash	1 MIN	Ambient
175	*Bond Wash Solution	Wash	1 MIN	Ambient

Step No.	Reagent	Step Type	Incubation Time	Temperature†
176	*Bond Wash Solution	Wash	1 MIN	Ambient
177	DAPI/Bond Wash‡	Reagent	10 MIN	Ambient
178	*De-ionized Water	Wash	0 MIN	Ambient
179	*De-ionized Water	Wash	0 MIN	Ambient
180	*De-ionized Water	Wash	0 MIN	Ambient
181	*De-ionized Water	Wash	0 MIN	Ambient

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

† Temperatures cannot be changed.

‡ 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.

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