USER MANUAL

BaseScope™ LS Duplex Fluorescent Kit

For use with BOND RX™ System, from Leica Biosystems

Document Number UM 323650

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UM 323650 RevA /07/22/2026

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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.

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Contents

1 Chapter 1. Product Information

Before using this product, read and understand the information in **Appendix E. 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 for using RNAscope™ Multiomic LS Expansion Pack: BaseScope with RNAscope Multiomic LS Reagents on formalin-fixed, paraffin embedded (FFPE) cell pellets and tissue sections. This assay workflow is supported on the BOND RX fully automated research stainers from Leica Biosystems.

Product description

The RNAscope Multiomic LS Expansion Pack: BaseScope enables the BaseScope LS Duplex Fluorescent Assay which uses a novel and proprietary method of *in situ* hybridization (ISH) to simultaneously visualize up to two short (50–300 nucleotides) RNA targets per cell in samples mounted on slides. The assay is based on Bio-Techne Spatial 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.

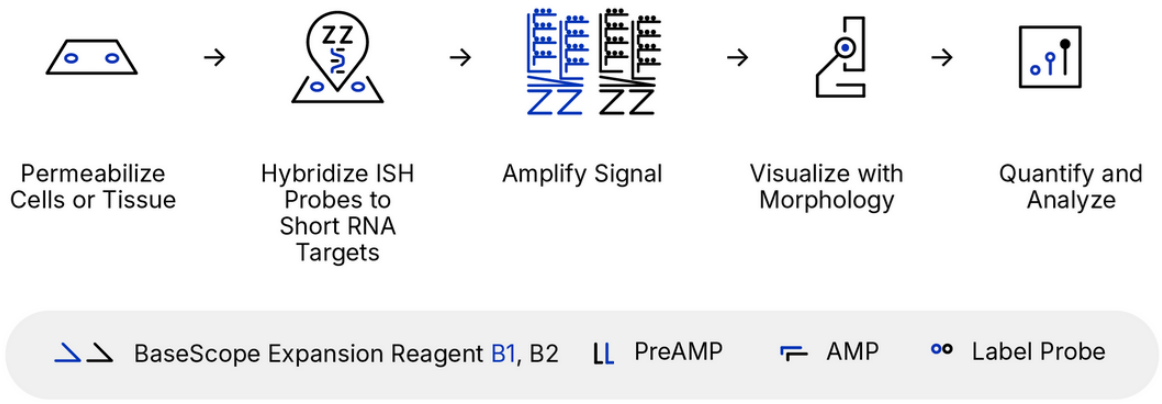


Figure 1. Multiomic LS Expansion Pack: BaseScope Workflow. The assay can be completed on the instrument in about 12–13 hours. Properly prepared and sectioned samples are permeabilized with target retrieval agents, followed by probes to allow binding to their short mRNA targets. Then the sections are incubated with amplification reagents to develop and detect the specific signal for up to two targets.

Kit contents and storage

Detection of the BaseScope LS Duplex Fluorescent signal requires the RNAscope Multiomic LS Expansion Pack: BaseScope and the RNAscope Multiomic LS CORE and C1 and C2 channel reagents both available from Advanced Cell Diagnostics. Additional reagents available from common laboratory suppliers and Leica Biosystems are also required.

Multiomic LS Expansion Pack: BaseScope

☑	Reagent	20-slide kit Cat. No. 323655 Quantity	Storage
	RNAscope Multiomic LS Expansion Pack: BaseScope	8 mL x 1 bottle	2°C to 8°C

RNAscope Multiomic LS Reagents

These kits provide enough reagents to stain ~ 20 or ~60 standard slides. The assay reagents are then used with TSA linked fluorophores and mounting medium.

All assay reagents are Ready-To-Use (RTU), however, the TSA buffer and the Multiomic Antibody Diluent are used for preparation of other materials for the assay (e.g, fluorophores). Storage conditions for each reagent are listed in the tables below.

RNAscope Multiomic LS CORE Reagents				
☑	Reagent	Quantity 60-slide kit Cat. No. 322930	Quantity 20-slide kit Cat. No. 323425	Storage
	RNAscope 2.5 LS Protease III	21 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic LS Rinse	29 mL x 3 bottles	11 mL x 2 bottles	2°C to 8°C
	RNAscope Multiomic LS AMP 1	21 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic LS AMP 2	21 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic LS AMP 3	21 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope PretreatPro™	21 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic LS Hydrogen Peroxide	21 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic LS DAPI	21 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic Antibody Diluent	29 mL x 3 bottles	14 mL x 3 bottles	2°C to 8°C
	RNAscope post hybridization wash	21 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C

RNAscope Multiomic C1 Channel Reagents				
☑	Reagent	Quantity	Quantity	Storage

		60-slide kit Cat. No. 322935	20-slide kit Cat. No. 323430	
	RNAscope Multiomic TSA Buffer	29 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic HRP Blocker	29 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic LS HRP C1	21 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
RNAscope Multiomic C2 Channel Reagents				
☑	**Reagent**	**Quantity 60-slide kit Cat. No. 322940**	**Quantity 20-slide kit Cat. No. 323435**	**Storage**
	RNAscope Multiomic TSA Buffer	29 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic HRP Blocker	29 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C
	RNAscope Multiomic LS HRP C2	21 mL x 1 bottle	8 mL x 1 bottle	2°C to 8°C

BaseScope Probes

The BaseScope Duplex LS Probes consist of user-specified Target Probe and the Positive and Negative Control Probes. Visit **https://www.bio-techne.com/probes** to find gene-specific Target Probe or appropriate control probes. (Important note: BaseScope LS Duplex Fluorescent Kit is NOT compatible with BaseScope LS C1/C2 probes) Each Target Probe contains a mixture of short oligonucleotides designed to bind to a specific target RNA and detectable in one of two color channels, B1 or B2. Signal detection is performed using Tyramide Signal Amplification (TSA) linked fluorophores.

Channel B1 target probes are Ready-To-Use (RTU), while channel B2 probes are shipped as a 50X concentrated stock. To independently detect multiple target RNAs, each target probe must be in a different channel.

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					
☑	**Reagent**	**Cat. No.**	**Content**	**Quantity**	**Storage**
	BaseScope LS Target Probe – *[species]* – *[gene]* – B1	Various	Ready-To-Use (RTU) probe for channel B1	16 mL x 1 bottle	2°C to 8°C
	BaseScope LS Target Probe – *[species]* – *[gene]* – B2	Various	50X probe for channel B2	320 µL x 1 tube	2°C to 8°C
Control Probes					
☑	**Reagent**	**Cat. No.**	**Content**	**Quantity**	**Storage**
	BaseScope LS Duplex Control Probe - BA-Hs-1zz (B1, B2)	322188	Human (Hs) B1-POLR2A-1ZZ / B2-PPIB-1ZZ	16 mL x 1 bottle	2°C to 8°C
	BaseScope LS Duplex Control Probe - BA-Mm-1zz (B1, B2)	324528	Mouse (Mm) B1-Polr2a-1ZZ / B2-Pbib-1ZZ	16 mL x 1 bottle	2°C to 8°C
	BaseScope LS Duplex Control Probe - BA-Hs-3zz (B1, B2)	322258	Human (Hs) B1-POLR2A-3ZZ / B2-PPIB-3ZZ	16 mL x 1 bottle	2°C to 8°C

	BaseScope LS Duplex Control Probe - BA-Mm-3zz (B1, B2)	322298	Mouse (Mm) B1-Polr2aA-3ZZ / B2-Ppib-3ZZ	16 mL x 1 bottle	2°C to 8°C
Control Probes					
☑	**Reagent**	**Cat. No.**	**Content**	**Quantity**	**Storage**
	BaseScope LS Duplex Negative Control Probe - DapB-1ZZ (B1, B2)	324538	Probe targeting bacterial gene dapB	16 mL x 1 bottle	2°C to 8°C
	BaseScope LS Duplex Negative Control Probe - DapB-3ZZ (B1, B2)	322308	Probe targeting bacterial gene dapB	16 mL x 1 bottle	2°C to 8°C

Required materials and equipment

Recommended fluorophores

The BaseScope LS Duplex Fluorescent Assay requires purchase of Opal fluorophore from Akoya Biosciences or ClariTSA™ fluorophores from ACD/Bio-Techne.

Dilute the fluorophores to the desired working concentration in the TSA Buffer provided in the RNAscope Multiomic C1 and C2 Channel Reagents. Choose a dilution factor for each fluorophore based on the recommendations below and your specific experimental conditions including target expression levels, tissue quality, or microscope setting.

Note: To reconstitute dyes, follow the manufacturer instructions available on the tube labels. Dilute the fluorophores in TSA buffer provided in the Channel Reagent kits.

See the following table for recommended fluorophores and dilution range.

TSA Fluorophores

☑	**Fluorophores**	**Quanterix Cat. No.**	**Recommended dilution range**
	Opal 520 Reagent Pack	FP1487001KT	1:500–1:1000
	Opal 570 Reagent Pack	FP1488001KT	1:500–1:1000
☑	**Fluorophores**	**ACD Cat. No.**	**Recommended dilution range**
	ClariTSA Fluorophore Kit 520	323271	1:1000–1:1500
	ClariTSA Fluorophore Kit 570	323272	1:1000–1:1500

Required slide scanner or microscope

A system with multispectral capabilities is recommended, especially 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).

Slide scanner or microscope	Optics
• Akoya PhenoImager HT • Leica DM series or equivalent • Zeiss Axio Imager, Axioscan or equivalent • Inverted microscope if optics and condenser meet requirements. • Required excitation/emission filter cube compatible with fluorophore used: DAPI/Opal 520/Opal 570	• 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

Required materials and equipment from Leica Biosystems

The BaseScope LS Duplex Fluorescent Assay is designed for the BOND RX and requires specific materials and equipment available from Leica Biosystems.

☑	Component	Cat. No.	Storage
	BOND RX System — automated slide stainer	—	—
	BOND 30 mL Open containers	OP309700	Room temp (20 to 25°C)
	BOND 6 mL Titration containers and inserts*	OPT9049	Room temp (20 to 25°C)
	BOND Research Detection System	DS9455	Room temp (20 to 25°C)
	BOND Universal Covertile	S21.4611	Room temp (20 to 25°C)
	BOND Epitope Retrieval Solution 1-1L (RTU)	AR9961	2°C to 8°C
	BOND Epitope Retrieval Solution 2-1L (RTU)	AR9640	2°C to 8°C
	BOND Dewax Solution – 1L (RTU)	AR9222	2°C to 8°C
	BOND Wash Solution 10X Concentrate – 1L	AR9590	2°C to 8°C
	BOND Aspirating Probe Cleaning System	CS9100	2°C to 8°C
	BOND Mixing Stations	S21.1971	Room temp (20 to 25°C)

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

Other user-supplied materials

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

☑	Description	Supplier	Cat. No.
	SuperFrost Plus Slides (required)	Fisher Scientific	12-550-15

☑	Description	Supplier	Cat. No.
	ProLong™ Gold Antifade Mountant	Thermo Fisher	P36930; P10144; P36934
	Opal dyes fluorophores	Akoya Biosciences	—
	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	—
	Vertical 24-slide racks (or other slide racks or holders)	American Master Tech Scientific/MLS	LWSRA24
	Vertical staining dishes (or similar containers)	American Master Tech Scientific/MLS	LWT4457EA
	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 laboratory supplier.

2 Chapter 2. Before You Begin

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

Become familiar with BOND RX Research Advanced Staining System from Leica Biosystems. Refer to the *BOND RX System User Manual*.

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 BaseScope LS Duplex Positive and Negative Control Probes.

Important procedural guidelines

- Start with appropriately fixed and prepared sections. Refer to the sample preparation and pretreatment chapters in this manual.
- Regularly maintain and clean your automated staining instrument.
- Do not substitute required materials included in the Kit. The assay has been validated with these materials only.
- Follow the protocol exactly for the best results.
- Do not let your sections dry out during the procedure.
- Use good laboratory practice and follow all necessary safety procedures. Refer to **Appendix E. Safety** for more information.

3 Chapter 3. Prepare Samples

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

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

Prepare FFPE sections

Materials required

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

Fix the sample

1. Immediately following dissection cut the tissue into blocks of 3–4 mm in thickness**.**
2. Place the tissue blocks into fixative within **1 HR** after 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 assay.

Dehydrate, embed, and cut the sample

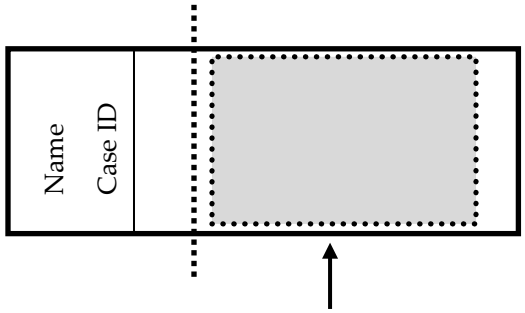
IMPORTANT! Use fresh reagents.

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

Note: Embedded samples may be stored at room temperature with desiccation.

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

5. 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 sections in the center of the slide.

6. Air dry slides **OVERNIGHT** at **RT**.

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

4 Chapter 4. Determine Pretreatment Conditions

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

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

Pretreat FFPE sections

Target retrieval

FFPE samples must be de-crosslinked with a target retrieval step. The BaseScope LS Duplex Fluorescent Assay specifically uses the BOND RX's ER2 solution for this step.

Permeabilization

Pretreatment for BaseScope LS Duplex Fluorescent assay in FFPE samples was validated using LS Protease III. Although LS Pretreat Pro is included in the RNAscope Multiomic LS CORE reagent kit, its use has not been optimized for the BaseScope LS Duplex Fluorescent Assay with RNAscope Multiomic LS Expansion Pack: BaseScope, and may result in reduced signal.

Tissue pretreatment recommendations

Use these conditions as starting points when FFPE tissues are prepared as described in **Chapter 3.** Prepare Samples**.** Depending on your tissue type, vary the amount of time for ER2 until the target probe signal is maximized with minimal or no negative control signal.

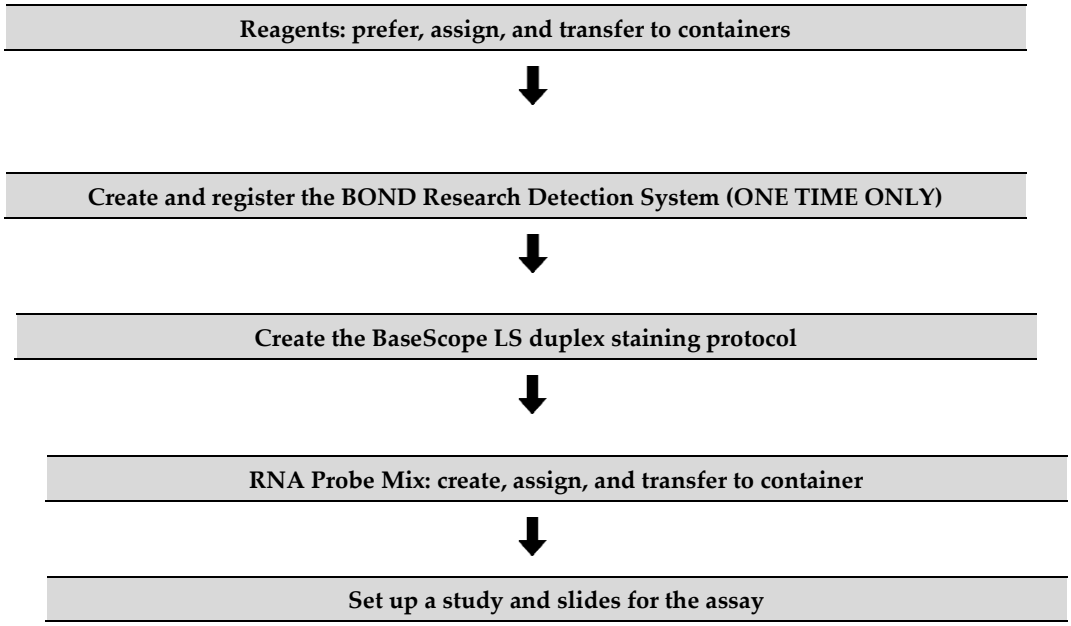
Reagent	Mild	Standard
BOND ER2	15 MIN at 88°C	15 MIN at 95°C
LS Protease III	15 MIN at 40°C	

* Sample types, such as certain xenografts and cell pellets, might require shorter incubation time. For these tissue types, reduce the BOND ER2 incubation time.

5 Chapter 5. Set Up Staining Protocols

In this section, you will find instructions on how to use protocols integrated into the Leica BXD software for BaseScope LS Duplex Fluorescent detection on the BOND RX.

Workflow



Create and register the BOND Research Detection System (one time only)

A BOND Research Detection System from Leica Biosystems is required to set up the BaseScope LS Duplex Fluorescent Assays. The BOND Research Detection System can stain up to two hundred tests.

1. Ensure that *Detection Wash reagent has been marked as **Preferred**.
2. Scan the barcode on the tray of a new BOND Research Detection System.
3. To set up a new detection system for the assay, enter **ACD LS Multiomic Detection Kit** in the Name text box.
4. Place one new BOND 30 mL Open container into position 1 of the Detection System tray.
5. Scan the container and select the registration name ***Detection Wash**. This container will be filled with 1X Bond Wash Solution
6. Select **Add.**

Notes:
When one Research Detection System is finished (up to two hundred tests), register a new detection system by scanning the barcode on the tray and select **ACD LS Multiomic Detection Kit** from the drop-down menu on the right. Creating the detection system needs to be performed only once on each BOND RX controller.

If you prefer to use a previously created Research Detection System, ensure that at least one reagent from the kit is included in each staining protocol and that the correct research kit is selected as the "Preferred Detection System."

Create BaseScope LS Duplex Fluorescent staining protocol

1. Go to the **Protocol** setup screen.
2. Set the **Protocol Group** filter at the bottom left to **Staining** and the Protocol Type to **ISH staining**.
3. Set the **Preferred** filter at the bottom right of the screen to **All**.
4. Copy "*ACD BaseScope Duplex Fluorescent". Remove the "*" symbol from the Name and Abbreviated Name.
5. Select your previously created BOND Research Detection System from the **Preferred Detection System** drop-down.
6. Ensure the **Preferred** box is selected.
7. Save the protocol.

Prepare reagents

Prefer the reagents

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

2. Select **All** for the filter at the bottom right of the screen.
3. Refer to the following table. Find these reagents in the Reagent Setup screen, open them, and mark them as **Preferred.**

Reagent Name	Abbreviated Name
*BaseScope LS Expansion B1, B2	*MOE-BA
*RNAscope Multiomic LS AMP 1	*MO-Amp1
*RNAscope Multiomic LS AMP 2	*MO-Amp2
*RNAscope Multiomic LS AMP 3	*MO-Amp3
*RNAscope Multiomic LS HRP C1	*MO-HRPC1
*RNAscope Multiomic LS HRP C2	*MO-HRPC2
*RNAscope Multiomic LS HRP Blocker	*HRPBK
*Multiomic TSA-F1	*MO-TSAF1
*Multiomic TSA-F2	*MO-TSAF2
*ACD Enzyme	*ACDE
*Open 0 Haz	*Open0H
*DAPI	*DAPI
*LS Rinse	*LS Rinse

4. Select **Save**.

Reagent volumes required

Refer to the following table 1 to calculate the volume of reagent required to run your assay. In addition to the slide volume, you need to add extra reagent to account for container dead volumes:

- 600 µL dead volume when using a BOND Titration container (6 mL)
- 1 mL dead volume when using a BOND 7 mL Open container.
- 2.5 mL dead volume when using a BOND 30 mL Open container.

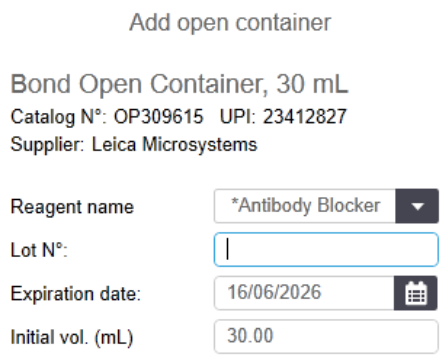
Table 1. Reagent volumes

Reagent name	BA Duplex assay (# of Dispenses/Total volume req.)
*LS Protease III	2/300 µL
*Open 0 Haz/ RNAscope Multiomic LS Hydrogen Peroxide	1/150 µL
*LS Rinse	6/900 µL
ACD RNA Probe (user-defined)	3/370 µL
BaseScope LS Expansion B1,B2	2/300 µL
*RNAscope Multiomic LS AMP 1	2/300 µL
*RNAscope Multiomic LS AMP 2	2/300 µL

Reagent name	BA Duplex assay (# of Dispenses/Total volume req.)
*RNAscope Multiomic LS AMP 3	2/300 µL
*RNAscope Multiomic LS HRP C1	2/300 µL
*RNAscope Multiomic LS HRP C2	2/300 µL
*RNAscope Multiomic LS HRP Blocker	4/600 µL
*Multiomic TSA-F1	2/300 µL
*Multiomic TSA-F2	2/300 µL
*DAPI	1/150 µL

Assign the reagents to Open Containers

1. Assay reagents need to be assigned to BOND Open Containers. Refer to your protocol to identify which reagents are required. Select an appropriately sized container, considering the volume required and whether reagents need to be freshly made for each run.
2. Label each BOND Open Container with the relevant reagent name.
3. Scan the front barcode of the BOND Open Container and select the reagent name from the drop-down menu.
4. Enter in a lot number (if required) and the expiration date.
5. Click on **Save**.



6. Repeat for each BOND Open Container.

Preparing the reagents

1. Carefully transfer the BaseScope LS Expansion B1, B2 reagent into its labeled, empty BOND Open container.
2. Carefully transfer all other RNAscope Multiomic LS kit reagents, excluding the TSA buffer, into their labeled, empty BOND Open containers. Transfer RNAscope Multiomic LS Hydrogen Peroxide into the *Open 0 Haz container.

Note: Before each run, make sure you have enough of each reagent. See the table 1 above 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 BaseScope LS target probe mix:
   a. Each slide requires 370 µL of probe mix. Make sure you add an extra amount for dead volume.
   b. Dilute the 50X B2 probe stocks 1:50 into the Ready-To-Use B1 probe. For example, add 320 µL of 50X B2 probe to a tube, then add enough B1 probe to bring the final volume to 16 mL.
   c. Transfer the RNAscope Multiomic LS probe mix into the labeled BOND open container.

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

4. Prepare the Opal fluorophore dilutions:
   a. Each slide requires 300 µL of each Opal fluorophore. Make sure you add an extra amount for dead volume.
   b. Dilute the Opal fluorophore stock using the TSA buffer provided in the reagent kit.
   c. Add the diluted fluorophores to the appropriate BOND open containers.

Reagents	Recommended dilution range
Opal 520*	1:500–1:1000 (in TSA buffer)
Opal 570*	1:500–1:1000 (in TSA buffer)
ClariTSA 520*	1:1000–1:1500 (in TSA buffer)
ClariTSA 570*	1:1000–1:1500 (in TSA buffer)

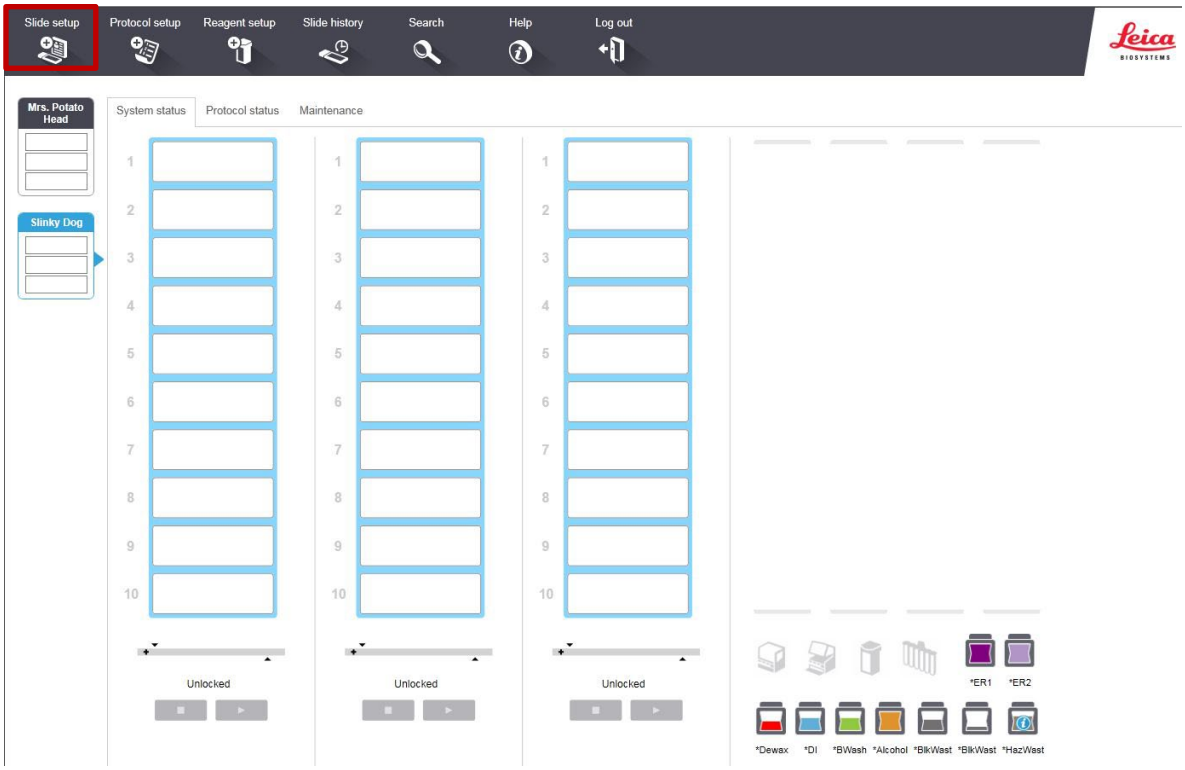
*Refer to manufacturer's website for instructions on Opal fluorophore stock reconstitution and preparation.

For best results, assign Opal 570 to the low expressor and Opal 520 to the high expressor.

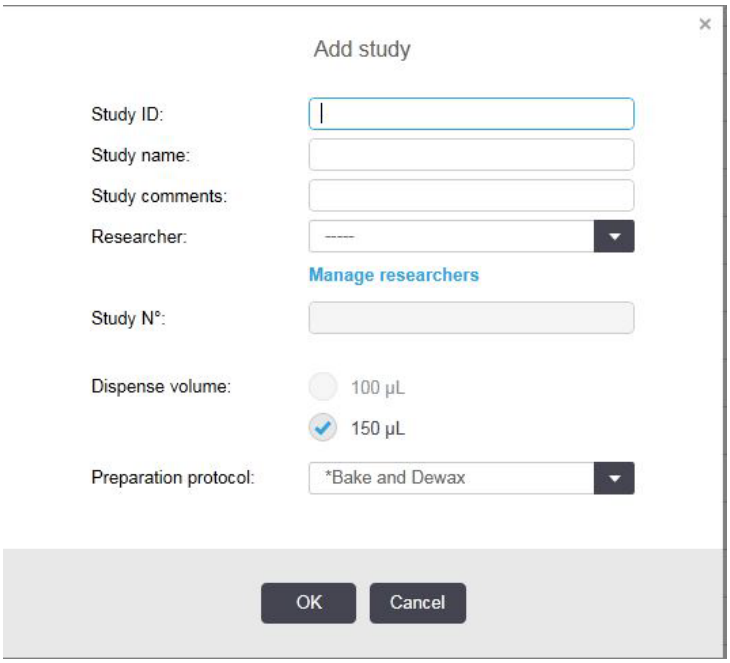
Setting up the study and slides

Creating the study

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



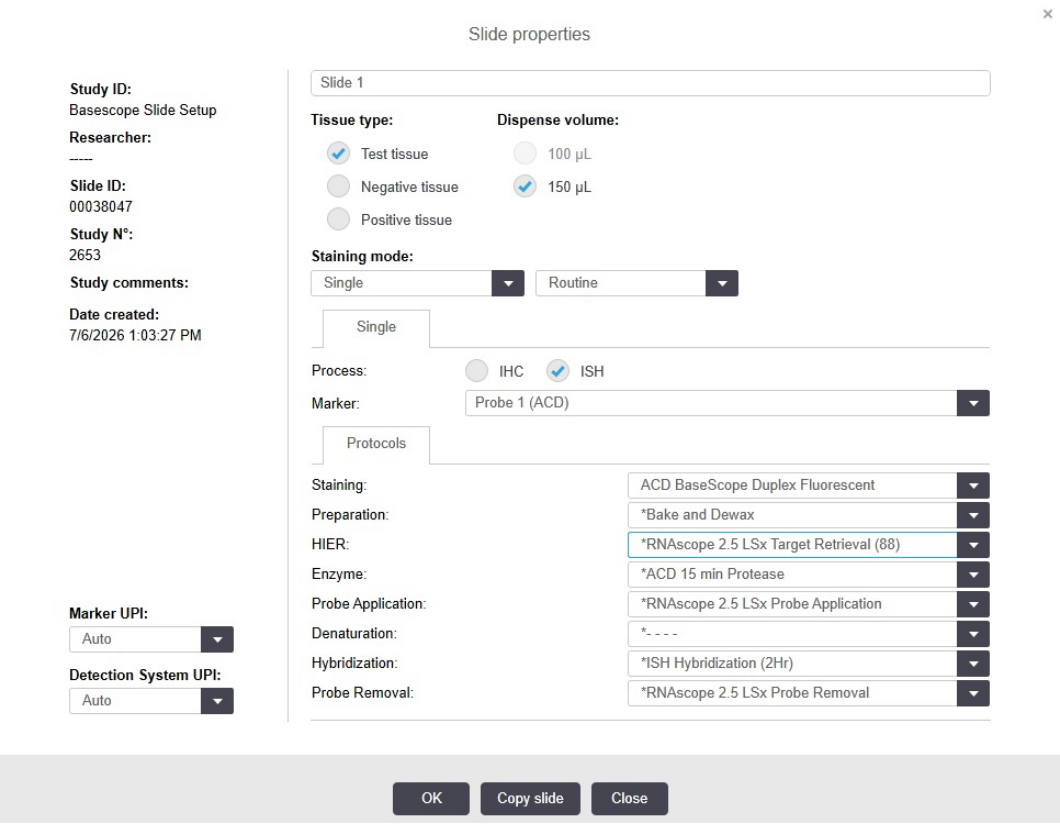
2. Select **Add study** and enter a name in the Study ID field (keep the Dispense volume at 150 µL as shown).



3. For FFPE tissues, select ***Bake and Dewax** as the Preparation protocol.
4. Select **OK**.

Creating the slides

1. Set up the slides for BaseScope LS Duplex Fluorescent Assay.
   a. Select **Add slide** to assign a protocol to each slide.
   b. Enter the tissue type and probe name under the Comments field.
   c. Keep **Single** as default from the Staining mode drop-down menu.
      i. Process = ISH
      ii. Marker = Probe created in **Prepare BaseScope LS target probe mix**
      iii. Staining protocol = Protocol created in **Create staining protocol**
      iv. Preparation = *Bake and Dewax
      v. HIER = *RNAscope 2.5 LSx Target Retrieval (88) or (95) – refer to **Determine Pretreatment Conditions**
      vi. Enzyme = *ACD 15min Protease
      vii. Probe Application = *RNAscope 2.5 LSx Probe Application
      viii. Denaturation = *----
      ix. Hybridization = *ISH Hybridization (2Hr)
      x. Probe Removal = *RNAscope 2.5 LSx Probe Removal



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

6 Chapter 6. Run the BaseScope LS Duplex Fluorescent Assay

Prepare the instrument

1. Fill the large containers located at the bottom of the instrument with the 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 Biosystems 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 barcoded labels to the slides and add the slides to the slide tray with the label sides facing up.
2. Add a covertile on top of each slide and verify placement and seating of each covertile.

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

3. Place the tray in the BOND RX and press the button to lock the slide tray onto the machine.
4. Once the slides have been scanned, select the **PLAY** (triangular) button on the software screen, located under the slide tray, to start the run. Alternatively, right-click on scanned label images, and select **Delayed Start** to start the run at a future time.

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

Complete the run and mount the samples

1. Slides must be unloaded from the instrument within 30 minutes of run completion.
2. After the run is complete, press the button on the front of the instrument to unlock the slide trays.
3. Remove the slide trays, followed by the covertile and slides.

4. Add a drop of ProLong Gold Antifade Mountant to each slide. Avoid introducing bubbles.
5. Carefully place a glass coverslip on the slides, and dry overnight in the dark.
6. Store the slides at **4˚C** in the dark for up to two weeks.

7 Chapter 7. Evaluate the Results

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. Set the light source and exposure time of image acquisition to acceptable background levels.
- Assess positive control signal strength using the same exposure time as negative control.

Scoring guidelines

When used for short RNA detection, the BaseScope LS Duplex Fluorescent assay enables a semiquantitative scoring guideline utilizing the estimated number of punctate dots present within each cell boundary. An example of how to develop such a guideline for semi-quantitative assessment of mRNA staining intensity is shown 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.

The BaseScope staining scoring is divided into five categories:

BaseScope staining score	Microscope objective scoring
0	No staining or less than 1 dot per 10 cells
1	1 dot per cell (visible at 20-40X magnification)
2	2-3 dots per cell. No or very few dot clusters (visible at 20-40X magnification)
3	4–10 dots per cell and/or <10% dots are in clusters (visible at 20-40X magnification)
4	>10 dots per cell and/or >10% dots are in clusters (visible at 20-40X magnification)

Example assay images

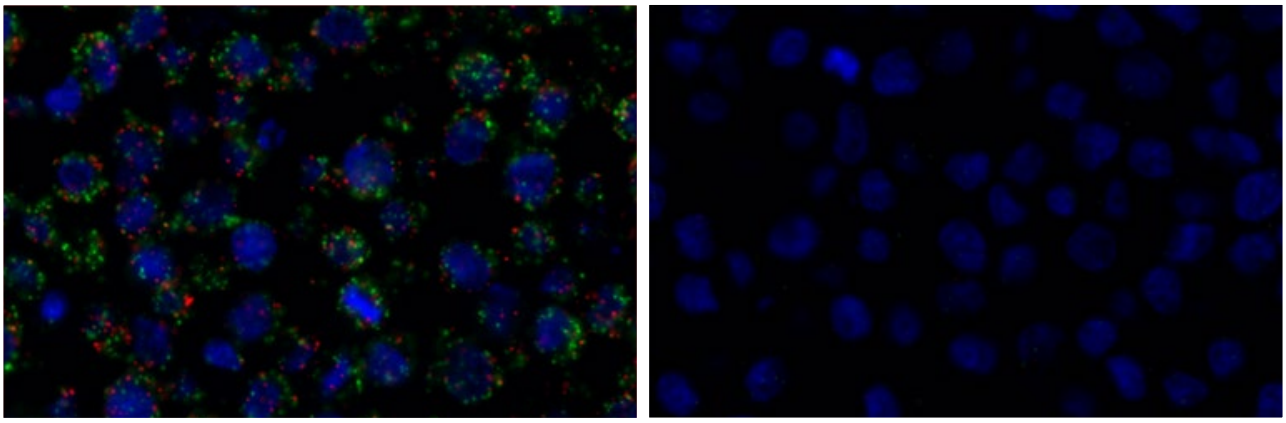


Figure 2. Left panel: BaseScope LS Duplex Fluorescent Assay detection of POLR2A (Red) and PPIB (Green) mRNA in HeLa cells using Hs-POLR2A-1zz and Hs-PPIB-1zz BaseScope Duplex LS probes. Right panel: Corresponding negative control using BaseScope LS Duplex Control Probe - BA-dapB-1zz.

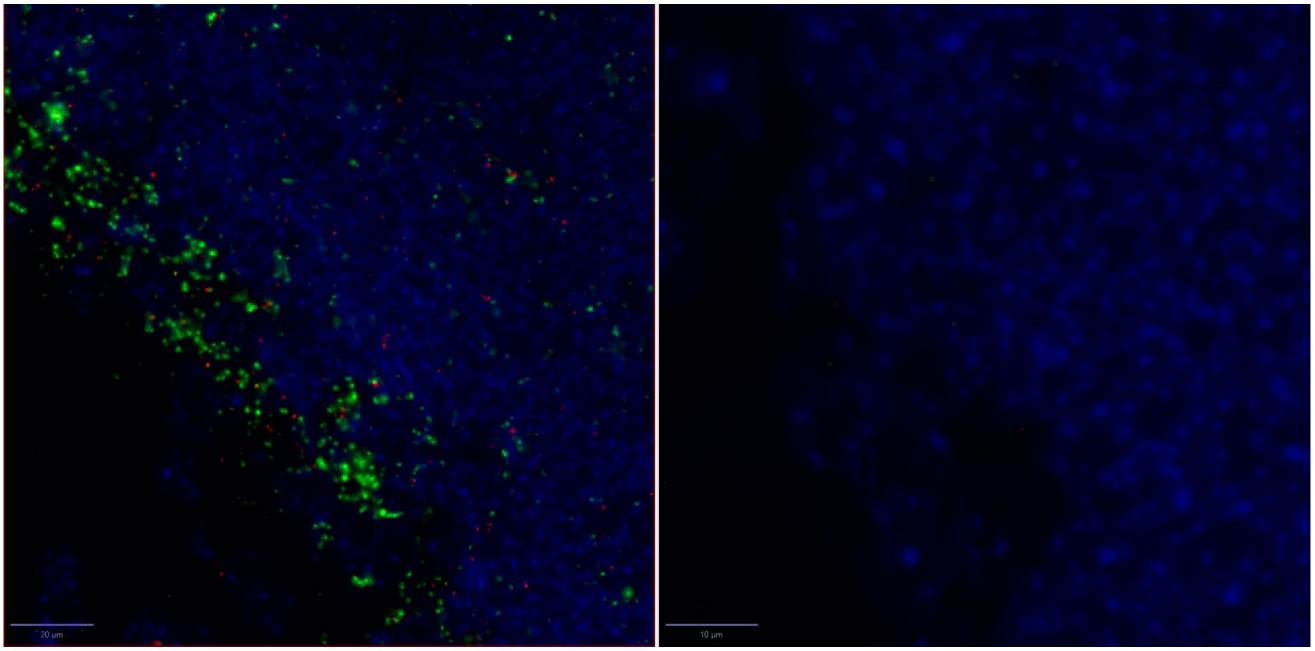


Figure 32. Left panel: BaseScope LS Duplex Fluorescent Assay detection of Polr2a (Red) and Ppib (Green) mRNA in mouse brain tissue using Mm-Polr2a-1zz and Mm-Ppib-1zz BaseScope Duplex LS probes. Right panel: Corresponding negative control using BaseScope LS Duplex Control Probe - BA-dapB-1zz.

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 fluorophore concentration.
- Use optimized fluorescence filter sets to reduce signal bleed-through. If you observe fluorescence bleed-through, reduce the fluorophore concentration of the affected channel and/or reduce the exposure time during image acquisition to avoid over-exposure.
- If your BaseScope signal cannot be distinguished from autofluorescence in tissues with high autofluorescence, increase the fluorophore concentration.
- Do not shake the contents in the dispensers as this will form bubbles and may lead to weak or no staining. If bubbles are present, leave the containers out at room temperature until the bubbles dissipate.
- For troubleshooting information, please contact technical support at **support.acd@bio-techne.com**.

A Appendix A. BaseScope LS Duplex Fluorescent Protocol

The following table displays the full software protocol.

Staining protocol

Step No.	Reagent	Step type	Incubation time	Temperature
1	*BaseScope LS Expansion B1, B2	Reagent	1 MIN	42°C
2	*BaseScope LS Expansion B1, B2	Reagent	30 MIN	42°C
3	*Bond Wash Solution	Wash	0 MIN	Ambient
4	*Bond Wash Solution	Wash	0 MIN	Ambient
5	*Bond Wash Solution	Wash	0 MIN	Ambient
6	*Bond Wash Solution	Wash	3 MIN	Ambient
7	*Bond Wash Solution	Wash	3 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	*RNAscope Multiomic LS Rinse	Reagent	5 MIN	Ambient
12	*RNAscope Multiomic LS Rinse	Reagent	5 MIN	Ambient
13	*Bond Wash Solution	Wash	0 MIN	Ambient
14	*Bond Wash Solution	Wash	0 MIN	Ambient
15	*Bond Wash Solution	Open Wash	0 MIN	Ambient
16	*Bond Wash Solution	Wash	0 MIN	Ambient
17	*RNAscope Multiomic LS Amp 1	Reagent	1 MIN	42°C
18	*RNAscope Multiomic LS Amp 1	Reagent	30 MIN	42°C
19	*Bond Wash Solution	Wash	0 MIN	Ambient
20	*Bond Wash Solution	Wash	0 MIN	Ambient
21	*Bond Wash Solution	Wash	0 MIN	Ambient
22	*Bond Wash Solution	Wash	3 MIN	Ambient
23	*Bond Wash Solution	Wash	3 MIN	Ambient
24	*Bond Wash Solution	Wash	0 MIN	Ambient
25	*Bond Wash Solution	Wash	0 MIN	Ambient
26	*Bond Wash Solution	Wash	0 MIN	Ambient

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

Step No.	Reagent	Step type	Incubation time	Temperature
63	*Bond Wash Solution	Wash	0 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	*Bond Wash Solution	Open Wash	1 MIN	Ambient
68	*Bond Wash Solution	Wash	1 MIN	Ambient
69	*RNAscope Multiomic TSA-F1	Reagent	1 MIN	Ambient
70	*RNAscope Multiomic TSA-F1	Reagent	30 MIN	Ambient
71	*Bond Wash Solution	Wash	0 MIN	Ambient
72	*Bond Wash Solution	Wash	0 MIN	Ambient
73	*Bond Wash Solution	Wash	0 MIN	Ambient
74	*Bond Wash Solution	Wash	1 MIN	Ambient
75	*Bond Wash Solution	Wash	1 MIN	Ambient
76	*Bond Wash Solution	Wash	1 MIN	Ambient
77	*Bond Wash Solution	Wash	1 MIN	Ambient
78	*RNAscope Multiomic LS HRP Blocker	Reagent	1 MIN	42°C
79	*RNAscope Multiomic LS HRP Blocker	Reagent	15 MIN	42°C
80	*Bond Wash Solution	Wash	0 MIN	Ambient
81	*Bond Wash Solution	Wash	0 MIN	Ambient
82	*Bond Wash Solution	Wash	0 MIN	Ambient
83	*Bond Wash Solution	Wash	1 MIN	Ambient
84	*Bond Wash Solution	Wash	1 MIN	Ambient
85	*Bond Wash Solution	Wash	1 MIN	Ambient
86	*Bond Wash Solution	Wash	1 MIN	Ambient
87	*RNAscope Multiomic HRP C2	Reagent	1 MIN	42°C
88	*RNAscope Multiomic HRP C2	Reagent	15 MIN	42°C
89	*Bond Wash Solution	Wash	0 MIN	Ambient
90	*Bond Wash Solution	Wash	0 MIN	Ambient
91	*Bond Wash Solution	Wash	0 MIN	Ambient
92	*Bond Wash Solution	Wash	1 MIN	Ambient
93	*Bond Wash Solution	Wash	1 MIN	Ambient
94	*Bond Wash Solution	Open Wash	1 MIN	Ambient
95	*Bond Wash Solution	Wash	1 MIN	Ambient
96	*RNAscope Multiomic TSA-F2	Reagent	1 MIN	Ambient
97	*RNAscope Multiomic TSA-F2	Reagent	30 MIN	Ambient
98	*Bond Wash Solution	Wash	0 MIN	Ambient

Step No.	Reagent	Step type	Incubation time	Temperature
99	*Bond Wash Solution	Wash	0 MIN	Ambient
100	*Bond Wash Solution	Wash	0 MIN	Ambient
101	*Bond Wash Solution	Wash	1 MIN	Ambient
102	*Bond Wash Solution	Wash	1 MIN	Ambient
103	*Bond Wash Solution	Wash	1 MIN	Ambient
104	*Bond Wash Solution	Wash	1 MIN	Ambient
105	*RNAscope Multiomic LS HRP Blocker	Reagent	1 MIN	42°C
106	*RNAscope Multiomic LS HRP Blocker	Reagent	15 MIN	42°C
107	*Bond Wash Solution	Wash	0 MIN	Ambient
108	*Bond Wash Solution	Wash	0 MIN	Ambient
109	*Bond Wash Solution	Wash	0 MIN	Ambient
110	*Bond Wash Solution	Wash	1 MIN	Ambient
111	*Bond Wash Solution	Wash	1 MIN	Ambient
112	*RNAscope Multiomic LS DAPI‡	Reagent	10 MIN	Ambient
113	*Bond Wash Solution	Wash	0 MIN	Ambient
114	*Bond Wash Solution	Wash	0 MIN	Ambient
115	*Bond Wash Solution	Wash	0 MIN	Ambient

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

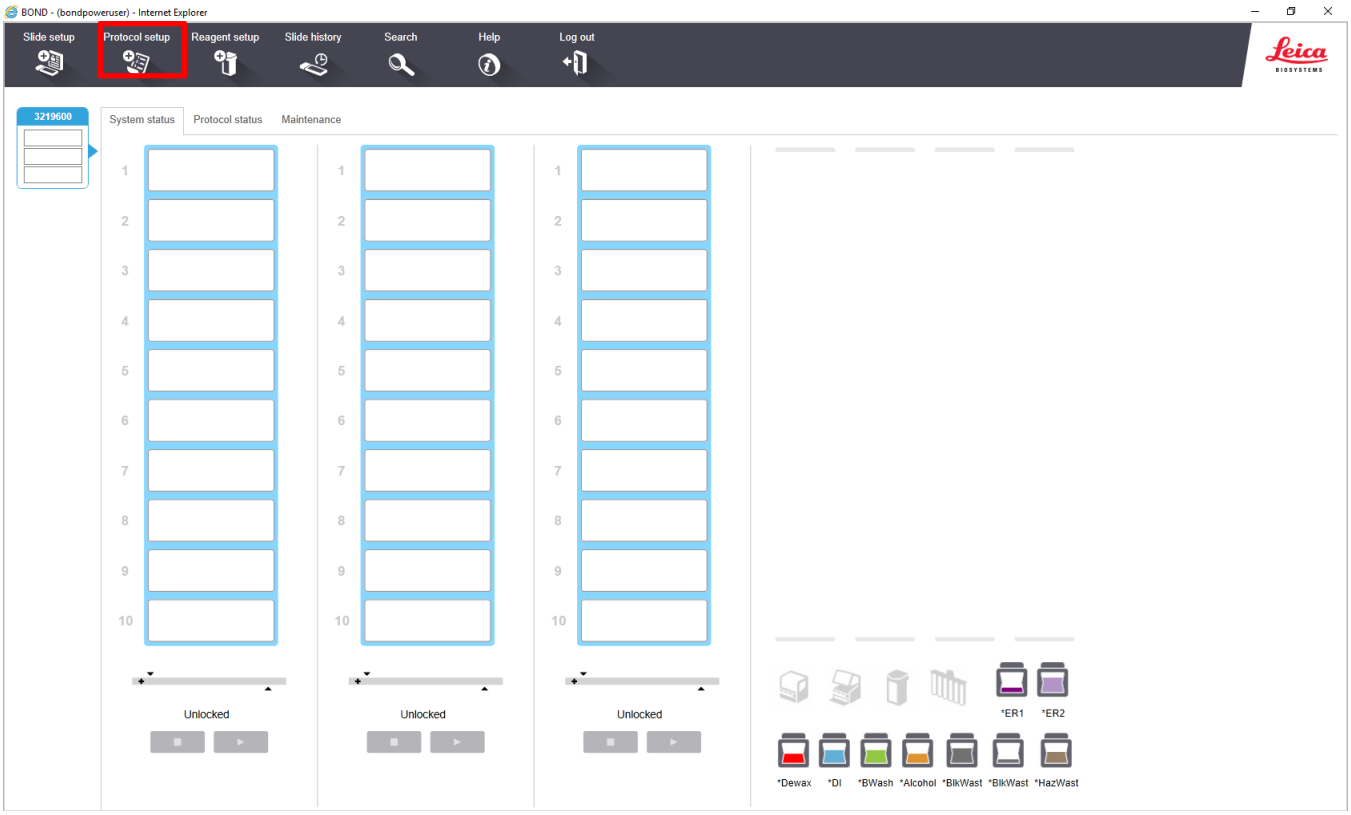
‡ The standard protocol uses DAPI. Use BOND Wash instead of DAPI, if you are using DAPI offline on your samples.

B Appendix B. Edit the Epitope Retrieval Protocol

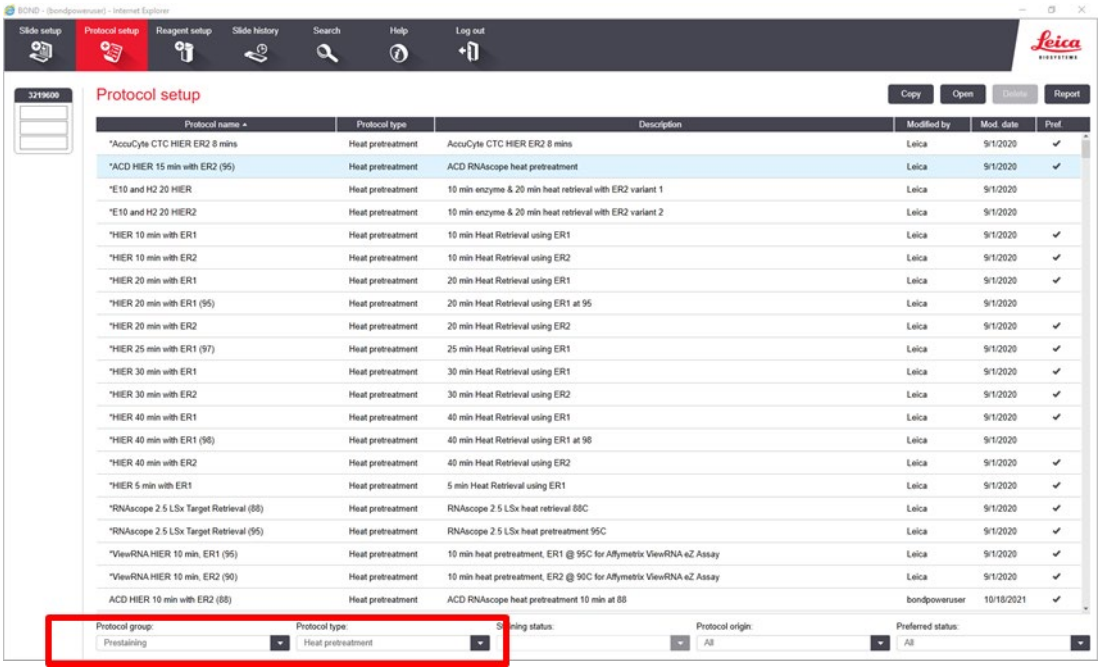
The following example shows how to edit the Epitope Retrieval procedure (95°C to 88°C) from within the software. Please refer to **Appendix D** for additional pretreatment guidance for FFPE samples.

Create a prestaining protocol

1. For RNAscope, ER 2 temperature varies between **100°C** and **92°C** depending on the tissue type used.
2. Open the BOND RX software and click on the **Protocol setup icon** as shown.



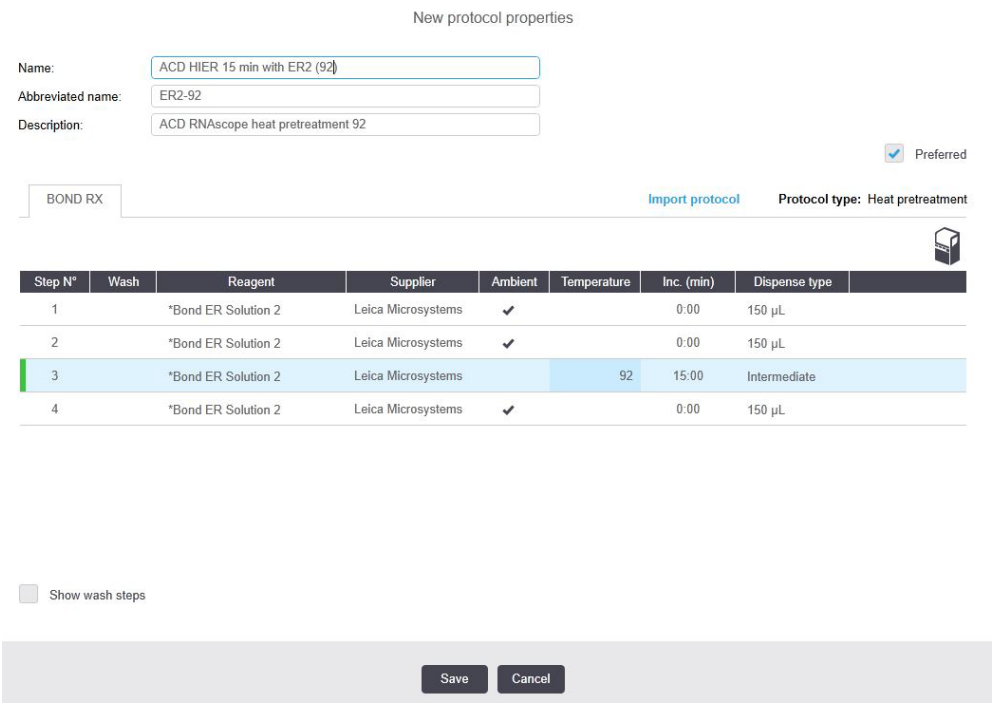
3. Select **Prestaining** under the Protocol group menu and **Heat pretreatment** under the Protocol type menu to access the heat pretreatment protocols.



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

Note: ER2 = Epitope Retrieval 2.

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



8. Highlight the third ***BOND ER Solution 2** step (see above) and change temperature to **88°C**.

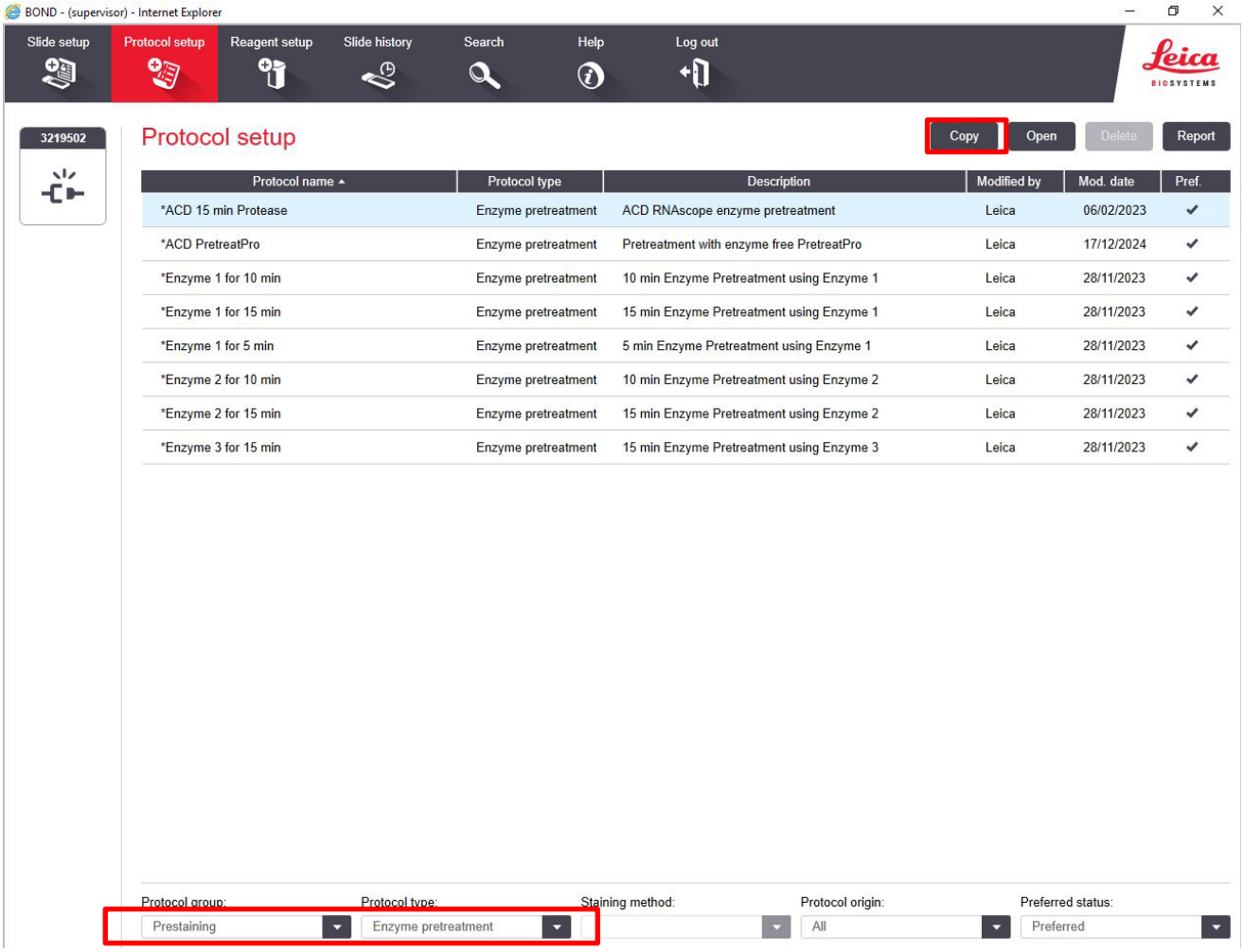
9. Select **Save**.
10. If needed, repeat Steps 1–8 to create new heating protocols for different incubation times and temperatures (for example, ACD 25min ER2).

C

Appendix C. Edit the Protease Protocol

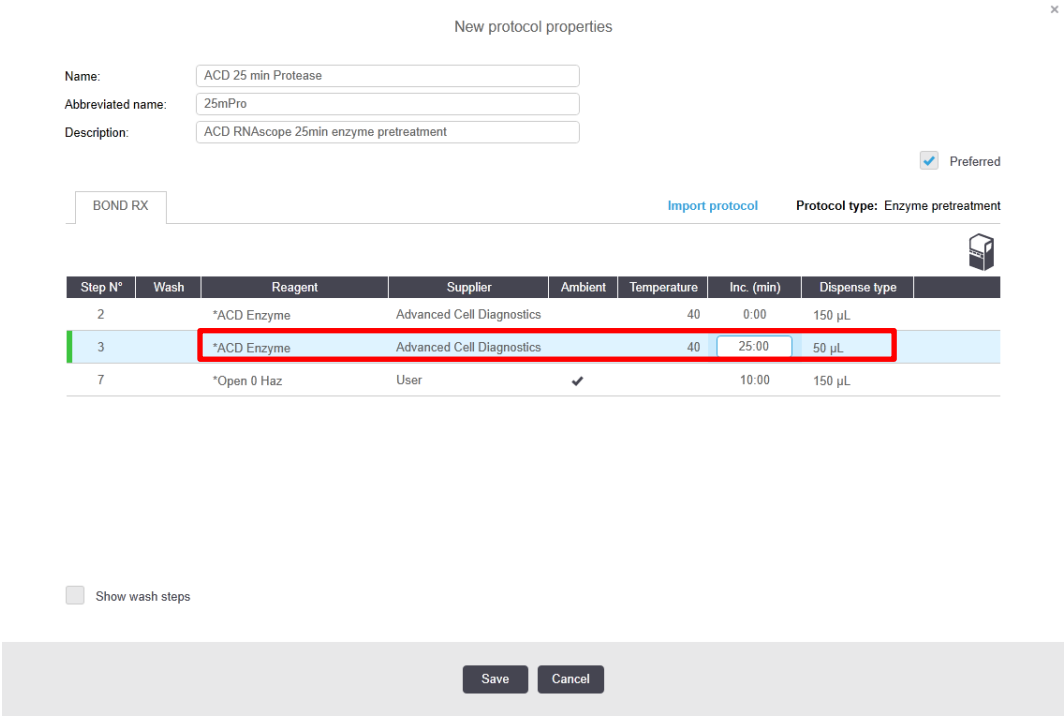
LS Protease III is recommended for the BaseScope LS Duplex Fluorescent Assay. The standard protease condition for Protease III (FFPE) is 15 minutes at 40°C, but the time can be adjusted if needed. The following example shows how to edit the protease procedure (15 min to 25 min) from within the software.

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



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

6. Highlight the second ***ACD Enzyme** step. Keep the temperature at **40°C** and set the enzyme incubation time to desired time (for example, 25min).



7. Select **Save**.
8. If needed, repeat steps 1–7 to create a new protease protocol for different sample types (for example, ACD 10min Protease or ACD 15min Protease at ambient temperature).

Appendix D. Pretreatment Guidance for FFPE Samples

Follow the recommended pretreatment conditions based on your tissue type for:

- Any new or previously untested FFPE tissue types
- Samples prepared differently than the sample preparation protocol found in **Chapter 3**.
- For specific guidance on other sample preparations contact ACD Support at **support.acd@bio-techne.com**

Tissue-specific pretreatment conditions

Refer to the following table for tissue specific FFPE pretreatment conditions. For information about species or tissue type not listed here, contact support at **support.acd@bio-techne.com.**

Species	Tissue type	Pathology	Pretreatment condition
Mouse/ Rat	Intestine	Normal	Standard
	Intestine	Tumor	Standard
	Embryo	Normal	Standard
	Brain	Normal	Standard
	Spleen	Normal	Standard
	Eye/Retina	Normal	Mild
	Liver	Normal	Standard
	Kidney	Normal	Standard
Human	Breast	Tumor	Standard
	Colon	Tumor	Standard
	Colon	Normal	Standard
	Lung	Tumor	Standard
	Lung	Normal	Standard
	Prostate	Tumor	Standard
	Prostate	Normal	Standard
	Lymph node	Tumor	Standard
	Lymph node	Normal	Mild
	Tonsil	Normal	Mild/Standard
	Pancreas	Normal	Standard
	Cervical	Cancer	Standard
	Cervical	Normal	Standard
	Cervical dysplasia	Abnormal	Standard
	Brain	Tumor	Standard

Species	Tissue type	Pathology	Pretreatment condition
Human	Neck	Cancer	Standard
	Liver	Cancer	Standard
	Liver	Normal	Standard
	Heart	Normal	Standard
	GI tract	Normal	Standard
	Kidney	Normal	Standard
	Skin	Normal	Standard
	Lymphoma	Cancer	Standard
	Thymus	Normal	Mild/Standard
	Melanoma	Tumor	Standard
	Nevus	Benign	Standard
	Placenta	Normal	Standard
	Skin (TMA*)	Normal	Standard
	Breast (TMA*)	Normal	Standard
	Melanoma (TMA*)	Normal	Standard
	Nevus (TMA)	Benign	Standard
	Stomach (TMA)	Normal	Standard
	Stomach (TMA)	Tumor	Standard
	Cell pellets, fixed with 10% NBF	—	Mild
	HeLa or 3T3 cells, fixed with 10% Formaldehyde	—	Mild

	Brain	Normal	Standard
	Head	Cancer	Standard

	/PBS/ACD Control		
	Xenograft tissue	—	Mild

Species	Tissue type	Pathology	Pretreatment condition
Cyno monkey	Spleen	Normal	Mild
	Lymph Node	Normal	Mild
	Tonsil	Normal	Mild
	Thymus	Normal	Mild
	Retina	Normal	Mild
	Prostate Gland	Normal	Standard/Mild
	Epididymis	Normal	Mild/Standard
	Testis	Normal	Mild/Standard
	Ovary	Normal	Mild/Standard
	Duodenum	Normal	Mild/Standard
	Jejunum	Normal	Mild/Standard
	Colon	Normal	Standard
	Adrenal Gland	Normal	Mild/Standard
Rat	Spleen	Normal	Mild
	Lymph Node	Normal	Mild
	Tonsil	Normal	N.A.
	Thymus	Normal	Mild
	Retina	Normal	Mild
	Prostate Gland	Normal	Standard/Mild
	Epididymis	Normal	Standard
	Testis	Normal	Standard
	Ovary	Normal	Standard
	Duodenum	Normal	Standard/Mild
	Jejunum	Normal	Standard
	Colon	Normal	Standard
	Adrenal Gland	Normal	N. A

Species	Tissue type	Pathology	Pretreatment condition
Dog	Spleen	Normal	Mild
	Lymph Node	Mild	Mild
	Tonsil	N.A.	N.A.
	Thymus	Mild	Mild
	Retina	Mild	Mild
	Prostate Gland	Mild	Mild
	Epididymis	Mild	Mild
	Testis	Mild/Standard	Mild/Standard
	Ovary	Mild/Standard	Mild/Standard
	Duodenum	Normal	Mild
	Jejunum	Normal	Mild
	Colon	Normal	Mild
	Adrenal Gland	Normal	Standard/Mild

E Appendix E. 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 **https://www.bio-techne.com/** and search for the product name.

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, Authorization and Restriction of Chemicals (REACH)

Documentation and Support

Obtaining SDSs

Safety Data Sheets (SDSs) are available at: **https://www.bio-techne.com/** in the documents download section of individual product pages. For the SDSs of chemicals not distributed by Advanced Cell Diagnostics, contact the chemical manufacturer.

Obtaining support

For support information, go to: **https://www.bio-techne.com/support/contact-us.**

Or for the latest resources and troubleshooting guides, go to**: https://www.bio-techne.com/resources.**

At the web pages notes above, you can:

- 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.

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 General Terms and Conditions of Sale found on the Bio-Techne website. If you have any questions, please contact Advanced Cell Diagnostics at: **https://www.bio-techne.com/support/contact-us**.

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7707 Gateway Blvd Suite 200, Newark, CA 94560

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

www.bio-techne.com