



RNAscope™ Plus smRNA-RNA LS Reagent Kit

For use with Leica Biosystems' BOND RX™ System

Document Number UM 322786

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

Trademarks

RNAscope, BaseScope, and miRNAscope are a registered trademark of Advanced Cell Diagnostics, Inc. Leica BOND RX is a registered trademark of Leica Biosystems. All other trademarks belong to their respective owners.

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

Advanced Cell Diagnostics, Inc. reserves the right to change its products and services at any time to incorporate technological developments. This manual is subject to change without notice.

Although this manual has been prepared with every precaution to ensure accuracy, Advanced Cell Diagnostics, Inc. assumes no liability for any errors, omissions, or for any damages resulting from the use of this information.

Copyright

© 2022. Advanced Cell Diagnostics, Inc. All rights reserved.

Contents

Chapter 1. Product Information	6
About this guide.....	6
Product description	6
Background.....	6
Overview.....	6
Kit contents and storage.....	7
RNAscope Plus smRNA-RNA LS Probes	7
RNAscope Plus smRNA-RNA LS Reagents	8
Required materials and equipment	9
Recommended Fluorophores.....	9
Required materials and equipment from Leica BOND RX.....	10
User-supplied materials.....	11
Chapter 2. Before You Begin	12
Important procedural guidelines.....	12
Chapter 3. Prepare and Pretreat Samples	13
Prepare FFPE sections	13
Materials required	13
Fix the sample	13
Dehydrate, embed, and cut the sample	13
Prepare fresh frozen sections.....	14
Materials required	14
Fix the sections	14
Dehydrate the sections.....	15
Chapter 4. Set Up and Run a Staining Protocol Using the Mock Probe (software version 5.2)	16
Workflow	16
Register the reagents.....	17
Create a one-minute probe hybridization protocol (if not created already)...	20
Create a staining protocol	22
Register the mock probe (if not already created).....	25
Prepare the instrument reagents.....	26
Scan containers into the software	27
Set up a study for the assay	28
Prepare the instrument.....	32
Start the run	32

Complete the run and mount the samples	33
Chapter 5. Set Up and Run a Staining Protocol (software versions 6.0 and 7.0).....	34
Workflow	34
Register the reagents.....	35
Create and register the BOND Research Detection System (if not registered already)	36
Create the smRNA-RNA probe hybridization protocol	37
Create a staining protocol	39
Prepare the instrument reagents.....	40
Scan containers into the software	41
Set up a study for the smRNA-RNA assay.....	41
Prepare the instrument.....	43
Start the run	44
Complete the run and mount the samples	44
Chapter 6. Evaluate the Results	45
Fluorescent Imaging Recommendations	45
Evaluate the samples	45
Scoring Guidelines	45
Control examples	48
Troubleshooting	49
Appendix A. smRNA RNA 4-plex Staining Protocol (using the Mock Probe workaround).....	50
Appendix B. smRNA RNA 4-plex Protocol with TSA-DIG and Opal 780 (using the Mock Probe workaround).....	56
Appendix C. Edit the Epitope Retrieval Protocol.....	62
Create a prestaining protocol	62
Appendix D. Edit the Protease Protocol.....	65
Appendix E. Dilute the Opal Fluorophore	67
Appendix F. Safety	69
Chemical safety	69
Biological hazard safety	69
Documentation and Support.....	71
Obtaining SDSs.....	71

Obtaining support.....	71
Contact information.....	71
Limited product warranty	71

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 Plus smRNA-RNA LS Fluorescent Reagent Kit for use with Leica Biosystems' BOND RX Research Advanced Staining System. RNAscope Plus smRNA-RNA LS Fluorescent Assays are compatible with a variety of sample types.

Product description

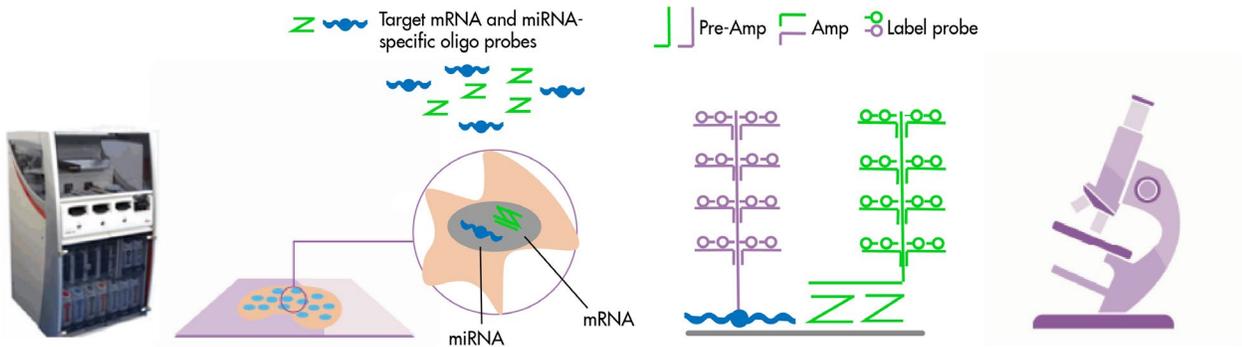
Background

The RNAscope Plus smRNA-RNA LS Assay uses a novel and proprietary method of *in situ* hybridization (ISH) to simultaneously visualize up to one smRNA (e.g. miRNA, siRNA or ASO) and up to three different RNA targets per cell in samples mounted on slides. The assay is based on ACD's patented signal amplification and background suppression technology, and incorporates signal amplification systems that enable users to investigate expression as well as positional relationship between multiple genes within a cellular context. The RNAscope Plus smRNA-RNA LS Assay allows users to automate the highly sensitive RNAscope Plus smRNA-RNA Assay using Leica Biosystems' BOND RX System.

Overview

Figure 1 on page 7 illustrates the RNAscope Plus smRNA-RNA LS Fluorescent Assay procedure, which can be completed on the instrument in ~15 hours. Starting with properly prepared samples, sections are first pretreated, and then smRNA and RNA-specific probes are hybridized to target RNA. The RNAscope Plus smRNA-RNA LS Fluorescent Assay employs four independent signal amplification systems each using a different fluorophore. The assay provides visualization of transcript expression as well as the positional relationship between four 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 tissue sections and pretreat to allow access to target RNA.	Hybridize multiple sets of gene-specific probe pairs to target RNAs.	Use up to four signal amplification systems to detect multiple target RNAs. Probes are hybridized to a cascade of signal amplification molecules, culminating in binding of dye-labeled probes visible in different fluorescent channels.	Visualize target RNA using a standard fluorescent microscope.

Kit contents and storage

The RNAscope Plus smRNA-RNA LS Assay requires the RNAscope Plus smRNA-RNA LS Probes and the RNAscope Plus smRNA-RNA LS Reagents, available from Advanced Cell Diagnostics.

RNAscope Plus smRNA-RNA LS Probes

The RNAscope Plus smRNA-RNA 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 smRNA and RNA, and detectable in one of four probe channels S1, C2, C3 or C4. Signal detection is performed using Tyramide (TSA) linked fluorophores. Different colors are assigned to the S1, C2, and C3, and C4 channel tags depending on the TSA Vivid™, Opal™ dye or TSA® plus fluorophore selected for that channel.

Channel S1 target probes are Ready-To-Use (RTU), while channels C2, C3 and C4 probes are shipped as a 50X concentrated stock. To independently detect three target RNAs, 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 to stain ~30 standard slides. The probes have a shelf life of two years from the manufacturing date when stored as indicated in the following tables:



Target Probes					
<input checked="" type="checkbox"/>	Reagent	Cat. No.	Content	Quantity	Storage
	RNAscope Plus smRNA-RNA LS Target Probe-S1	Various	Ready-To-Use (RTU) probe for channel S1	16 mL x 1 bottle	2–8°C
	RNAscope Plus smRNA-RNA LS Target Probe – [species] – [gene] – C2	Various	50X probe for channel C2	320 µL x 1 tube	2–8°C
	RNAscope Plus smRNA-RNA LS Target Probe – [species] – [gene] – C3	Various	50X probe for channel C3	320 µL x 1 tube	2–8°C
	RNAscope Plus smRNA-RNA LS Target Probe – [species] – [gene] – C4	Various	50X probe for channel C4	320 µL x 1 tube	2–8°C
Control Probes					
<input checked="" type="checkbox"/>	Reagent	Cat. No.	Content	Quantity	Storage
	RNAscope Plus smRNA-RNA LS Positive 4-plex Control Probe	Various	RNAscope Plus smRNA-RNA LS Positive Control Probe for the RNAscope Plus smRNA-RNA LS Fluorescent Assay – <i>RNU6</i> (S1 channel), <i>POLR2A</i> (C2 channel), <i>PPIB</i> (C3 channel), <i>UBC</i> (C4 channel). <i>RNU6</i> is nuclear and highly expressed. <i>UBC</i> has the highest relative expression levels in cytoplasm, followed by <i>PPIB</i> and <i>POLR2A</i> in that order.	16 mL x 1 bottle	2–8°C
	RNAscope Plus smRNA-RNA LS 4-plex Negative Control Probe	323398	RNAscope Plus smRNA-RNA LS Negative Control Probe for the RNAscope Plus smRNA-RNA LS Fluorescent Assay – Scramble (S1 channel), <i>dapB</i> (<i>Bacillus subtilis</i> strain, C2-C4 channels).	16 mL x 1 bottle	2–8°C

RNAscope Plus smRNA-RNA LS Reagents

The RNAscope Plus smRNA-RNA LS Fluorescent Reagent Kit (Cat. No. 322786) contains all the reagents needed to run the RNAscope Plus smRNA-RNA LS Fluorescent Assay on Leica Biosystems' BOND RX System, except for the RNA-specific probes, TSA linked 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 Plus smRNA-RNA LS Detection Kit (Cat. No. 322770)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
<input checked="" type="checkbox"/>	RNAscope Plus smRNA-RNA LS Hydrogen Peroxide	21 mL x 1 bottle	2–8°C
<input checked="" type="checkbox"/>	RNAscope Plus smRNA-RNA LS Protease III	21 mL x 1 bottle	2–8°C
<input checked="" type="checkbox"/>	RNAscope Plus smRNA-RNA LS Rinse	29 mL x 2 bottles	2–8°C
<input checked="" type="checkbox"/>	RNAscope Plus smRNA-RNA LS AMP 1	21 mL x 1 bottle	2–8°C
<input checked="" type="checkbox"/>	RNAscope Plus smRNA-RNA LS AMP 2	21 mL x 1 bottle	2–8°C
<input checked="" type="checkbox"/>	RNAscope Plus smRNA-RNA LS AMP 3	21 mL x 1 bottle	2–8°C
<input checked="" type="checkbox"/>	RNAscope Plus smRNA-RNA LS HRP S1	21 mL x 1 bottle	2–8°C
<input checked="" type="checkbox"/>	RNAscope Plus smRNA-RNA LS HRP C2	21 mL x 1 bottle	2–8°C
<input checked="" type="checkbox"/>	RNAscope Plus smRNA-RNA LS HRP C3	21 mL x 1 bottle	2–8°C
<input checked="" type="checkbox"/>	RNAscope Plus smRNA-RNA LS HRP C4	21 mL x 1 bottle	2–8°C
<input checked="" type="checkbox"/>	RNAscope Plus smRNA-RNA LS HRP Blocker	29 mL x 4 bottles	2–8°C
<input checked="" type="checkbox"/>	RNAscope Plus smRNA-RNA LS DAPI	21 mL x 1 bottle	2–8°C
RNAscope TSA Buffer Pack (Cat. No. 322810)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
<input checked="" type="checkbox"/>	RNAscope TSA Buffer Pack	29 mL x 3 bottles	2–8°C

Required materials and equipment

The following materials and equipment are needed to perform the RNAscope Assay.

Recommended Fluorophores

The RNAscope Plus smRNA-RNA LS Reagent Kit (Cat. No. 322786) requires purchase of TSA Vivid™ from ACD or Opal™ dye or TSA® Plus fluorophores from Akoya Biosciences.

For simplicity, TSA Vivid dyes are recommended when working with a 3-Plex experimental design and Opal dyes are recommended when using a 4-Plex design. Choose a combination of fluorophores that will work best with your imaging system while considering native autofluorescence of tissue.

Dilute the fluorophores to the desired working concentration in the TSA Buffer provided in the RNAscope Kit. 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 fluorophores. We cannot guarantee assay results if you use other fluorescent dyes.

Note: The TSA Vivid Dyes are brighter than the Opal dyes.



3-Plex assay

Fluorophores*	Cat. No.	Recommended dilution range
TSA Vivid Fluorophore 520	323271	1:750–1:3000
TSA Vivid Fluorophore 570	323272	1:750–1:3000
TSA Vivid Fluorophore 650	323273	1:750–1:3000

* To reconstitute dyes, follow the manufacturer instructions available on the tube labels. Dilute the fluorophores in TSA buffer provided in the RNAscope Plus LS Reagent Kit.

4- Plex assay

Fluorophores	Part number (Akoya Biosciences)*	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 650 Reagent Pack	FP1496001KT	1:750–1:3000
Opal 690 Reagent Pack	FP1497001KT	1:750–1:3000
Opal Polaris 780 Reagent Pack †	FP1501001KT	TSA-DIG: 1:750–1:3000
		Polaris 780: 1:187.5–1:750

* To reconstitute dyes, follow the manufacturer instructions available on the tube labels. Dilute the fluorophores in TSA buffer provided in the RNAscope Plus LS 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 Antibody Diluent/Block from Akoya Biosciences (PN: ARD1001EA)

† We recommend keeping the dilution factors of Opal TSA-DIG and Opal Polaris 780 at a constant ratio. For example, when using 1:1500 dilution for Opal TSA-DIG, use 1:375 dilution for Opal Polaris 780. When using 1:750 dilution for Opal TSA-DIG, use 1:187.5 dilution for Opal Polaris 780.

Required materials and equipment from Leica BOND RX

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

<input checked="" type="checkbox"/>	Component	Cat. No.	Storage
	Leica Biosystems' BOND RX System – automated slide stainer	–	–
	BOND 30 mL Open containers	OP309700	Room temp (20–25°C)
	BOND 6 mL Titration containers*	OPT9049	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



<input checked="" type="checkbox"/>	Component	Cat. No.	Storage
	BOND Mixing Stations	S21.1971	Room temp (20–25°C)

* BOND 7 mL Containers may be used instead but offer less flexibility.

User-supplied materials

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

<input checked="" type="checkbox"/>	Description	Supplier	Cat. No.
	SuperFrost Plus Slides (required)	Fisher Scientific	12-550-15
	Prolong Gold Antifade	Thermo Fisher	P36930; P10144; P36934
	Opal dyes or TSA Plus fluorophores (if not using TSA Vivid Dyes from Bio-Techne)	Akoya Biosciences	—
	Either Primary Antibody Diluent BOND or Antibody Diluent/Block (if Opal Polaris 780 is used)	Leica Akoya Biosciences	AR9352 ARD1001EA
	Xylene	Fisher Scientific/MLS	X3P-1GAL
	100% alcohol (EtOH)	American Master Tech Scientific/MLS*	ALREACS
	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	—
	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.

Chapter 2. Before You Begin

Prior to running the RNAscope Plus smRNA-RNA LS 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 Control Slides (Cat. No. 310045 for Human HeLa Cell Pellet, and Cat. No. 310023 for Mouse 3T3 Cell Pellet) using the RNAscope Plus smRNA-RNA LS Positive and Negative smRNA-RNA Control Probes.

Important procedural guidelines

- Start with properly fixed and prepared sections. Refer to **Chapter 3. Prepare and Pretreat Samples** for preparation of FFPE and fresh frozen 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 E. Safety** for more information.

Chapter 3. Prepare and Pretreat Samples

This chapter describes two tissue sample preparation methods: formalin-fixed, paraffin-embedded (FFPE) sample preparation and pretreatment and fresh-frozen sample preparation and pretreatment. For other sample types and preparation methods, contact support.acd@bio-techne.com for the latest protocols and guidelines.

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
 - 100% alcohol (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 Plus smRNA-RNA LS Fluorescent Assay.

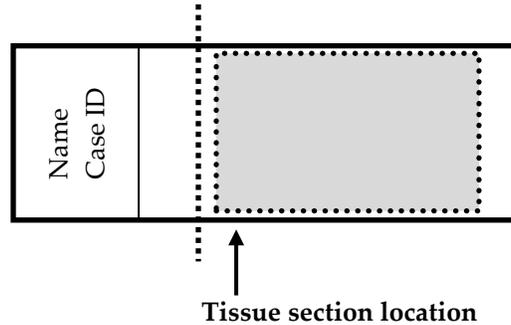
Dehydrate, embed, and cut the sample

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:



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.

10. To prevent any signal issues, proceed *immediately* to **Chapter 4 or Chapter 5** and begin your run.

Prepare fresh frozen sections

Materials required

-
- 1X PBS
 - 10% Neutral Buffered Formalin (NBF) or 4% Paraformaldehyde (PFA)
 - 100% alcohol (EtOH)
 - Tissue-Tek Vertical 24 Slide Rack
 - Tissue-Tek Staining Dishes
-

Fix the sections

1. Remove fresh-frozen tissue slides from 80°C and place in a Tissue Tek Slide Rack.
2. *Immediately* immerse the slides in 200 mL of 10% NBF or freshly prepared 4% PFA.
3. Incubate the slides for at least **90 MIN** at **ROOM TEMPERATURE (RT)**.

Note: Formalin that has been stored for more than six months, exposed to air for more than a week, or used repeatedly may result in suboptimal tissue fixation. 4% PFA must be freshly prepared for each experiment.



Dehydrate the sections

Reagents may be prepared ahead of time. Ensure all containers remain covered.

1. Prepare 200 mL 50% ethanol, 200 mL 70% ethanol, and 2X 200 mL 100% ethanol in Tissue Tek Staining Dishes.
2. Place the slides in 50% ethanol for **5 MIN** at **RT**.
3. Place the slides in 70% ethanol for **5 MIN** at **RT**.
4. Place the slides in 100% ethanol for **5 MIN** at **RT**.
5. Place slides in fresh 100% ethanol for **5 MIN** at **RT**.
6. Remove slides from ethanol, and let them dry for **5 MIN** at **RT**.
7. To prevent any signal issues, proceed *immediately* to **Chapter 4** or **Chapter 5** and begin your run.

4

Chapter 4. Set Up and Run a Staining Protocol Using the Mock Probe (software version 5.2)

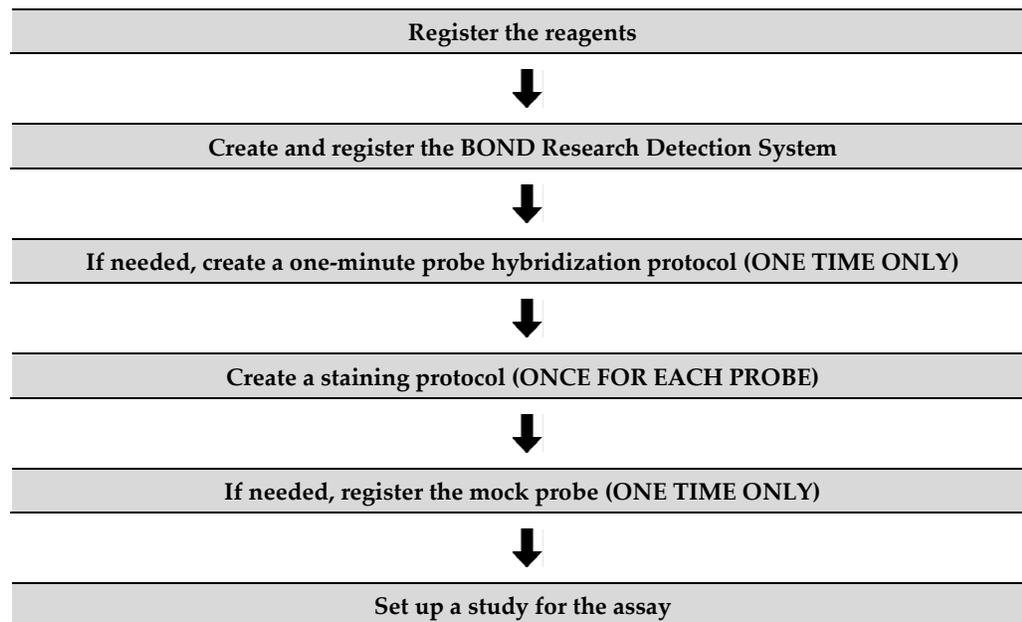
Use the instructions in this chapter to set up the RNAscope Plus smRNA-RNA LS Assay using the mock probe workaround, recommended for LS software version 5.2. To set up the assay without the mock probe, proceed to **Chapter 5**.

IMPORTANT! BXD11 or higher is required to run the following setup on software version 5.2. Please contact your Leica FAS to upgrade to BXD11 before starting the assay.

IMPORTANT! We strongly recommend you run the Control Slides (Cat. No. 310045 for human or Cat. No. 310023 for mouse) using the RNAscope Plus smRNA-RNA LS positive and negative control probes along with your samples in every run.

IMPORTANT! Make sure you have software version 5.2 or higher before setting up the assay. The RNAscope Plus smRNA-RNA LS Assay is not supported by software version 4.0.

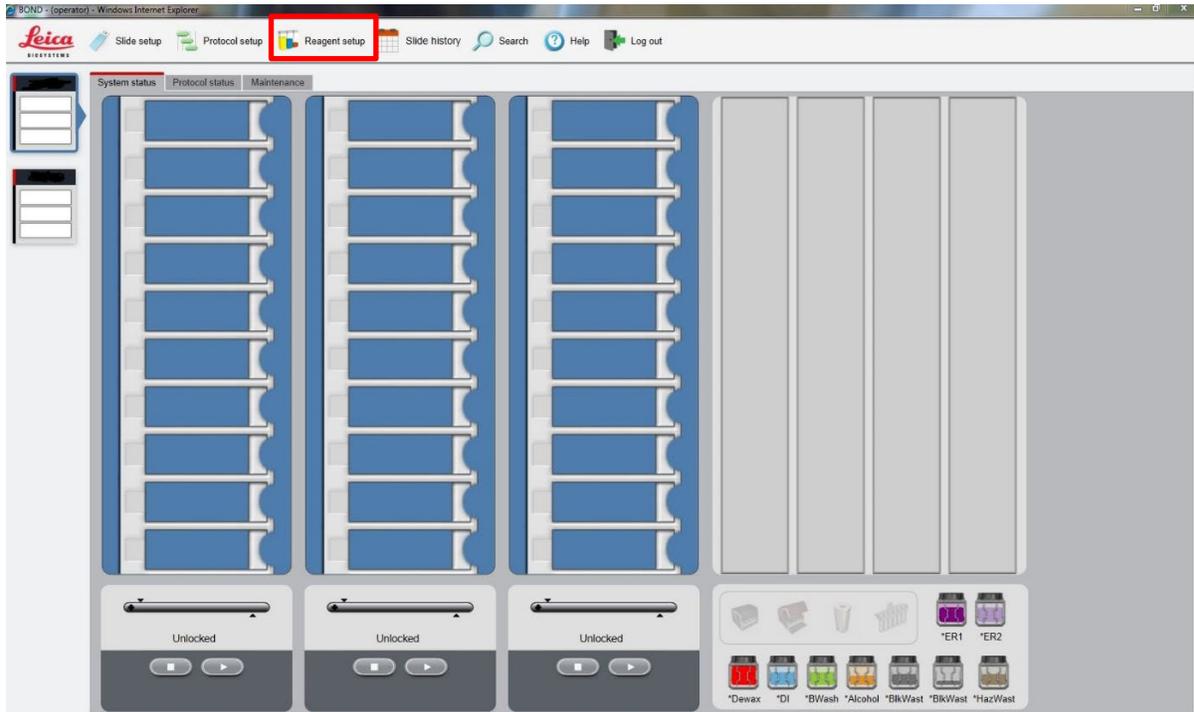
Workflow



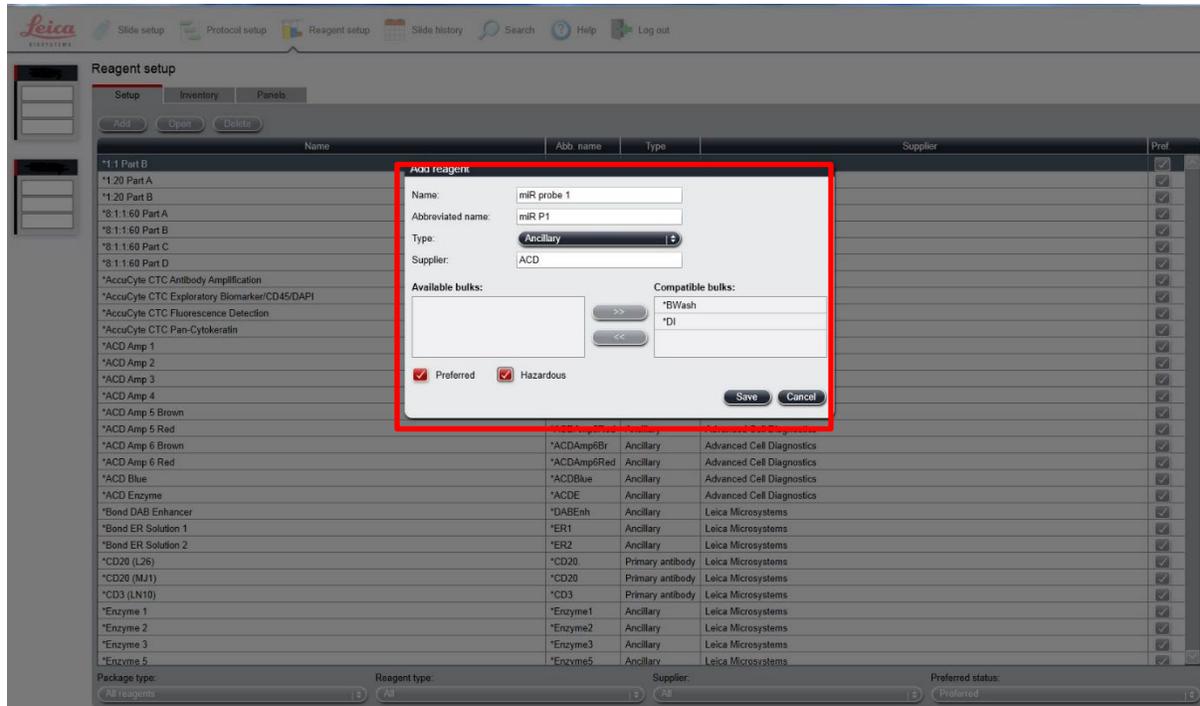
Register the reagents

This step is a “workaround” to the existing 5.2 software to accommodate the RNAscope Plus smRNA-RNA LS 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. Enter a reagent name (for example, smRNA-RNA probe 1) in the Name text box.
4. Enter **smRNA-RNA P1** (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 the probes, RNAscope Plus smRNA-RNA Amp 1, and RNAscope Plus smRNA-RNA Amp 3 only).
8. Select **Save**.
9. Repeat Steps 2–7 for each probe and for the rest of the reagents using the container names in the following table. Reagents with a “*” are pre-filled in software and do not need to be added:

Reagents	Container Name	Abbreviated Name	Volume required per slide (not accounting for dead volume)
RNAscope Plus smRNA-RNA LS AMP 1	ACD smRNA-RNA Amp 1	smAmp1	300 µL
RNAscope Plus smRNA-RNA LS AMP 2	ACD smRNA-RNA Amp 2	smAmp2	300 µL
RNAscope Plus smRNA-RNA LS AMP 3	ACD smRNA-RNA Amp 3	smAmp3	300 µL
RNAscope Plus smRNA-RNA LS HRP S1	ACD smRNA-RNA HRP-S1	smHRPs1	300 µL
RNAscope Plus smRNA-RNA LS HRP C2	ACD smRNA-RNA HRP-C2	smHRPc2	300 µL
RNAscope Plus smRNA-RNA LS HRP C3	ACD smRNA-RNA HRP-C3	smHRPc3	300 µL
RNAscope Plus smRNA-RNA LS HRP C4	ACD smRNA-RNA HRP-C4	smHRPc4	300 µL



Reagents	Container Name	Abbreviated Name	Volume required per slide (not accounting for dead volume)
RNAscope Plus smRNA-RNA LS HRP Blocker	ACD smRNA-RNA HRP blocker	smHRPbk	900 µL
Opal-fluorophore 1 (user to dilute in TSA buffer)	ACD Multiplex TSA-F1	MuxTSAf1	300 µL
Opal-fluorophore 2 (user to dilute in TSA buffer)	ACD Multiplex TSA-F2	MuxTSAf2	300 µL
Opal-fluorophore 3 (user to dilute in TSA buffer)	ACD Multiplex TSA-F3	MuxTSAf3	300 µL
Opal-fluorophore 4 (user to dilute in TSA buffer)	ACD Multiplex TSA-F4	MuxTSAf4	300 µL
Opal TSA-DIG † (if Opal 780 is used)	TSA-DIG	TSA-DIG	300 µL
Opal 780† (if Opal 780 is used)	Opal 780	Opal780	300 µL
LS smRNA-RNA probe mix	(user's choice but must be in container registered as "Ancillary" in software)		450 µL for 5.2 software
RNAscope Plus smRNA-RNA LS DAPI	DAPI	DAPI	150 µL
1X BOND Wash	Bond Wash	BondWash	150 µL
1X BOND Wash	Mock Probe (registered as "Probe RNA" in software)	ACDMock	270 µL
RNAscope Plus smRNA-RNA LS Hydrogen Peroxide	*Open 0 Haz		150 µL
RNAscope Plus smRNA-RNA LS Protease III	*ACD Enzyme		200 µL
RNAscope Plus smRNA-RNA LS Rinse	*LS Rinse		600 µL

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

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

Create and register the BOND Research Detection System (if not registered already)

A BOND Research Detection System from Leica is required to setup the RNAscope smRNA-RNA LS 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 Plus smRNA-RNA LS 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.

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

Reagent system inventory details

Name:

Show Available Empty Expired

UPI	Lot N°	Expiration date	Registered	First used	Marked empty	Tests remaining
20029225		12/31/2026	8/15/2022			200

Pstrn.	UPI	Reagent	Vol. (mL)
1	20028937	Bond Wash	30.00
2	20028965	DAPI	30.00

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

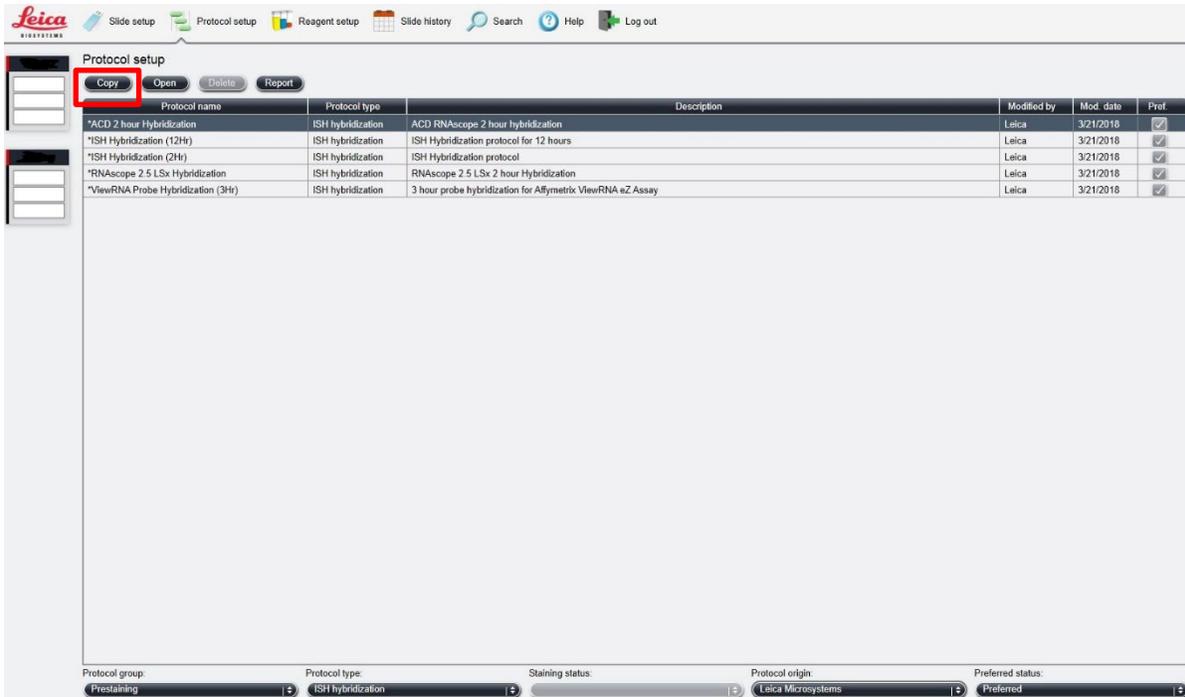
Note: If you prefer not to use DAPI on the instrument or want to perform immunohistochemistry (IHC) steps after the assay, you may use BOND Wash in place of DAPI in the staining protocol.

- Scan the second container and select the registration name **Bond Wash**.
- 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.
- 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.
- Select **OK**.

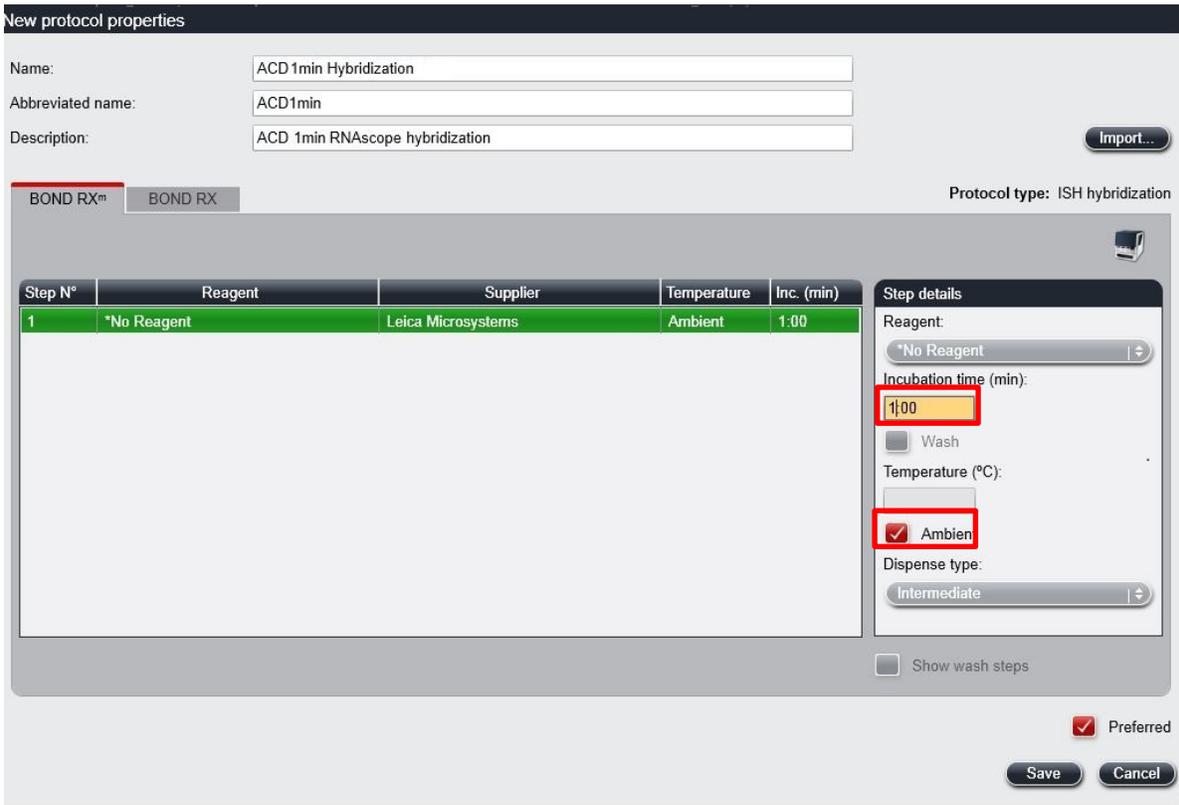
Create a one-minute probe hybridization protocol (if not created already)

A mock probe hybridization step must be created as a part of the software workaround, if using software version below 6.0 for the RNAscope Plus smRNA-RNA LS Assay. The following example copies the existing two hour hybridization protocol and changes the incubation time to one minute.

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



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

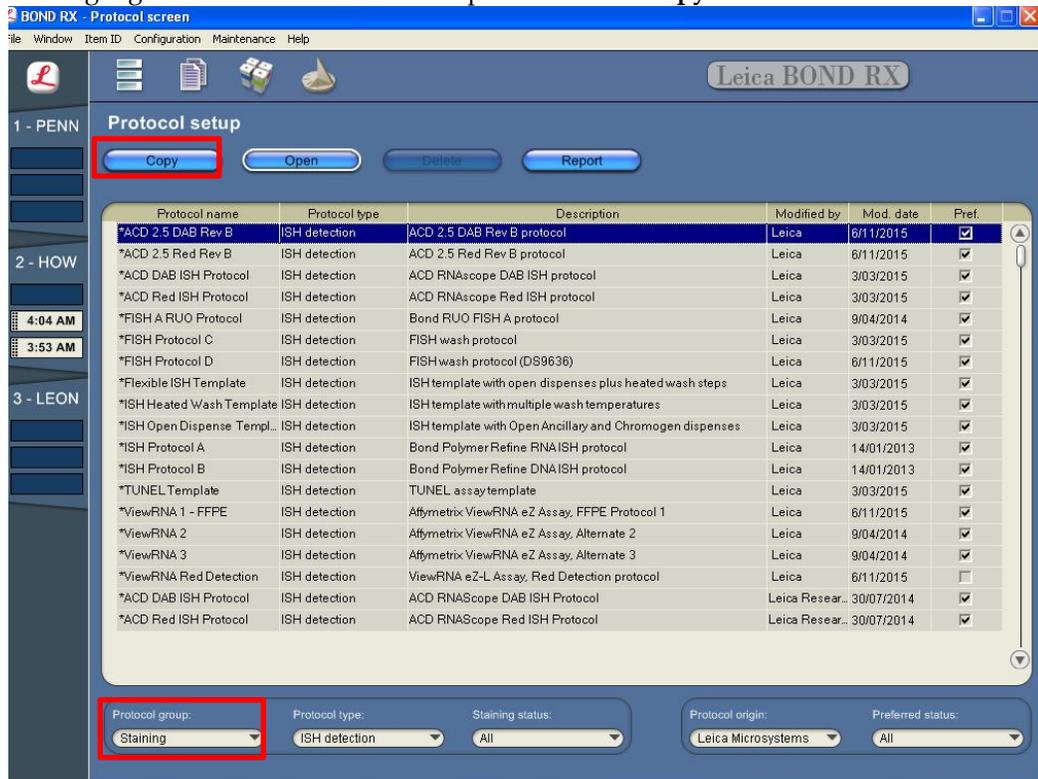


4. Highlight the ***No Reagent** step.
5. Change the incubation time to **1 MIN** and select **Ambient** as Temperature (°C).
6. Select **Save**.

Create a staining protocol

Due to the software workaround for the RNAscope Plus smRNA-RNA LS 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 smRNA-RNA Protocol P1** in the Name text box, **Multi_P1** in the Abbreviated name text box, and **ACD smRNA-RNA Protocol P1** in the Description text box.
 - b. Select **ACD LS Multiplex Detection Kit** from the Preferred detection system menu.
 - c. For Steps 1 –3, change to the desired probe container. In the example, the probe mix is in the **Open 1** container.

IMPORTANT! To assure the success of the assay, the temperature of Step 3 must be 37°C.

Note: The next four figures display all reagent steps using TSA-DIG and Polaris 780 to label –C4.

Edit protocol properties

Name: ACD smRNA-RNA Protocol P1
 Abbreviated name: Multi_P1
 Description: ACD smRNA-RNA Protocol P1

Protocol type: ISH detection

BOND RX™ BOND RX

Preferred detection system: Bond Research Detection Kit

Step N°	Reagent	Supplier	Temperature	Inc. (min)
1	*Open 1	User	Ambient	0:00
2	*Open 1	User	Ambient	0:00
3	*Open 1	User	37	120:00
15	smRNA-RNA Amp1		42	1:00
16	smRNA-RNA Amp1		42	30:00
25	*LS Rinse	Advanced Cell Diagnostics	Ambient	5:00
26	*LS Rinse	Advanced Cell Diagnostics	Ambient	5:00
31	smRNA-RNA Amp2		42	1:00
32	smRNA-RNA Amp2		42	30:00
41	*LS Rinse	Advanced Cell Diagnostics	Ambient	5:00
42	*LS Rinse	Advanced Cell Diagnostics	Ambient	5:00
47	smRNA-RNA Amp3		42	1:00

Step details: Reagent: *Open 1, Incubation time (min): 0:00, Wash: , Temperature (°C): , Ambient, Dispense type: 150 µL

Staining method: Single First Second Preferred

Buttons: Save, Cancel, Import...

Edit protocol properties

Name: ACD smRNA-RNA Protocol P1
 Abbreviated name: Multi_P1
 Description: ACD smRNA-RNA Protocol P1

Protocol type: ISH detection

BOND RX™ BOND RX

Preferred detection system: Bond Research Detection Kit

Step N°	Reagent	Supplier	Temperature	Inc. (min)
47	smRNA-RNA Amp3		42	1:00
48	smRNA-RNA Amp3		42	15:00
57	smRNA-RNA HRP-S1		42	1:00
58	smRNA-RNA HRP-S1		42	15:00
67	ACD Multiplex TSA-F1	ACD	Ambient	1:00
68	ACD Multiplex TSA-F1	ACD	Ambient	30:00
76	smRNA-RNA HRPblocker		42	1:00
77	smRNA-RNA HRPblocker		42	15:00
85	smRNA-RNA HRP C2		42	1:00
86	smRNA-RNA HRP C2		42	15:00
94	ACD Multiplex TSA-F2	ACD	Ambient	1:00
95	ACD Multiplex TSA-F2	ACD	Ambient	30:00

Step details: Reagent: *Open 1, Incubation time (min): 0:00, Wash: , Temperature (°C): , Ambient, Dispense type: 150 µL

Staining method: Single First Second Preferred

Buttons: Save, Cancel, Import...

Edit protocol properties

Name: ACD smRNA-RNA Protocol P1
 Abbreviated name: Multi_P1
 Description: ACD smRNA-RNA Protocol P1 Import...

BOND RX™ **BOND RX** Protocol type: ISH detection

Preferred detection system: Bond Research Detection Kit

Step N°	Reagent	Supplier	Temperature	Inc. (min)
103	smRNA-RNA HRPblocker		42	1:00
104	smRNA-RNA HRPblocker		42	15:00
112	smRNA-RNA HRPC3		42	1:00
113	smRNA-RNA HRPC3		42	15:00
121	ACD Multiplex TSA-F3	ACD	Ambient	1:00
122	ACD Multiplex TSA-F3	ACD	Ambient	30:00
130	smRNA-RNA HRPblocker		42	1:00
131	smRNA-RNA HRPblocker		42	15:00
139	smRNA-RNA HRPC4		42	1:00
140	smRNA-RNA HRPC4		42	15:00
148	TSA Dig		Ambient	1:00
149	TSA Dig		Ambient	30:00
157	smRNA-RNA HRPblocker		Ambient	10:00

Insert wash Insert reagent Delete step Show wash steps

Staining method: Single First Second Preferred

Save Cancel

Edit protocol properties

Name: ACD smRNA-RNA Protocol P1
 Abbreviated name: Multi_P1
 Description: ACD smRNA-RNA Protocol P1 Import...

BOND RX™ **BOND RX** Protocol type: ISH detection

Preferred detection system: Bond Research Detection Kit

Step N°	Reagent	Supplier	Temperature	Inc. (min)
122	ACD Multiplex TSA-F3	ACD	Ambient	30:00
130	smRNA-RNA HRPblocker		42	1:00
131	smRNA-RNA HRPblocker		42	15:00
139	smRNA-RNA HRPC4		42	1:00
140	smRNA-RNA HRPC4		42	15:00
148	TSA Dig		Ambient	1:00
149	TSA Dig		Ambient	30:00
157	smRNA-RNA HRPblocker		Ambient	10:00
158	smRNA-RNA HRPblocker		Ambient	10:00
166	Polaris 780		Ambient	1:00
167	Polaris 780		Ambient	30:00
175	DAPI	ACD	Ambient	10:00

Insert wash Insert reagent Delete step Show wash steps

Staining method: Single First Second Preferred

Save Cancel

4. Click **Show wash** steps to also view the wash steps. Insert BOND Washes to match each of the protocol steps shown.
5. Compare and confirm the on-screen protocol with the protocol listed in **Appendix A**.
 - a. The Appendices show steps for 4-plex. If 3-plex is desired, delete the HRP-C4/TSA-F4/HRP Blocking, steps 137 (Bond Wash) through 159 (Bond Wash)
 - b. If you use Opal Polaris 780, refer to **Appendix B** instead.
6. Select **Preferred** in the bottom right corner of the window.

Edit protocol properties

Name: ACD smRNA-RNA Protocol P1
 Abbreviated name: Multi_P1
 Description: ACD smRNA-RNA Protocol P1 Import...

BOND RX™ **BOND RX** Protocol type: ISH detection

Preferred detection system: ACD LS smRNA-RNA Detection kit

Step N°	Reagent	Supplier	Temperature	Inc. (min)
1	*ACD 2.5 P1	Advanced Cell Diagnostics	Ambient	0:00
2	*ACD 2.5 P1	Advanced Cell Diagnostics	Ambient	0:00
3	*ACD 2.5 P1	Advanced Cell Diagnostics	37	120:00
15	smRNA-RNA Amp1		42	1:00
16	smRNA-RNA Amp1		42	30:00
25	*LS Rinse	Advanced Cell Diagnostics	Ambient	5:00
26	*LS Rinse	Advanced Cell Diagnostics	Ambient	5:00
31	smRNA-RNA Amp2		42	1:00
32	smRNA-RNA Amp2		42	30:00
41	*LS Rinse	Advanced Cell Diagnostics	Ambient	5:00
42	*LS Rinse	Advanced Cell Diagnostics	Ambient	5:00
47	smRNA-RNA Amp3		42	1:00

Step details

Reagent: *ACD 2.5 P1
 Incubation time (min): 0:00
 Wash
 Temperature (°C):
 Ambient
 Dispense type: 150 µL

Insert wash Insert reagent Delete step Show wash steps

Staining method: Single First Second Preferred

Save Cancel

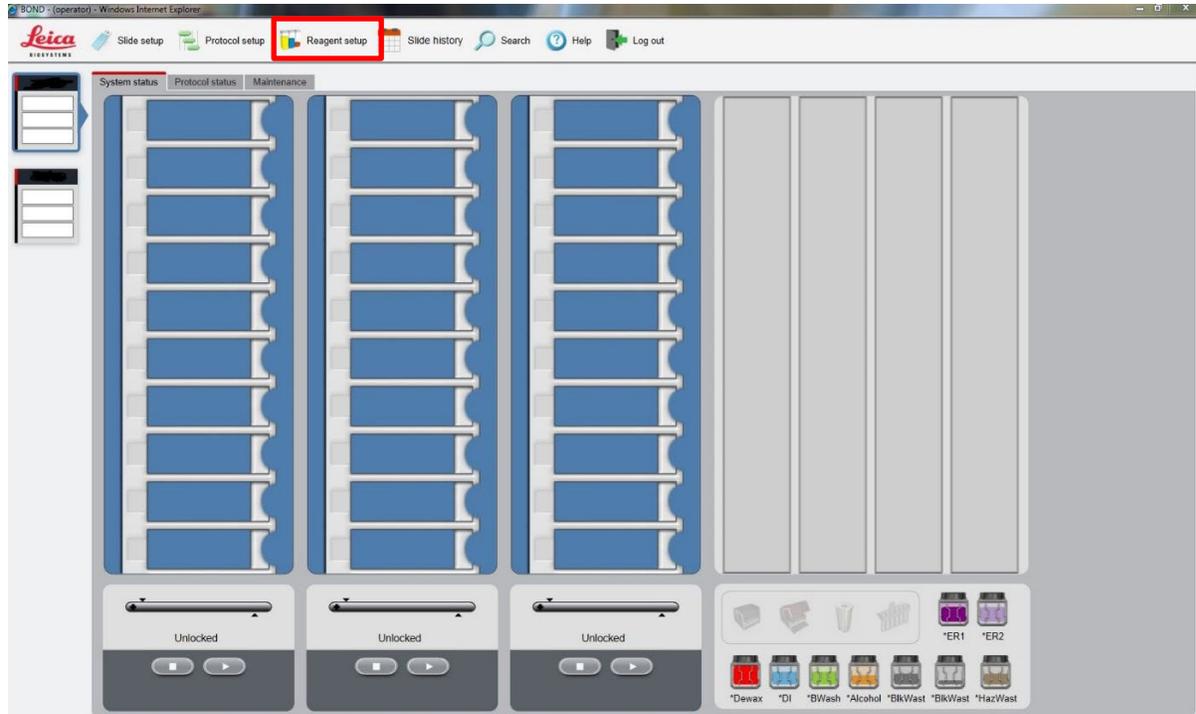
7. Select **Save**.
8. Click **Next** to proceed. Ignore any pop-ups that may appear on the screen.
9. Create a new probe protocol.

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

Register the mock probe (if not already created)

Create a mock probe in the reagent set up.

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



2. Select **Add**.
3. Enter **Mock Probe** in the Name and **ACD Mock** in Abbreviated name text boxes.
4. Select **Probe RNA** in the Type drop-down menu. Enter **ACD** in the Supplier text box.
5. Check RNA for Probe Type.
6. Select **ACD smRNA-RNA Protocol P1** (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**.
12. Fill a 30 mL Open container with 1X Bond Wash from the instrument's bulk container and label it **Mock Probe**. This container will be registered as a "Probe RNA" in the software.

Prepare the instrument reagents

1. Fill the DAPI container with DAPI and the Bond Wash container with Leica Biosystems' 1X Bond Wash. The kit requires 150 μ L DAPI or Bond Wash per slide.
2. Carefully transfer all other RNAscope LS kit reagents *except for the TSA buffer* into empty 30 mL BOND Open containers.
3. If you haven't already done so, fill a 30 mL Open container with 1X Bond Wash and register this container as **Mock Probe** in the software.

Note: Before each run, make sure you have enough of each reagent. See the table on page 18 for the reagent volume required per slide.



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.

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

4. Prepare the LS smRNA-RNA target probe mix:
 - a. Determine the volume of probe needed (volume needed for the total number of slides plus container dead-volume). If using Mock Probe workaround, you will require 450 μ L of RNAscope probe mix per slide plus dead volume for the container. 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 μ L dead-volume when using a BOND Titration container (6 mL).
 - b. Dilute the 50X C2 and C3 and C4 probe stocks 1:50 into the Ready-To-Use S1 probe. For example, add 320 μ L 50X C2 probe and 320 μ L 50X C3 probe to a tube, then add enough S1 probe to bring the final volume to 16 mL.
 - c. Transfer the LS smRNA-RNA probe mix into the appropriate Bond container, probe containers must be registered as “Ancillary” in the software.

Note: The LS smRNA-RNA probe mix is stable for one year at 2–8°C.

5. Prepare the Opal fluorophore dilutions:
 - a. Determine the volume of Opal fluorophore needed (see Appendix E. Dilute the Opal Fluorophore), and make sure to add dead volume per container (0.6–2.5 mL for 6 mL Titration container or 30 mL Open container, respectively).
 - b. Dilute the 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
Opal 690*	1:750–1:3000 (in TSA buffer)
Opal 520*	1:750–1:3000 (in TSA buffer)
Opal 570*	1:750–1:3000 (in TSA buffer)
Opal 620*	1:750 -1:3000 (in TSA buffer)
Opal TSA-DIG* (if using Opal 780)	1:750–1:3000 (in TSA buffer)
Opal Polaris 780* (if using Opal 780)	1:187.5–1:750+ (in Akoya or Bond diluent)

* Reconstitute all Opals (except Opal Polaris 780) with 75 μ L Dimethylsulfoxide (DMSO).

Reconstitute Opal Polaris 780 with 300 μ L double distilled water (ddH₂O).

† We recommend keeping the dilution factors of Opal TSA-DIG and Opal Polaris 780 at a constant ratio. For example, when using 1:1500 dilution for Opal TSA-DIG, use 1:375 dilution for Opal Polaris 780. When using 1:750 dilution for Opal TSA-DIG, use 1:187.5 dilution for Opal Polaris 780.

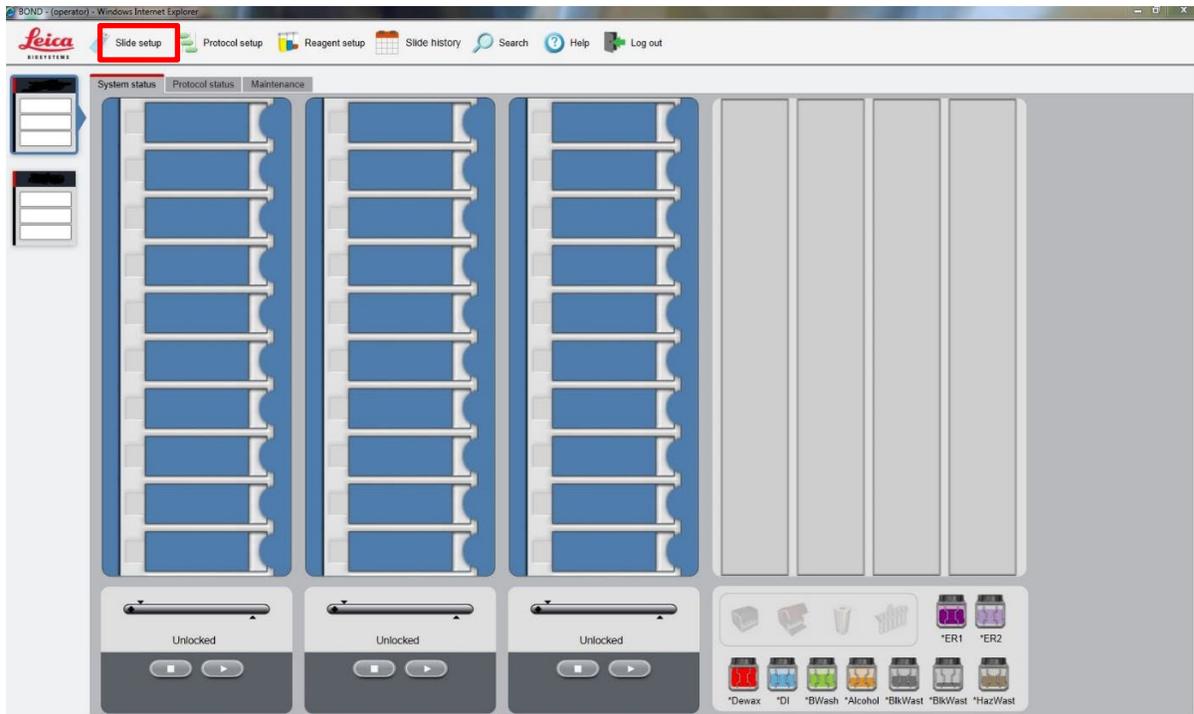
Scan containers into the software

1. Using the Barcode Scanner, scan the barcode located on the front of the BOND Open container. A window appears.
2. From the drop down menu, select the corresponding name of the reagent as shown in the table on page 17 under **Container name**.

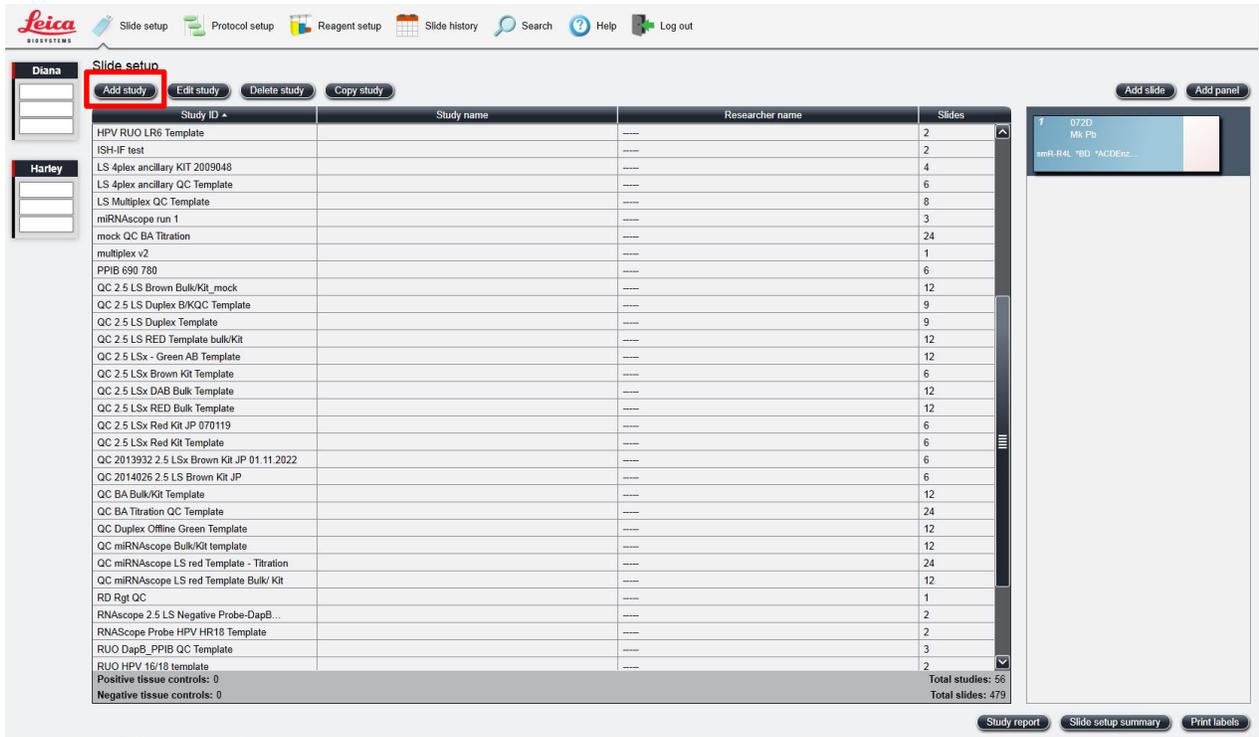
Set up a study for the assay

IMPORTANT! To be able to use a maximum of two trays for the assay (four channels), strictly follow the protocol steps listed in **Appendix A. and Appendix B. smRNA-RNA Protocol**. Adding any additional steps will prevent the system from running all three trays.

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



2. Select **Add study**.



3. Enter a name in the Study ID field (keep the Dispense volume at 150 μ L as shown).

Add study

Study ID:

Study name:

Study comments:

Researcher:

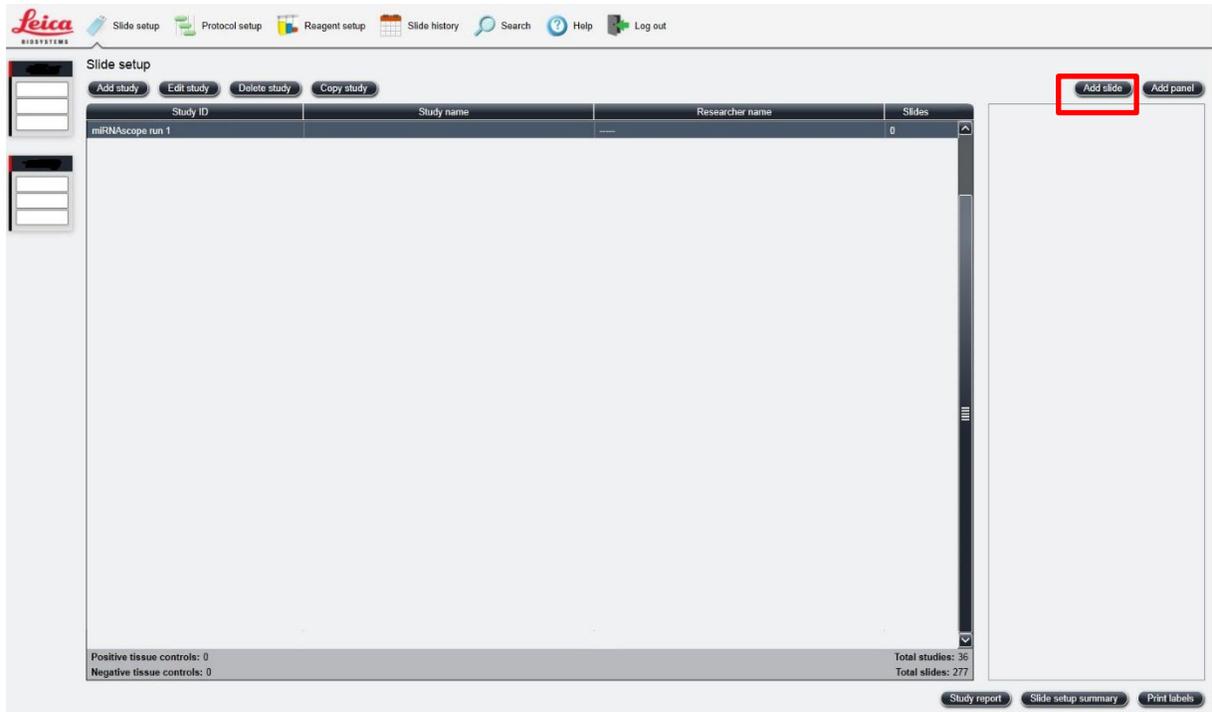
Study N°:

Dispense volume:
 100 μ L
 150 μ L

Preparation protocol:

- a. For FFPE tissues, select ***Bake and Dewax** as the Preparation protocol.
- b. For fresh frozen tissues, select ***----** instead.

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.

Add slide

Slide ID:

Study N°:

Study name:

Study comments:

Study ID:

Comments:

Tissue type:

Test tissue

Negative tissue

Positive tissue

Dispense volume:

100 µL

150 µL

Staining mode:

Single

Process: IHC ISH

Marker:

Protocols

Staining:

Preparation:

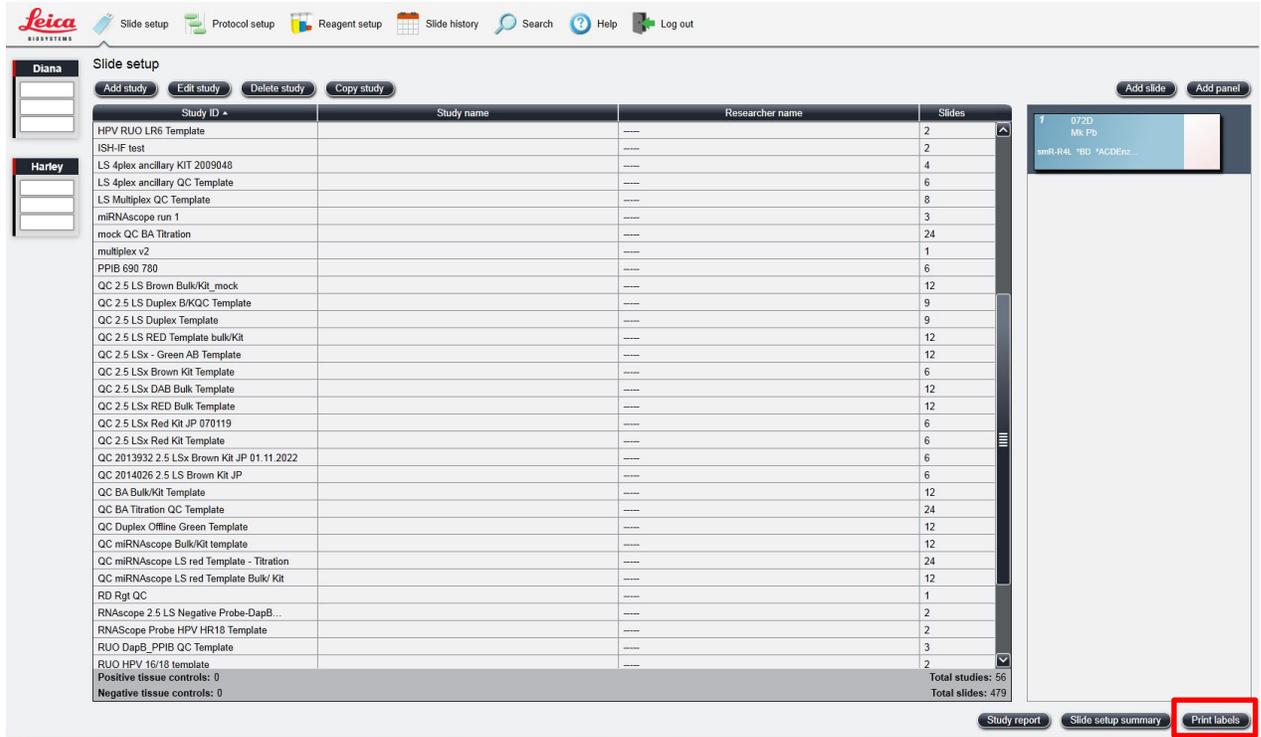
HIER:

Enzyme:

Denaturation:

Hybridization:

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 smRNA-RNA 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.
 - 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.



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.

Start the run

1. Attach the barcodes to the slides, and add the slides to the slide tray with the label sides facing up.
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.

2. Place the tray in the Leica BOND RX, and press the button to load the tray onto the machine.



3. 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. Do not use Delayed Start with fresh-frozen tissue.

Note: The total run time for LS smRNA-RNA Fluorescent Assay is 14–15 hrs depending on the number of slides.

IMPORTANT! Before leaving the instrument unattended, ensure that the instrument is running successfully.

Complete the run and mount the samples

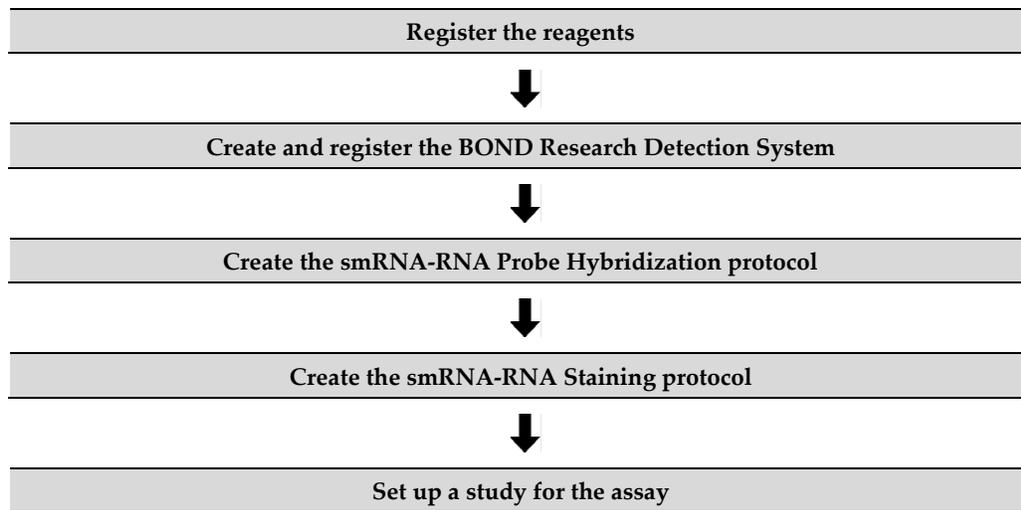
1. After the run is complete, press the button on the front of the instrument to unload the slides.
2. Remove the covertiles.
3. Add a drop of Prolong Gold Antifade mounting media to each slide. Avoid introducing bubbles.
4. Carefully place a glass coverslip on the slides, and dry overnight in the dark.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

5

Chapter 5. Set Up and Run a Staining Protocol (software versions 6.0 and 7.0)

Use the instructions in this chapter to set up the RNAscope Plus smRNA-RNA LS Assay without mock probe workaround, recommended for LS software versions 6.0 and 7.0. **To set up the assay with the Mock Probe, or if you have software version 5.2, see Chapter 4.**

Workflow





Register the reagents

Your ACD Field Application Specialist (FAS) should implement this procedure.

1. Select the **Reagent Setup** icon at the top of the screen.
2. Select **Add** to enter reagent information for components that are not pre-filled in the Leica software.
3. Enter a reagent name in the Name text box (see below Table)
4. Select **Ancillary** in the Type drop-down menu.
5. Enter **ACD** in the Supplier text box.
6. Check both the **Preferred** and **Hazardous** boxes (for RNAscope Plus smRNA-RNA Amp 1, and RNAscope Plus smRNA-RNA Amp 3 only).
7. Select **Save**.
8. Repeat Steps 2–7 for the rest of the reagents using the container names in the following table:

Reagents	Container Name	Abbreviated Name	Volume required per slide (not accounting for dead volume)
RNAscope Plus smRNA-RNA LS AMP 1	ACD smRNA-RNA Amp 1	smAmp1	300 µL
RNAscope Plus smRNA-RNA LS AMP 2	ACD smRNA-RNA Amp 2	smAmp2	300 µL
RNAscope Plus smRNA-RNA LS AMP 3	ACD smRNA-RNA Amp 3	smAmp3	300 µL
RNAscope Plus smRNA-RNA LS HRP S1	ACD smRNA-RNA HRP-S1	smHRPs1	300 µL
RNAscope Plus smRNA-RNA LS HRP C2	ACD smRNA-RNA HRP-C2	smHRPc2	300 µL
RNAscope Plus smRNA-RNA LS HRP C3	ACD smRNA-RNA HRP-C3	smHRPc3	300 µL
RNAscope Plus smRNA-RNA LS HRP C4	ACD smRNA-RNA HRP-C4	smHRPc4	300 µL
RNAscope Plus smRNA-RNA LS HRP Blocker	ACD smRNA-RNA HRP blocker	smHRPbk	900 µL
Opal-fluorophore 1 (user to dilute in TSA buffer)	ACD Multiplex TSA-F1	MuxTSAf1	300 µL
Opal-fluorophore 2 (user to dilute in TSA buffer)	ACD Multiplex TSA-F2	MuxTSAf2	300 µL
Opal-fluorophore 3 (user to dilute in TSA buffer)	ACD Multiplex TSA-F3	MuxTSAf3	300 µL
Opal-fluorophore 4 (user to dilute in TSA buffer)	ACD Multiplex TSA-F4	MuxTSAf4	300 µL
Opal TSA-DIG † (if Opal 780 is used)	TSA-DIG	TSA-DIG	300 µL
Opal 780† (if Opal 780 is used)	Opal 780	Opal780	300 µL



Reagents	Container Name	Abbreviated Name	Volume required per slide (not accounting for dead volume)
LS smRNA-RNA probe mix	(you can use any container but make sure that the container is registered as "Probe RNA" in the software)	—	370 µL for 6.0/7.0 software
RNAscope Plus smRNA-RNA LS DAPI	DAPI	DAPI	150 µL
BOND Wash	Bond Wash	BondWash	150 µL
RNAscope Plus smRNA-RNA LS Hydrogen Peroxide	*RNAscope 2.5 LSx H2O2	—	150 µL
RNAscope Plus smRNA-RNA LS Protease III	*RNAscope 2.5 LSx Protease	—	200 µL
RNAscope Plus smRNA-RNA LS Rinse	*LS Rinse	—	600 µL

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

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

Create and register the BOND Research Detection System (if not registered already)

A BOND Research Detection System from Leica is required to setup the RNAscope Plus smRNA-RNA LS 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 Plus smRNA-RNA LS 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.

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

Add research reagent system x

Name:

UPI:

Lot N°:

Expiration date:

Reagents

Pstn.	UPI	Reagent	Vol. (mL)
1		DAPI	
2		Bond Wash	

Add reagent | Remove reagent

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.

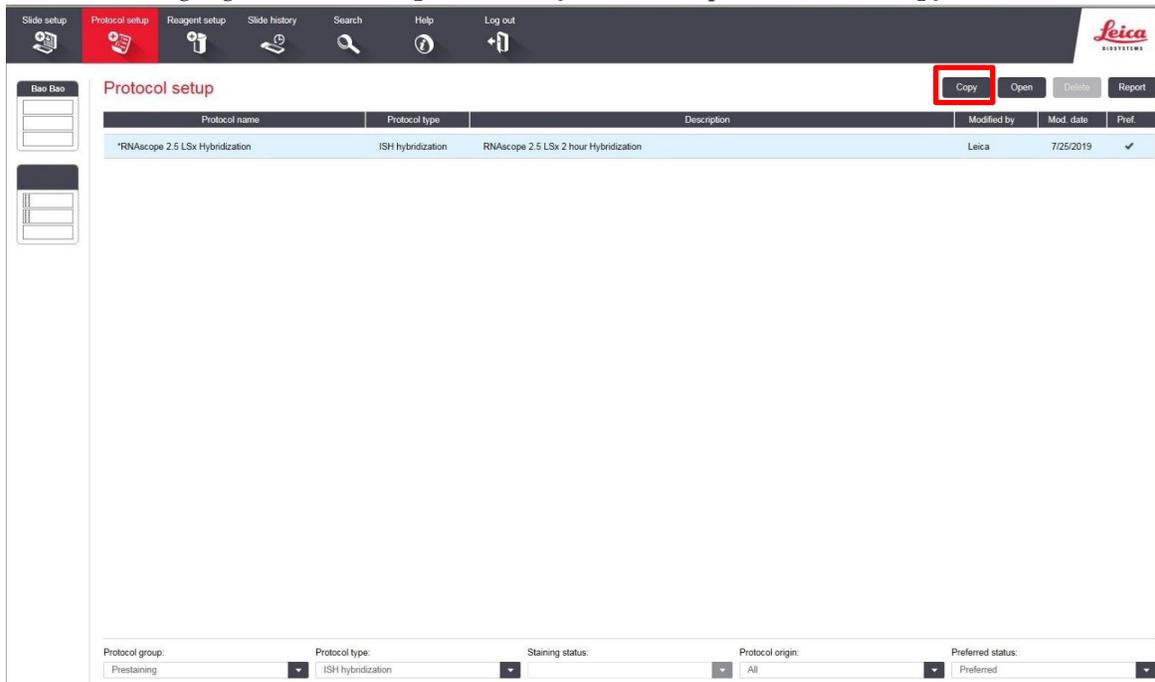
Note: If you prefer not to use DAPI on the instrument or want to perform immunohistochemistry (IHC) steps after the assay, you may use BOND Wash in place of DAPI in the protocol.

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.
7. Select **Add**.

Create the smRNA-RNA probe hybridization protocol

1. In the Protocol setup screen, select **Prestaining** from the Protocol group menu and **ISH Hybridization** from the Protocol type menu.

2. Highlight the *RNAscope 2.5 LSx Hybridization protocol. Select Copy.



The screenshot shows the 'Protocol setup' page in the Leica Biosystems software. The interface includes a top navigation bar with icons for Slide setup, Protocol setup (highlighted in red), Reagent setup, Slide history, Search, Help, and Log out. The Leica Biosystems logo is in the top right corner. On the left, there is a sidebar with a 'Bao Bao' icon and a list of protocols. The main area displays a table of protocols with the following data:

Protocol name	Protocol type	Description	Modified by	Mod. date	Pref.
*RNAscope 2.5 LSx Hybridization	ISH hybridization	RNAscope 2.5 LSx 2 hour Hybridization	Leica	7/25/2019	✓

At the top right of the table, there are buttons for 'Copy', 'Open', 'Delete', and 'Report'. The 'Copy' button is highlighted with a red box. At the bottom of the page, there are several dropdown menus for filtering: Protocol group (Prestaining), Protocol type (ISH hybridization), Staining status (All), Protocol origin (All), and Preferred status (Preferred).

New protocol properties

Name: ACD smRNA-RNA 37C
 Abbreviated name: ACDsmR37
 Description: ACD smRNA-RNA Hybridization 37C

Preferred

BOND RX Import protocol Protocol type: ISH hybridization

Step N°	Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
1		*No Reagent	Leica Microsystems		37	120.00	Intermediate

Show wash steps

3. Change the Name to **ACD smRNA-RNA 37C**, the Abbreviated Name to **ACDsmR37**, and the Description to **ACD smRNA-RNA hybridization 37C**.
4. Change the temperature to **37°C**.

IMPORTANT! To assure the success of the assay, the temperature must be **37°C**.

5. Select **Save**.

Create a staining protocol

Your ACD Field Application Specialist (FAS) should implement this procedure.

1. In the Protocol setup screen, select **Staining** under the Protocol drop down menu.
2. Highlight the ***ACD 2.5 DAB Rev B protocol**. Select **Copy**.
3. Change the protocol name to **ACD smRNA-RNA Protocol** in the Name text box, **Multi_P1** in the Abbreviated name text box, and **ACD smRNA-RNA Protocol** in the Description text box.
4. Select ACD LS Multiplex Detection Kit from the Preferred detection system menu
5. Compare and confirm the on-screen protocol with the protocol listed in **Appendix A**. If you use Opal Polaris 780, refer to **Appendix B** instead.
6. Highlight and select each step to edit. Click **Show wash** steps to also view the wash steps. Insert BOND Washes to match each of the protocol steps shown.
7. Once the staining protocol matches the steps in Appendix A or B, delete steps 1–14. **ACD smRNA RNA Amp1** should be steps 1 and 2.
8. Select **Preferred** in the bottom right corner of the window.
Select **Save**.



Prepare the instrument reagents

1. Fill the DAPI container with DAPI and the Bond Wash container with Leica Biosystems' 1X BOND Wash. The kit requires 150 µL DAPI or Bond Wash per slide.
2. Carefully transfer all other RNAscope LS kit 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 on page 35 for the reagent volume required per slide.

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.

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

3. Prepare the LS smRNA-RNA target probe mix:
 - a. Determine the volume of probe needed (volume needed for the total number of slides plus container dead-volume). For software 6.0/7.0 you will require 370ul of probe per slide plus dead volume for the container. 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 and C4 probe stocks 1:50 into the Ready-To-Use S1 probe. For example, add 320 µL 50X C2 probe and 320 µL 50X C3 probe to a tube, then add enough S1 probe to bring the final volume to 16 mL.
 - c. Transfer the LS smRNA-RNA probe mix into the appropriate Bond container, probe containers must be registered as "Probe RNA" in the software.

Note: The LS smRNA-RNA probe mix is stable for one year at 2–8°C.

4. Prepare the Opal fluorophore dilutions:
 - a. Determine the volume of Opal fluorophore needed (see **Appendix E**. Dilute the Opal Fluorophore), and make sure to add dead volume per container (0.6–2.5 mL for 6 mL Titration container or 30 mL Open container, respectively).
 - b. Dilute the 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
Opal 690*	1:750–1:3000 (in TSA buffer)
Opal 520*	1:750–1:3000 (in TSA buffer)
Opal 570*	1:750–1:3000 (in TSA buffer)
Opal 620*	1:750 -1:3000 (in TSA buffer)
Opal TSA-DIG* (if using Opal 780)	1:750–1:3000 (in TSA buffer)
Opal Polaris 780* (if using Opal 780)	1:187.5–1:750+ (in Akoya or Bond diluent)

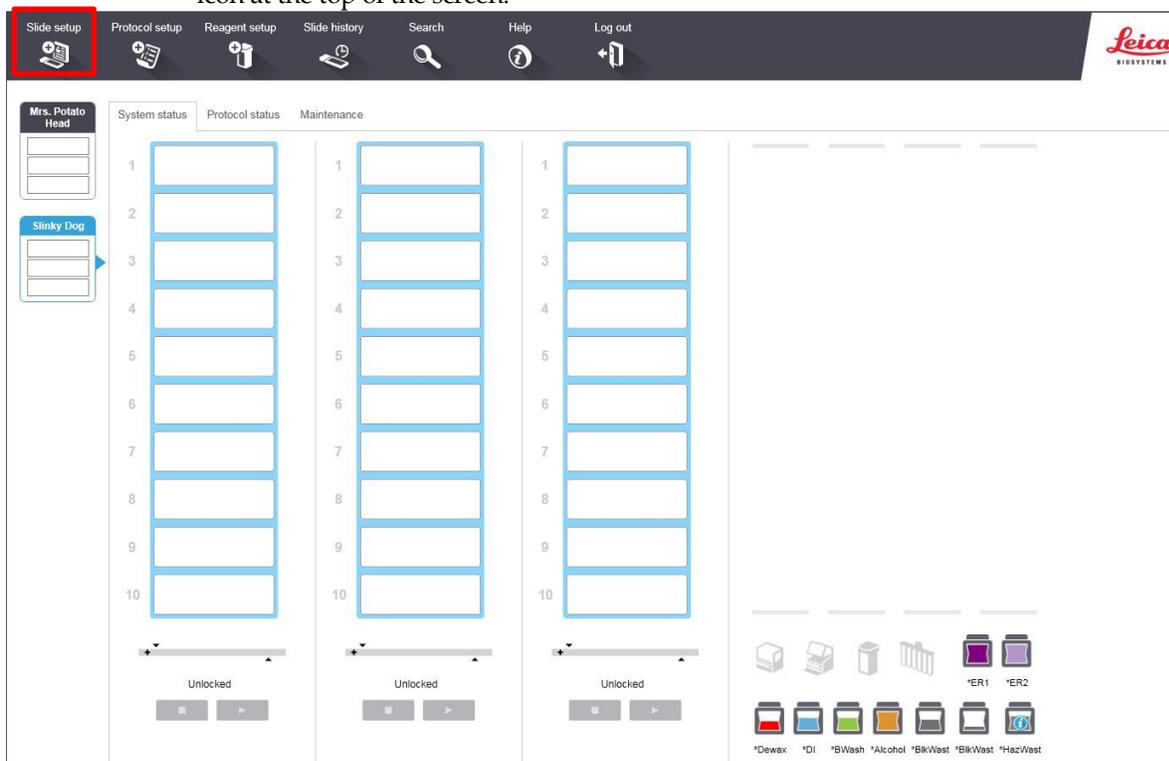
* Reconstitute all Opals (except Opal Polaris 780) with 75 μ L Dimethylsulfoxide (DMSO). Reconstitute Opal Polaris 780 with 300 μ L double distilled water (ddH₂O).
 † We recommend keeping the dilution factors of Opal TSA-DIG and Opal Polaris 780 at a constant ratio. For example, when using 1:1500 dilution for Opal TSA-DIG, use 1:375 dilution for Opal Polaris 780. When using 1:750 dilution for Opal TSA-DIG, use 1:187.5 dilution for Opal Polaris 780.

Scan containers into the software

1. Using the Barcode Scanner, scan the barcode located on the front of the BOND Open container. A window appears.
2. From the drop-down menu, select the corresponding name of the reagent as shown in the table on pg. 34 under **Container name**.

Set up a study for the smRNA-RNA assay

1. To build a study for the RNAscope Plus smRNA-RNA LS 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 μ L as shown).

3. For FFPE tissues, select ***Bake and Dewax** as the Preparation protocol. For fresh frozen tissues, select ***----** instead.
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 **the appropriate probe** from the Marker drop down menu.

9. Under the **Protocols** tab, do the following:

- a. Select the appropriate protocol from the Staining drop down menu (for example, ACD smRNA-RNA Protocol).
 - b. For standard FFPE tissues, select the protocol ***Bake and Dewax** from the Preparation drop down menu. For fresh frozen tissues, select ***----** instead.
 - c. Select ***RNAscope 2.5 LSx Target Retrieval (95)** as the HIER protocol or the appropriate HIER protocol for your tissue.
 - d. Select ***RNAscope 2.5 LSx Enzyme**, or the appropriate enzyme protocol for your tissue.
 - e. Select ***RNAscope 2.5 LSx Probe Application** from the Probe Application dropdown menu
 - f. Select ***----** for Denaturation
 - g. Select **ACD smRNA-RNA 37C** for Hybridization.
 - h. Select ***RNAscope 2.5 LSx Probe Removal** for Probe Removal
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.

The screenshot displays the 'Slide setup' interface in the Leica Biosystems software. At the top, there is a navigation bar with icons for Slide setup, Protocol setup, Reagent setup, Slide history, Search, Help, and Log out. Below this, the main area is titled 'Slide setup' and contains a table of studies. The table has the following columns: Study ID, Study name, Researcher name, and Slides. The 'Print labels' button is highlighted with a red box at the bottom right of the interface.

Study ID	Study name	Researcher name	Slides
miR-124 and Training	miR124_Training	----	4
MultiplexV2-Tocris TSA C1-C3-MMT	MuxV2-Tocris C1-3	----	5
MultiplexV2-Tocris TSA C1-C3-Hela	MuxV2-Tocris C1-3	----	5
Applications ICW		----	1
miRNA 6Plex COPY	miRNA 6Plex COPY	----	10
miRNA 6Plex_1	miRNA 6Plex-1	----	20
20220516_NewConf_mux	4-plex new	----	8
SPD13829 test ICW 2		----	0
20220511_RNAremoval-1 COPY	RNAremoval1-1 COPY	----	24
Hplex QC v2 053122		----	14
New Config_QC_TMA COPY	New Config_TMA and QC COPY	----	12
20220609_RNAremoval-online	RNAremovalonline	----	24
20220608_RNAremovalonline1	RNAremovalonline1	----	4
RNA Removal Time Titration COPY	RNA Removal Time Titration COPY	----	10
New ConfigTMA	New Config_TMA	----	18
New ConfigTMA-miRs	New Config_TMA Mir	----	3
Test Study		----	1

At the bottom of the interface, there are buttons for 'Study report', 'Slide setup summary', and 'Print labels' (highlighted with a red box). The total number of studies is 129 and the total number of slides is 1099.

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.

Start the run

1. Attach the barcodes to the slides, and add the slides to the slide tray with the label sides facing up.
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.

2. Place the tray in the Leica BOND RX, and press the button to load the tray onto the machine.
3. 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. Do not use Delayed Start with fresh-frozen tissue.

Note: The total run time for LS smRNA-RNA Fluorescent Assay is 14–15 hrs depending on the number of slides.

IMPORTANT! Before leaving the instrument unattended, ensure that the instrument is running successfully.

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
<ul style="list-style-type: none"> Leica DM series or equivalent Zeiss Axio Imager or equivalent Inverted microscope, if optics and condenser meet requirements Required excitation/emission filter cube: DAPI/FITC/Cy3/Cy5/Cy7 (if Opal Polaris 780 is used) 	<ul style="list-style-type: none"> 20X (N.A. 0.75) air 40X (N.A. 0.8) air (recommended) 40X (N.A. 1.3) oil 63X (N.A. 1.3) oil – use for low expression targets, if needed Use 20X and 40X to visualize high expression genes and low expression genes, respectively 	<ul style="list-style-type: none"> Microscope with camera for fluorescence capturing. Multispectral microscope/camera system (eg. Nuance® EX, Mantra, Vectra and Polaris) recommended for imaging tissue with high autofluorescence. 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.

Scoring Guidelines

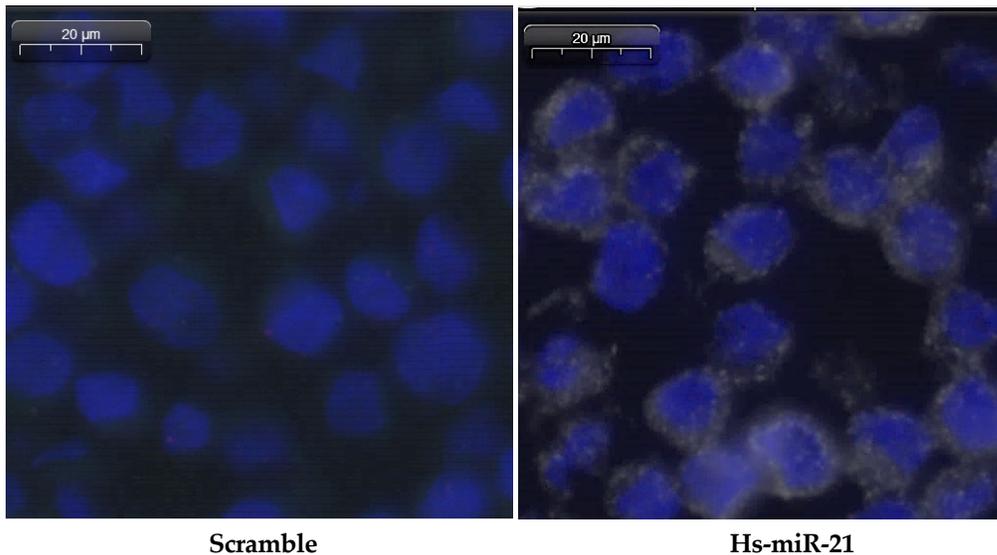
RNAScope Plus smRNA-RNA LS Fluorescent Assay enables a semi-quantitative scoring guideline utilizing the estimated number of punctate dots present within each cell boundary. miRNA and mRNA targets follow different scoring guidelines. An example of how to develop such a guideline for semi-quantitative assessment of miRNA and mRNA staining intensity is presented below for a gene with expression level varying between 1 to > 10 dots per cell.

Note: If your gene expression level is higher or lower than this range, you may need to scale the criteria accordingly.

miRNA scoring is divided into four categories:

Staining Score	Microscope Objective Scoring
0	No staining or less than 1 dot per cell
1	2–10 dots per cell, no or very few cell clusters
2	11–20 dots per cell and/or <25% dots are in clusters
3	>20 dots per cell and/or >25% dots are in clusters

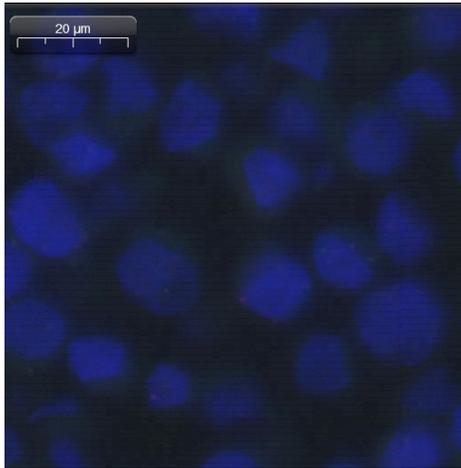
Figure 2: miRNA staining examples



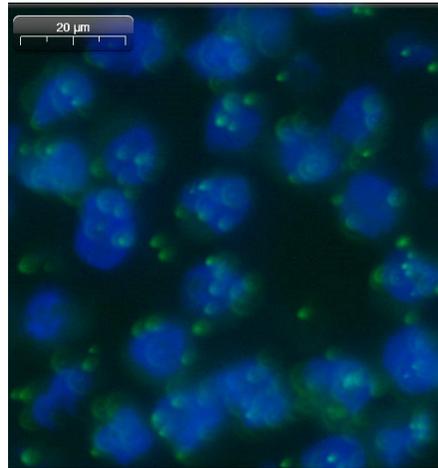
mRNA scoring is divided into five categories:

Staining Score	Microscope Objective Scoring
0	No staining or less than 1 dot per 10 cells
1	1–3 dots per cell
2	4–9 dots per cell, no or very few dot clusters
3	10–15 dots per cell and/or <10% dots are in clusters
4	>15 dots per cell and/or >10% dots are in clusters

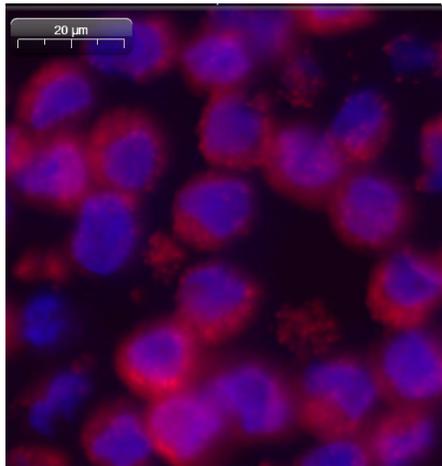
Figure 3: mRNA staining examples



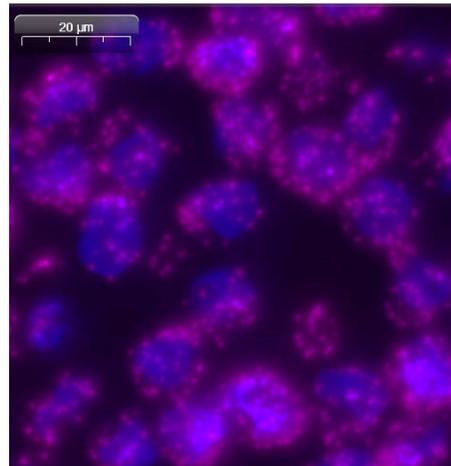
DapB



Hs-TBP



Hs-PP1B



Hs-Polr2a

Control examples

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

Figure 4. RNAscope Plus smRNA-RNA LS Fluorescent Assay detection of RNU6 (white)-miRNA, and POLR2A (green), PPIB (red), and 18S (pink) mRNA in HeLa FFPE tissue at 40X magnification.

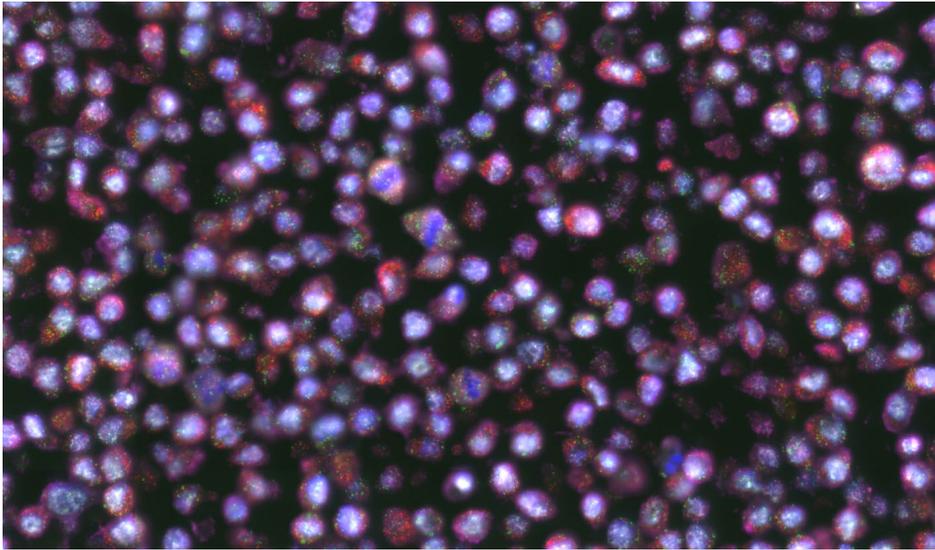
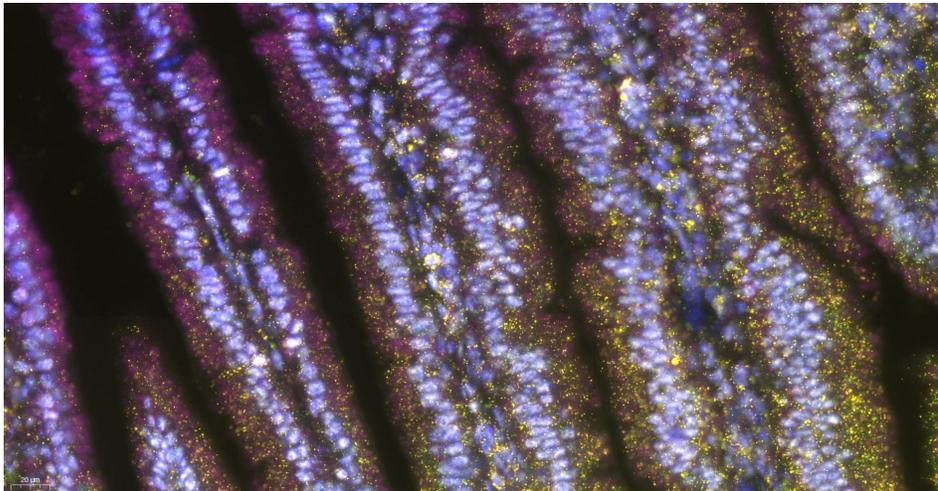


Figure 5. RNAscope Plus smRNA_RNA LS Assay detection of RNU6 (white) miRNA, Polr2a (green), PPIB (yellow), UBC (pink) mRNA in mouse intestine FFPE tissue at 40X magnification.





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 Opal fluorophore concentration.
- Use optimized fluorescence filter sets to reduce signal bleed-through. If you observe fluorescence bleed-through, reduce the 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 Opal fluorophore concentration.
- If you observe the presence of background staining, limit the sensitivity of image acquisition or reduce the corresponding 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 D**. Edit the Protease Protocol). We recommend increasing the Enzyme time first.
- Use the above process for over-fixed tissues.
- The RNAscope smRNA-RNA LS 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.



Appendix A. smRNA RNA 4-plex Staining Protocol (using the Mock Probe workaround)

The following table displays the full software protocol. For use of TSA-DIG /Opal 780 see **Appendix B**.

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 two trays for the smRNA-RNA assay (four channels), follow the protocol steps listed in the following table. Adding any additional steps will prevent the system from running all the 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	37°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 smRNA-RNA Amp 1	Reagent	1 MIN	42°C
16	ACD smRNA-RNA 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



Step No.	Reagent	Step Type	Incubation Time	Temperature†
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
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 smRNA-RNA Amp 2	Reagent	1 MIN	42°C
32	ACD smRNA-RNA 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 smRNA-RNA Amp 3	Reagent	1 MIN	42°C
48	ACD smRNA-RNA 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 smRNA-RNA HRP-S1	Reagent	1 MIN	42°C



Step No.	Reagent	Step Type	Incubation Time	Temperature†
58	ACD smRNA-RNA HRP-S1	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
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 smRNA-RNA HRP blocker	Reagent	1 MIN	42°C
77	ACD smRNA-RNA 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 smRNA-RNA HRP-C2	Reagent	1 MIN	42°C
86	ACD smRNA-RNA 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



Step No.	Reagent	Step Type	Incubation Time	Temperature†
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
102	*Bond Wash Solution	Wash	1 MIN	Ambient
103	ACD smRNA-RNA HRP blocker	Reagent	1 MIN	42°C
104	ACD smRNA-RNA 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 smRNA-RNA HRP-C3	Reagent	1 MIN	42°C
113	ACD smRNA-RNA 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



Step No.	Reagent	Step Type	Incubation Time	Temperature†
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 smRNA-RNA HRP blocker	Reagent	1 MIN	42°C
131	ACD smRNA-RNA 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	*Bond Wash Solution	Wash	1 MIN	Ambient
138	ACD smRNA-RNA HRP-C4	Reagent	1 MIN	42°C
139	ACD smRNA-RNA HRP-C4	Reagent	15 MIN	42°C
140	*Bond Wash Solution	Wash	0 MIN	Ambient
141	*Bond Wash Solution	Wash	0 MIN	Ambient
142	*Bond Wash Solution	Wash	0 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	ACD Multiplex TSA-F4	Reagent	1 MIN	Ambient
147	ACD Multiplex TSA-F4	Reagent	30 MIN	Ambient
148	*Bond Wash Solution	Wash	0 MIN	Ambient
149	*Bond Wash Solution	Wash	0 MIN	Ambient
150	*Bond Wash Solution	Wash	0 MIN	Ambient
151	*Bond Wash Solution	Wash	1 MIN	Ambient
152	*Bond Wash Solution	Wash	1 MIN	Ambient
153	ACD smRNA-RNA HRP blocker	Reagent	1 MIN	42°C
154	ACD smRNA-RNA HRP blocker	Reagent	15 MIN	42°C
155	*Bond Wash Solution	Wash	0 MIN	Ambient
156	*Bond Wash Solution	Wash	0 MIN	Ambient
157	*Bond Wash Solution	Wash	0 MIN	Ambient
158	*Bond Wash Solution	Wash	1 MIN	Ambient
159	*Bond Wash Solution	Wash	1 MIN	Ambient



Step No.	Reagent	Step Type	Incubation Time	Temperature†
160	DAPI/Bond Wash‡	Reagent	10 MIN	Ambient
161	*De-ionized Water	Wash	0 MIN	Ambient
162	*De-ionized Water	Wash	0 MIN	Ambient
163	*De-ionized Water	Wash	0 MIN	Ambient

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

‡ 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. smRNA RNA 4-plex Protocol with TSA-DIG and Opal 780 (using the Mock Probe workaround)

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	37°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 smRNA-RNA Amp 1	Reagent	1 MIN	42°C
16	ACD smRNA-RNA 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 smRNA-RNA Amp 2	Reagent	1 MIN	42°C
32	ACD smRNA-RNA 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 smRNA-RNA Amp 3	Reagent	1 MIN	42°C
48	ACD smRNA-RNA 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 smRNA-RNA HRP-S1	Reagent	1 MIN	42°C
58	ACD smRNA-RNA HRP-S1	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	*Bond Wash Solution	Wash	1 MIN	Ambient
76	ACD smRNA-RNA HRP blocker	Reagent	1 MIN	42°C
77	ACD smRNA-RNA 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 smRNA-RNA HRP-C2	Reagent	1 MIN	42°C
86	ACD smRNA-RNA 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



Step No.	Reagent	Step Type	Incubation Time	Temperature†
99	*Bond Wash Solution	Wash	1 MIN	Ambient
100	*Bond Wash Solution	Wash	1 MIN	Ambient
101	*Bond Wash Solution	Wash	1 MIN	Ambient
102	*Bond Wash Solution	Wash	1 MIN	Ambient
103	ACD smRNA-RNA HRP blocker	Reagent	1 MIN	42°C
104	ACD smRNA-RNA 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 smRNA-RNA HRP-C3	Reagent	1 MIN	42°C
113	ACD smRNA-RNA 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 smRNA-RNA HRP blocker	Reagent	1 MIN	42°C
131	ACD smRNA-RNA HRP blocker	Reagent	15 MIN	42°C



Step No.	Reagent	Step Type	Incubation Time	Temperature†
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	*Bond Wash Solution	Wash	1 MIN	Ambient
138	ACD smRNA-RNA HRP-C4	Reagent	1 MIN	42°C
139	ACD smRNA-RNA HRP-C4	Reagent	15 MIN	42°C
140	*Bond Wash Solution	Wash	0 MIN	Ambient
141	*Bond Wash Solution	Wash	0 MIN	Ambient
142	*Bond Wash Solution	Wash	0 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	TSA-DIG	Reagent	1 MIN	Ambient
147	TSA-DIG	Reagent	30 MIN	Ambient
148	*Bond Wash Solution	Wash	0 MIN	Ambient
149	*Bond Wash Solution	Wash	0 MIN	Ambient
150	*Bond Wash Solution	Wash	0 MIN	Ambient
151	*Bond Wash Solution	Wash	1 MIN	Ambient
152	*Bond Wash Solution	Wash	1 MIN	Ambient
153	ACD smRNA-RNA HRP blocker	Reagent	1 MIN	42°C
154	ACD smRNA-RNA HRP blocker	Reagent	15 MIN	42°C
155	*Bond Wash Solution	Wash	0 MIN	Ambient
156	*Bond Wash Solution	Wash	0 MIN	Ambient
157	*Bond Wash Solution	Wash	0 MIN	Ambient
158	*Bond Wash Solution	Wash	1 MIN	Ambient
159	*Bond Wash Solution	Wash	1 MIN	Ambient
160	Opal 780	Reagent	1 MIN	Ambient
161	Opal 780	Reagent	30 MIN	Ambient
162	*Bond Wash Solution	Wash	0 MIN	Ambient
163	*Bond Wash Solution	Wash	0 MIN	Ambient
164	*Bond Wash Solution	Wash	0 MIN	Ambient
165	*Bond Wash Solution	Wash	1 MIN	Ambient
166	*Bond Wash Solution	Wash	1 MIN	Ambient



Step No.	Reagent	Step Type	Incubation Time	Temperature†
167	DAPI/Bond Wash‡	Reagent	10 MIN	Ambient
168	*De-ionized Water	Wash	0 MIN	Ambient
169	*De-ionized Water	Wash	0 MIN	Ambient
170	*De-ionized Water	Wash	0 MIN	Ambient

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

‡ 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.

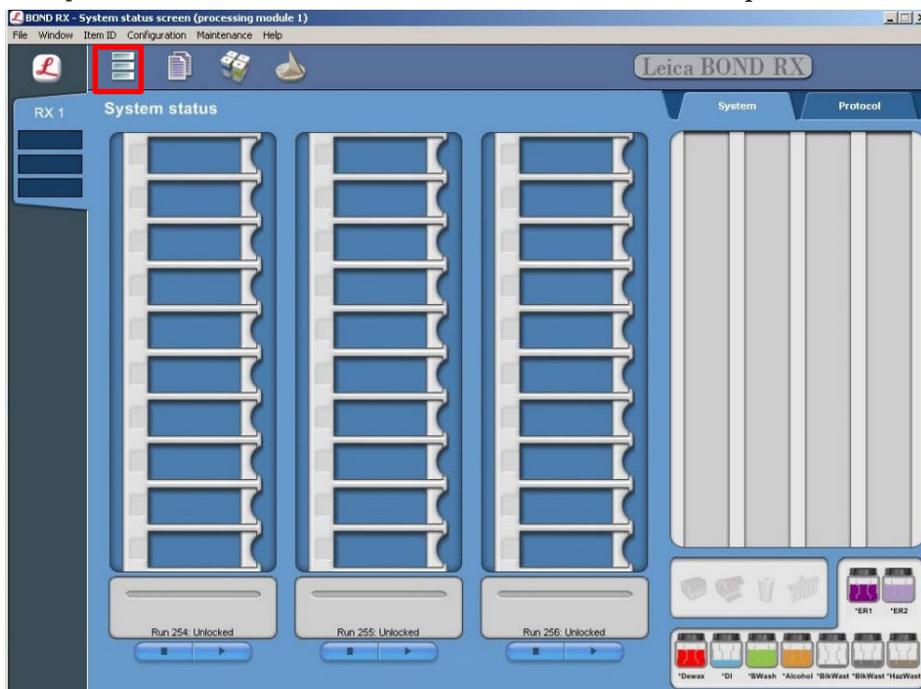


Appendix C. Edit the Epitope Retrieval Protocol

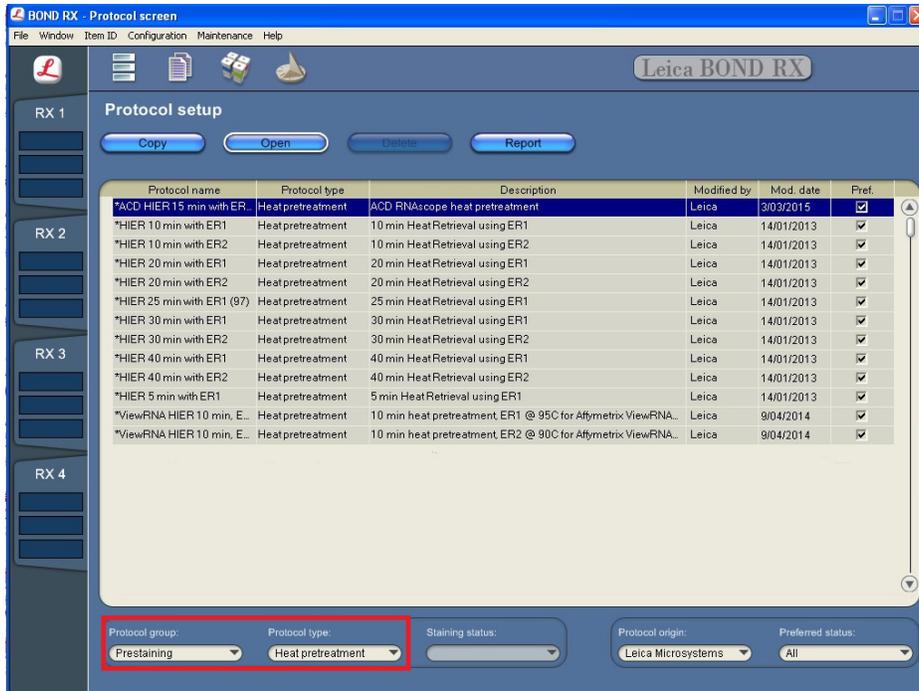
The following example shows how to edit the Epitope Retrieval procedure from within the software. The same pretreatment conditions can be used for LS single-plex, duplex, and multiplex assays.

Create a prestaining protocol

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



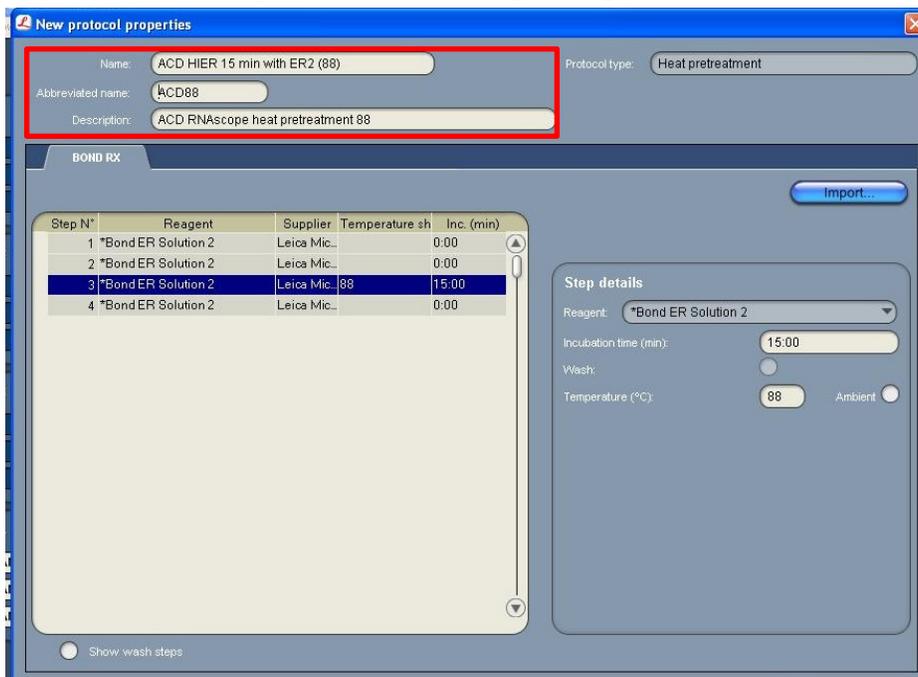
2. Select **Prestaining** under the Protocol group menu and **Heat pretreatment** under the Protocol type menu to access the heat pretreatment protocols.



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

Note: ER2 = Epitope Retrieval 2.

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



- 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.

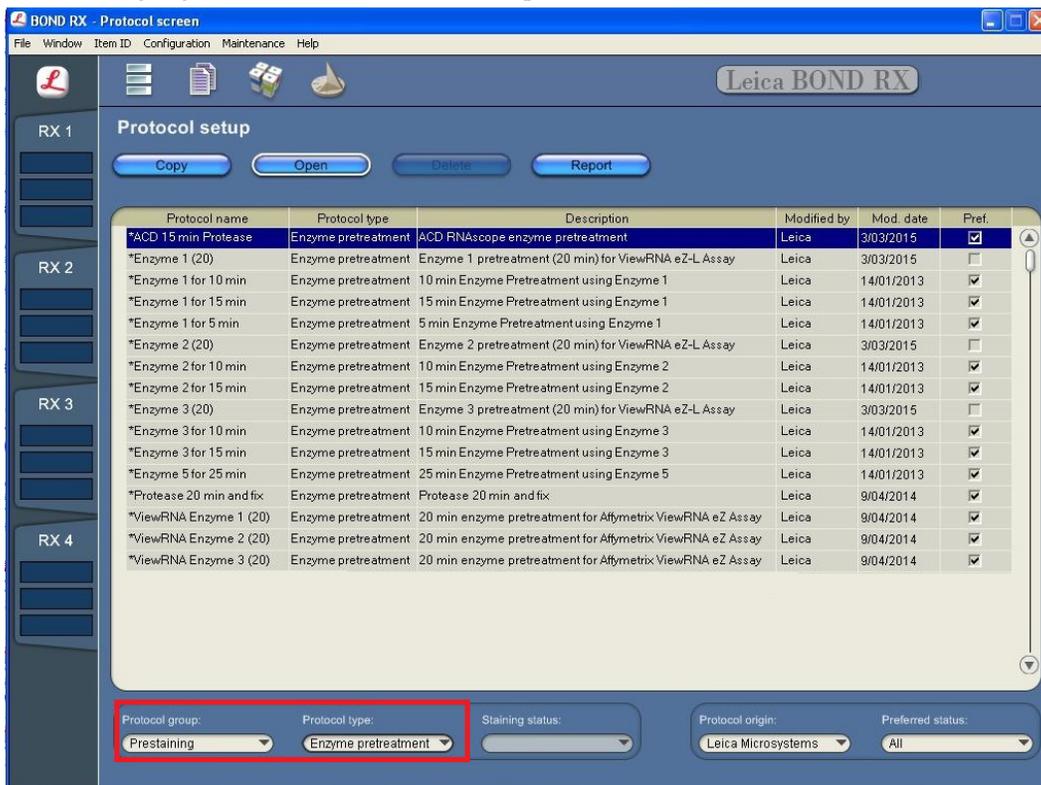
8. Select **Save** to create a protocol for ER2 pretreatment at 88°C.
If needed, repeat Steps 1–8 to create a new heating protocol (for example, ACD 25min ER2).

D

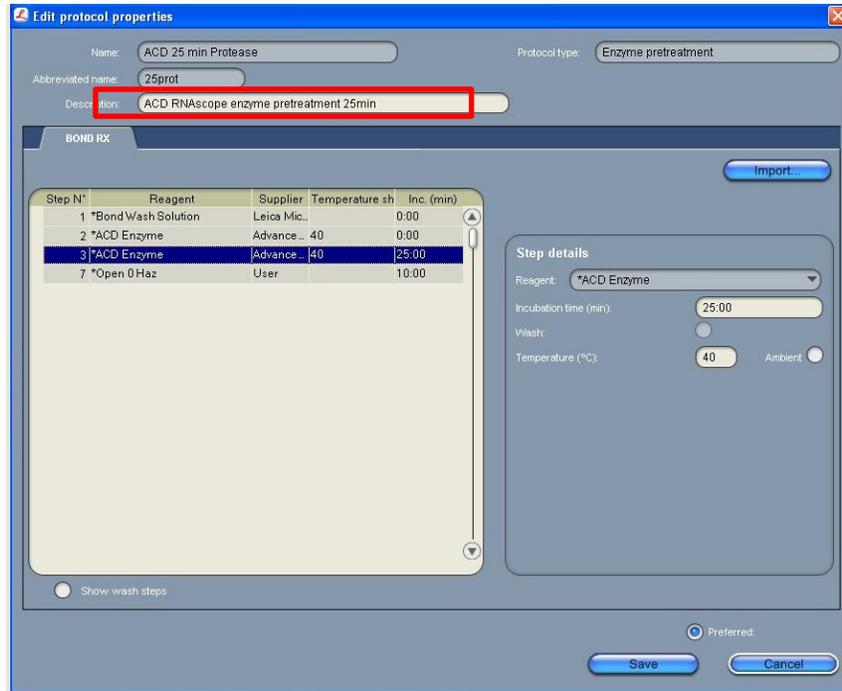
Appendix D. Edit the Protease Protocol

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). Highlight the ***ACD 15min Protease** protocol. Select **Copy**.



2. Rename the protocol to **ACD 25min Protease**.
3. Rename the Abbreviated name to **25prot**.
4. Rename the Description to **ACD RNAscope enzyme pretreatment 25min** (see the next figure).



5. Highlight the second ***ACD Enzyme** step. Keep the temperature at **40°C** and set the enzyme incubation time to **25 MIN**.
6. Select **Save**.
7. If needed, repeat Steps 1–7 to create a new protease protocol for different sample types.
8. 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

Note: 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.

E

Appendix E. Dilute the Opal Fluorophore

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

1. Reconstitute lyophilized Opal fluorophore stock according to manufacturer instructions.
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 μ L 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	Opal-fluorophore solution per channel after dilution (μ L)	Opal-fluorophore stock needed if using 1:750 dilution (μ L)	Opal-fluorophore stock needed if using 1:1500 dilution (μ L)	Opal-fluorophore stock needed if using 1:3000 dilution (μ L)
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
24	7200	9.6	4.8	2.4
25	7500	10.0	5.0	2.5



Number of slides	Opal-fluorophore solution per channel after dilution (µL)	Opal-fluorophore stock needed if using 1:750 dilution (µL)	Opal-fluorophore stock needed if using 1:1500 dilution (µL)	Opal-fluorophore stock needed if using 1:3000 dilution (µL)
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 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 can use the TSA buffer to make a small amount of 1:10 stock. Use the 1:10 stock to make the final dilution.



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](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

Advanced Cell Diagnostics, Inc.
7707 Gateway Blvd Suite 200
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>.

Headquarters

7707 Gateway Blvd Suite 200, Newark, CA 94560
For support, email support.acd@bio-technne.com
www.acdbio.com

Phone 1-510-576-8800

Toll Free 1-877-576-3636

