RNAscope™ 2.5 LS Reagent Kit – RED
User Manual

For use with Leica Biosystems’ BOND RX System
For Research Use Only. **Not for use in diagnostic procedures.**

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**Citing RNAscope in Publications**

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Chapter 1. Product Information

Before using this product, read and understand the information in Appendix F. Safety in 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 2.5 LS Reagent Kit with Leica Biosystems’ BOND RX Research Advanced Staining System. RNAscope 2.5 LS Assays are compatible with a variety of sample types.

Product description

Background

The RNAscope 2.5 LS Assays use a novel and proprietary method of in situ hybridization (ISH) to visualize single RNA molecules per cell in formalin-fixed, paraffin-embedded (FFPE), fixed-frozen, and fresh-frozen tissue mounted on slides. The assays are based on Advanced Cell Diagnostic’s patented signal amplification and background suppression technology and can detect RNA molecules in archival samples and partially degraded specimens. The RNAscope 2.5 LS Assay allows users to automate the highly sensitive RNAscope Assay using Leica Biosystems’ BOND RX System.

Overview

Figure 1 illustrates the RNAscope 2.5 LS Assay procedure, which can be completed on the instrument in ~9–10 hours. Starting with properly prepared samples, sections are first pretreated, and then RNA-specific probes are hybridized to target RNA. The signal is amplified using multiple steps, followed by the hybridization of alkaline phosphatase (AP)-labeled probes and detection using the Fast Red chromogenic substrate. Each single RNA transcript appears as a distinct dot of chromogen precipitate visible using a common bright-field microscope.
Kit contents and storage

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

RNAscope 2.5 LS Probes


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

<table>
<thead>
<tr>
<th>Target Probes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent</td>
</tr>
<tr>
<td>RNAscope 2.5 LS Target Probe – {species} – {gene}</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Control Probes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent</td>
</tr>
<tr>
<td>RNAscope 2.5 LS — Positive Control Probe – {species} – PPIB</td>
</tr>
<tr>
<td>RNAscope 2.5 LS — Negative Control Probe – dapB</td>
</tr>
</tbody>
</table>
RNAscope 2.5 LS Reagents

The RNAscope 2.5 LS Reagent Kit – RED (Cat. No. 322150) contains all the reagents needed to run the RNAscope 2.5 LS Assay on Leica Biosystems’ BOND RX System, except for the RNA-specific probes. The kits provide enough reagents to stain ~60 standard slides. The reagents are Ready-To-Use (RTU) and are stored as indicated in the following table:

<table>
<thead>
<tr>
<th>Component</th>
<th>Cat. No.</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNAscope 2.5 LS Hydrogen Peroxide</td>
<td></td>
<td>2–8°C</td>
</tr>
<tr>
<td>RNAscope 2.5 LS Protease III</td>
<td></td>
<td>2–8°C</td>
</tr>
<tr>
<td>RNAscope 2.5 LS PretreatPro™</td>
<td></td>
<td>2–8°C</td>
</tr>
<tr>
<td>RNAscope 2.5 LS AMP 1</td>
<td></td>
<td>2–8°C</td>
</tr>
<tr>
<td>RNAscope 2.5 LS AMP 2</td>
<td></td>
<td>2–8°C</td>
</tr>
<tr>
<td>RNAscope 2.5 LS AMP 3</td>
<td></td>
<td>2–8°C</td>
</tr>
<tr>
<td>RNAscope 2.5 LS AMP 4 - RED</td>
<td></td>
<td>2–8°C</td>
</tr>
<tr>
<td>RNAscope 2.5 LS AMP 5 – RED</td>
<td></td>
<td>2–8°C</td>
</tr>
<tr>
<td>RNAscope 2.5 LS AMP Pro™</td>
<td></td>
<td>2–8°C</td>
</tr>
<tr>
<td>RNAscope 2.5 LS AMP 6 – RED</td>
<td></td>
<td>2–8°C</td>
</tr>
<tr>
<td>RNAscope 2.5 LS Rinse</td>
<td></td>
<td>2–8°C</td>
</tr>
<tr>
<td>RNAscope 2.5 LS Bluing Reagent*</td>
<td></td>
<td>2–8°C</td>
</tr>
</tbody>
</table>

* Bluing is optional.

**IMPORTANT!** Use only RNAscope 2.5 LS Probes. Do not substitute the reagent components of the RNAscope 2.5 LS Reagent Kit with those of any RNAscope Reagent Kits.

Required materials from Leica BOND RX

The RNAscope 2.5 LS Assay requires specific materials and equipment available only from Leica Biosystems.

<table>
<thead>
<tr>
<th>Component</th>
<th>Cat. No.</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOND Open Containers 30 mL</td>
<td>Op309700</td>
<td>Room temp (20–25°C)</td>
</tr>
<tr>
<td>BOND Universal Covertiles 100 pack</td>
<td>S21.2001</td>
<td>Room temp (20–25°C)</td>
</tr>
<tr>
<td>BOND Epitope Retrieval Solution 1-1L (RTU)</td>
<td>AR9961</td>
<td>2–8°C</td>
</tr>
<tr>
<td>BOND Epitope Retrieval Solution 2-1L (RTU)</td>
<td>AR9640</td>
<td>2–8°C</td>
</tr>
<tr>
<td>BOND Dewax Solution – 1L (RTU)</td>
<td>AR9222</td>
<td>2–8°C</td>
</tr>
<tr>
<td>BOND Wash Solution 10X Concentrate – 1L</td>
<td>AR9590</td>
<td>2–8°C</td>
</tr>
<tr>
<td>BOND Polymer Refine Red Detection*</td>
<td>DS9390</td>
<td>2–8°C</td>
</tr>
<tr>
<td>BOND Aspirating Probe Cleaning System</td>
<td>CS9100</td>
<td>2–8°C</td>
</tr>
<tr>
<td>BOND Mixing Stations</td>
<td>S21.1971</td>
<td>Room temp (20–25°C)</td>
</tr>
</tbody>
</table>

* Do not substitute with any other chromogen kit.
Equipment

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
<th>Supplier</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leica Biosystems’ BOND RX System — automated slide stainer</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

User-supplied materials

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

<table>
<thead>
<tr>
<th>Description</th>
<th>Supplier</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>95% Ethanol (EtOH)</td>
<td>American Master Tech Scientific/MLS*</td>
<td>ALREA95</td>
</tr>
<tr>
<td>Xylene</td>
<td>Fisher Scientific/MLS</td>
<td>X3P-1GAL</td>
</tr>
<tr>
<td>10% neutral-buffered formalin (NBF)</td>
<td>MLS</td>
<td>—</td>
</tr>
<tr>
<td>Paraffin wax</td>
<td>MLS</td>
<td>—</td>
</tr>
<tr>
<td>1X PBS</td>
<td>MLS</td>
<td>—</td>
</tr>
<tr>
<td>Microtome</td>
<td>MLS</td>
<td>—</td>
</tr>
<tr>
<td>Drying oven, capable of holding temperature at 60 +/- 1°C (optional)</td>
<td>MLS</td>
<td>—</td>
</tr>
<tr>
<td>Water bath or incubator, capable of holding temperature at 40 +/- 1°C</td>
<td>MLS</td>
<td>—</td>
</tr>
<tr>
<td>BioCare EcoMount or Vector Labs Vectamount</td>
<td>ACD (or directly from manufacturer)</td>
<td>320409 or 321584</td>
</tr>
<tr>
<td>Tissue-Tek® Vertical 24 Slide Rack</td>
<td>American Master Tech Scientific/MLS</td>
<td>LWS2124</td>
</tr>
<tr>
<td>Tissue-Tek Staining Dish (4 required)</td>
<td>American Master Tech Scientific/MLS</td>
<td>LWS20WH</td>
</tr>
<tr>
<td>Tissue-Tek Clearing Agent Dish, xylene resistant (2 required)</td>
<td>American Master Tech Scientific/MLS</td>
<td>LWS20GR</td>
</tr>
<tr>
<td>Cover Glass 24 x 50 mm</td>
<td>Fisher Scientific/MLS</td>
<td>12-545-F</td>
</tr>
<tr>
<td>Distilled water</td>
<td>MLS</td>
<td>—</td>
</tr>
<tr>
<td>Fume hood</td>
<td>MLS</td>
<td>—</td>
</tr>
</tbody>
</table>

* 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 2.5 LS Assay on your samples for the first time, we recommend that you:

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

Important procedural guidelines

- Start with properly fixed and prepared sections. Refer to Chapter 3. Prepare Samples for preparation of FFPE, fixed-frozen or 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 the best results.
- Use good laboratory practices and follow all necessary safety procedures. Refer to Appendix F. Safety for more information.
Chapter 3. Prepare Samples

IMPORTANT! The following protocols describe formalin-fixed, paraffin-embedded (FFPE), fixed frozen and fresh frozen sample preparation. We highly recommend following these guidelines. We cannot guarantee assay results with other preparation methods.

Prepare FFPE sections

Materials required

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

Fix the sample

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

CAUTION! Handle biological specimens appropriately.

IMPORTANT! Fixation for <16 HRS or >32 HRS will impair the performance of the RNAscope 2.5 LS 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. To better preserve RNA quality over a long period (>1 yr), storing at 2–8°C with desiccation is recommended.
4. Trim paraffin blocks as needed and cut embedded tissue into 5 +/- 1 \( \mu \text{m} \) 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:

![Diagram of tissue section location]

**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.

---

### Prepare fixed-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
- Drying oven

**Bake and fix the sections**

1. Remove fixed-frozen tissue slides from -80°C and place in a Tissue TekSlide Rack. Bake in drying oven for 60 MIN at 60°C.
2. *Immediately* immerse the slides in prechilled 10% NBF or 4% PFA for 15–60 MIN at 4°C.

**Dehydrate and dry 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. Remove the slides from the 10% NBF or 4% PFA, and immerse them in 50% EtOH 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.

**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.
Chapter 4. Determine Pretreatment Conditions

The following protocols describe formalin-fixed, paraffin-embedded (FFPE), fixed frozen and fresh-frozen sample pretreatment. For other sample types and preparation methods, contact support.acd@bio-tecne.com for the latest protocols and guidelines.

**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 RNAscope 2.5 LS Assay uses the BOND RX's ER2 solution exclusively for this step.

**Permeabilization**

Two options are available:

- Protease-based permeabilization is recommended for experiments that stain only RNA. This option uses LS Protease III.
- Protease-free permeabilization uses the LS PretreatPro reagent which is free of protease enzyme. This allows co-detection of RNA and proteins that were previously incompatible with protease on the same tissue section using immunohistochemistry (IHC). Please refer to Appendix D to implement the use of LS PretreatPro reagent on the BOND Rx.
To determine the correct permeabilization option, please refer to the following flowchart:

1. **Is your experiment?**
   - **RNA only**
     - Low expression level
     - Medium to High expression level
   - **RNA + Protein**

2. **Is the protein antigen protease-sensitive?**
   - **NO**
     - Test sensitivity using RNAscope protease with IHC
   - **DON'T KNOW**
   - **YES**
     - Is morphology more critical than sensitivity for data
     - If **NO**, use LS Protease
     - If **YES**, **Use LS PretreatPro and LS AMP Pro**

For Research Use Only. Not for use in diagnostic procedures.
** For optimizing ISH signal strength for low RNA expressors in the protease free workflow, please refer to the Optimization Note in Appendix D on page 50

Tissue pretreatment recommendations

Use these conditions as a starting point when tissues are prepared as described in Chapter 3. Depending on your tissue type, vary the amount of time for the ER2 and LS Protease III, or LS PretreatPro until positive RNA control signal is maximized with minimal/no negative RNA control signal (see Appendix A for a list of tissues).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Mild</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOND ER2</td>
<td>15 MIN @88C</td>
<td>15 MIN @95C</td>
</tr>
<tr>
<td>LS Protease III</td>
<td>15 MIN @40C</td>
<td>15 MIN @40C</td>
</tr>
<tr>
<td>OR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LS PretreatPro* (see Appendix D)</td>
<td>30 MIN @40C</td>
<td>30 MIN @40C</td>
</tr>
</tbody>
</table>

*Sample types, such as certain xenografts and cell pellets, might require shorter incubation time. For these tissue types, reduce the BOND ER2 incubation time. LS Protease III and LS PretreatPro incubation times can also be adjusted but is rarely needed. If you have a tissue type not listed, contact ACD Support at support.acd@bio-techne.com.

Pretreat fixed-frozen sections

Target Retrieval

Fixed-frozen samples must be gently de-crosslinked with a target retrieval step. The RNAscope 2.5 LS Assay uses the BOND Rx’s ER2 solution exclusively for this step.

Permeabilization

Only LS Protease III has been tested for use with fixed-frozen sections. Check with ACD Support for any updates.

Tissue pretreatment recommendations

Use these conditions as a starting point when tissues are prepared as described in Chapter 3. Depending on your tissue type, vary the amount of time for the ER2 and/or LS Protease III until positive control RNA signal is maximized with minimal or no negative RNA control signal (see Appendix E for details).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOND ER2</td>
<td>5 MIN @95C*</td>
</tr>
<tr>
<td>LS Protease III†</td>
<td>15 MIN @40C</td>
</tr>
</tbody>
</table>

* You might need to create this heat treatment protocol. Please refer to Appendix B for further instructions.
†Sample types, such as certain xenografts and cell pellets, might require shorter incubation time. For these tissue types, reduce the BOND ER2 incubation time. LS Protease III time can also be adjusted but is rarely needed.
Pretreat fresh-frozen sections

Target Retrieval

Fresh-frozen sections do not need target retrieval.

Permeabilization

Only LS Protease IV has been tested for use with fresh-frozen sections. Check with ACD Support for any updates.

Tissue pretreatment recommendations

Use these conditions as a starting point when tissues are prepared as described in Chapter 3. Depending on your tissue type, vary the amount of time for the Protease IV until positive RNA control signal is maximized with minimal/no negative RNA control signal (see Appendix E for details).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS Protease IV (ACD Part Number 322140)</td>
<td>30 MIN @Ambient*</td>
</tr>
</tbody>
</table>

* You might need to create this heat treatment protocol. Please refer to Appendix C for further instructions.
Chapter 5. Set Up Software
Version 5.2

Use the instructions in this chapter to set up the RNAscope 2.5 LS Assay using software version 5.2. To set up the assay using software version 6.0, proceed to Chapter 6.

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 or Cat. No. 310023) using the RNAscope 2.5 LS positive and negative control probes along with your samples in every run.

This workflow is a “workaround” in the existing 5.2 software to accommodate the RNAscope 2.5 LS Assay. It performs probe hybridization during the staining protocol to enable the use of a high probe volume required for optimal assay performance. In summary, a probe is created as an ancillary reagent and added to the staining protocol. A ‘Mock Probe’ and mock one-minute hybridization protocol are also created to fulfill BOND RX software requirements when performing any staining run. Follow the steps in this chapter to enable the workflow. Your ACD Field Application Specialist (FAS) can help you implement this procedure.

Workflow
Register the reagents

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, probe1 2.5) in the Name text box.

4. Enter P1 2.5 (for example) in the Abbreviated name text box.

5. Select Ancillary in the Type drop-down menu.

6. Enter ACD in the Supplier text box.

7. Check both the Preferred and Hazardous boxes (for all probe reagents).

8. Select Save.

Note: Standard Amp reagents are pre-loaded into the database for this chemistry and do not need to be registered.

Create a one-minute probe hybridization protocol (one time only)
Error! Reference source not found. (ONE TIME ONLY)
Register the reagents

9. Select the Reagent Setup icon at the top of the screen.

10. Select Add to enter reagent information.

11. Enter a reagent name (for example, probe1 2.5) in the Name text box.

12. Enter P1 2.5 (for example) in the Abbreviated name text box.
13. Select Ancillary in the Type drop-down menu.
14. Enter ACD in the Supplier text box.
15. Check both the Preferred and Hazardous boxes (for all probe reagents).
16. Select Save.

Note: Standard Amp reagents are pre-loaded into the database for this chemistry and do not need to be registered.

Create a one-minute probe hybridization protocol (one time only)

A mock probe hybridization protocol must be created as part of the version 5.2 software workaround for the RNAscope 2.5 LS assay. The following example copies the existing two-hour hybridization protocol and changes the incubation time to one minute and the temperature to ambient.

If an existing one-minute probe hybridization protocol already exists, skip to the next section.

8. In the Protocol setup screen, select Prestaining from the Protocol group menu and ISH Hybridization from the Protocol type menu.
9. Highlight the *ACD 2 hour Hybridization protocol. Select Copy.

10. When using 5.2 software ensure that you have the correct instrument tab highlighted before making the following edits.
11. Change the Name to ACD 1min Hybridization, the Abbreviated Name to ACD1min, and the Description to ACD 1min RNAscope hybridization.
12. Highlight the *No Reagent step.
13. Change the incubation time to 1 MIN and select Ambient as Temperature (°C).
14. Select Save.

Create a staining protocol

Due to the 5.2 software workaround for the RNAscope 2.5 LS assay, a unique staining protocol must be created for each probe.

1. In the Protocol setup screen, select Staining from the Protocol group menu.
2. Highlight the *ACD 2.5 Red Rev B protocol. Select Copy.
3. Change the protocol name for your first probe to ACD 2.5 Red P1 Rev B in the Name text box, 2.5RRBPI in the Abbreviated name text box, and ACD 2.5 Red Rev B protocol P1 in the Description text box OR use your own naming convention.

4. When editing the staining protocol, make sure to select the correct tab that matches your instrument type (BOND RX®m or BOND RX).
5. Next highlight the default probe reagent lines (Lines 1-3: *ACD 2.5 P1) and choose the appropriate Ancillary probe reagent created earlier from the Reagent drop-down list on the right.
6. Select **Preferred** in the bottom right corner of the window.
7. Select **Save**.
8. Click on **Next** to proceed. Ignore any pop-up warnings that may appear on the screen.

**Register the mock probe (one time only)**

Create a mock probe in the reagent set up.

1. Click on the **Reagent setup** icon.
2. Select Add.
3. Enter Mock Probe in the Name and Abbreviated name text boxes.
4. Select Probe RNA in the Type drop-down menu.
5. Enter ACD in the Supplier text box.
6. For Single/Double Stain: Select Single/Sequential DS.
7. Select a commonly used ACD staining protocol as the Default staining protocol. During slide set up, you can select other protocols.
8. Select *ACD HIER 15min with ER2 (95) or as the Default HIER protocol.
9. Select *ACD 15min Protease as the Default enzyme protocol.
10. Leave the Default denaturation protocol blank.
11. Select ACD 1 min Hybridization as the Default hybridization protocol.
12. Select Preferred, then select Save.
Set up a study

1. To build a study, select the **Slide setup** icon at the top of the screen.

2. Select **Add study** and enter a name in the Study ID field (keep the Dispense volume at 150 µl as shown).
3. For FFPE tissues, select *Bake and Dewax as the Preparation protocol. For alternative tissue preps, see Appendix E. for details).
4. Select OK.
5. Select Add slide to assign a protocol to each slide.

6. Enter the tissue type and probe name in the Comments field.
7. Keep **Single** as default from the Staining mode drop down menu.
8. Select **ISH** for Process and **Mock Probe (ACD)** from the Marker drop down menu.
9. In the **Protocols** tab, do the following:
   a. For each distinct probe, select a different protocol from the Staining drop down menu (for example, ACD 2.5 Red Rev B P1).
   b. For FFPE tissues, select the protocol **Bake and Dewax** from the Preparation drop down menu.
   c. For FFPE tissues, select **ACD HIER 15 min with ER2 (95)** as the HIER protocol.
   d. For FFPE tissues, select **ACD 15 min Protease** for Enzyme, or the appropriate enzyme protocol for your tissue (see Appendix C to edit the enzyme treatment protocol).
   e. Select **ACD 1 min Hybridization** for Hybridization.
   
   **Note:** HIER protocol time and temperature varies depending on the tissue type (see Appendix A).
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.

13. Proceed to Error! Reference source not found..
Chapter 6. Set Up Software Version 6.0 or higher

Use the instructions in this chapter to set up the RNAscope 2.5 LS Assay using software version 6.0. To set up the assay using software version 5.2, see Chapter 5.

**IMPORTANT!** BXD20 or higher is required to run the following setup on software version 6.0. Please contact your Leica FAS to upgrade to BXD20 before starting the assay.

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

This workflow uses the standard software setup for the Bond RX instruction control software. Probes are registered in the software as Probe RNA and a universal staining protocol is created and used for all staining runs of this type. Follow the steps in this chapter to enable the workflow. Your ACD Field Application Specialist (FAS) can help you implement this procedure.

**Workflow**

- **Register the probes**

- **Create a Universal RED staining protocol (ONE TIME ONLY)**
Register the probes

If you have software version 6.0, you can register the probes as Probe RNA. Contact your ACD Field Application Scientist (FAS) to assist you if needed.

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

2. Select **Add** to enter reagent information.
3. Enter Target Probe 1 (for example) in the Abbreviated name text box.
4. Select Probe RNA in the Type drop-down menu.
5. Enter ACD in the Supplier text box.
7. Select *ACD 2.5 Red Rev B or other common protocol as the Default Staining protocol.
8. Select *RNAiscope 2.5 LSx Target Retrieval (95) as the Default HIER protocol.
9. Select *RNAiscope 2.5 LSx Enzyme as the Default enzyme protocol.
10. Leave the Default denaturation protocol blank.
11. Select *RNAiscope 2.5 LSx Hybridization as the Default hybridization protocol.
12. Check both the Preferred and Hazardous boxes.
13. Select Save.

Create the universal staining protocol (one time only)

For software version 6.0 or higher, you can use the same staining protocol for all RNAiscope probes but first edit the *ACD 2.5 Red Rev B protocol.

1. In the Protocol setup screen, select Staining from the Protocol group menu.
2. Highlight the *ACD 2.5 Red Rev B protocol. Select Copy.
3. Change the protocol name to ACD 2.5 Red Rev B P1 (for example) in the Name text box, 25RRBP1 in the Abbreviated name text box, and ACD 2.5 Red Rev B protocol P1 - mock probe lines removed in the Description text box.
4. Next highlight the default probe reagent lines (Lines 1-3: *ACD 2.5 P1) and delete those steps.
5. Select Preferred in the top right corner of the window.
6. Select Save.
7. Click on Next to proceed. Ignore any pop-up warnings that may appear on the screen.

**Note:** Use this protocol to perform all RNAscope Red assays.

### Set up a study

1. To build a study, select the **Slide setup** icon at the top of the screen.

2. Select **Add study** and enter a name in the Study ID field (keep the Dispense volume at 150 µl as shown).
3. For FFPE tissues, select *Bake and Dewax* as the Preparation protocol. (For alternative tissue preps, see Appendix E).
4. Select OK.
5. Select Add slide to assign a protocol to each slide.
6. Enter the tissue type and probe name in the Comments field.
4. Keep **Single** as default from the Staining mode drop down menu.
5. Select **ISH** for Process and the appropriate Marker from the drop-down menu.
6. For RNAscope 2.5 LS assays, under the **Protocols** tab, do the following:
   a. Staining: Select the universal protocol created in the previous step.
   b. Preparation: For FFPE tissues, select **Bake and Dewax**. For alternative tissue preps, see Appendix E for details).
   c. HIER: For FFPE tissues, select **RNAscope 2.5 LSx Target Retrieval (95)** or **(88)**. See Appendix A. (For alternative tissue preparations, see Appendix E).
   d. Enzyme: For FFPE tissues, select **RNAscope 2.5 LSx Enzyme**. (For alternative tissue preparations, see Appendix E).
   e. Probe Application: Select **RNAscope 2.5 LSx Probe Application**.
   f. Denaturation: Select **,...**
   g. Hybridization: Select **RNAscope 2.5 LSx Hybridization**.
   h. Probe Removal: Select **RNAscope 2.5 LSx Probe Removal**.

**Note:** HIER protocol time and temperature varies depending on tissue type.

10. Select **Add slide**.
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.

13. Proceed to **Chapter 7. Run the RNAscope 2.5 LS Assay**.
Chapter 7. Run the RNAscope 2.5 LS Red Assay

Workflow

<table>
<thead>
<tr>
<th>Prepare the instrument reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td>↓</td>
</tr>
<tr>
<td>Start the run</td>
</tr>
<tr>
<td>↓</td>
</tr>
<tr>
<td>Complete the run</td>
</tr>
<tr>
<td>↓</td>
</tr>
<tr>
<td>Dry and mount the samples</td>
</tr>
</tbody>
</table>
Materials required

<table>
<thead>
<tr>
<th>Materials provided by Advanced Cell Diagnostics</th>
<th>Materials provided by Leica Biosystems</th>
<th>Materials provided by User</th>
</tr>
</thead>
<tbody>
<tr>
<td>• RNAscope 2.5 LS Target Probe</td>
<td>• Leica Biosystems’ BOND RX System</td>
<td>• Distilled water</td>
</tr>
<tr>
<td>• RNAscope 2.5 LS Positive Control Probe</td>
<td>• Stainer</td>
<td>• Drying oven</td>
</tr>
<tr>
<td>• RNAscope 2.5 LS Negative Control Probe</td>
<td>• Bulk Reagents</td>
<td>• Fume hood</td>
</tr>
<tr>
<td>• RNAscope 2.5 LS Hydrogen Peroxide</td>
<td>• BOND Wash Solution, 10X</td>
<td>• Tissue-Tek Staining Dish</td>
</tr>
<tr>
<td>• RNAscope 2.5 LS Protease III</td>
<td>• BOND Dewax Solution</td>
<td>• Tissue-Tek Clearing Agent</td>
</tr>
<tr>
<td>• RNAscope 2.5 LS PretreatPro</td>
<td>• BOND Epitope Retrieval Solution 1</td>
<td>• Dish, xylene-resistant (2)</td>
</tr>
<tr>
<td>• RNAscope 2.5 LS AMP 1</td>
<td>• BOND Epitope Retrieval Solution 2</td>
<td>• Tissue-Tek Vertical 24</td>
</tr>
<tr>
<td>• RNAscope 2.5 LS AMP 2</td>
<td></td>
<td>• Slide Rack</td>
</tr>
<tr>
<td>• RNAscope 2.5 LS AMP 3</td>
<td></td>
<td>• Drying oven</td>
</tr>
<tr>
<td>• RNAscope 2.5 LS AMP 4 – RED</td>
<td></td>
<td>• Ecomount or VectaMount</td>
</tr>
<tr>
<td>• RNAscope 2.5 LS AMP 5 – RED</td>
<td></td>
<td>• Cover Glass, 24 mm x 50</td>
</tr>
<tr>
<td>• RNAscope 2.5 LS AMP 6 – RED</td>
<td></td>
<td>mm</td>
</tr>
<tr>
<td>• RNAscope 2.5 LS AMP Pro</td>
<td>• BOND Polymer Refine Red Detection</td>
<td></td>
</tr>
<tr>
<td>• RNAscope 2.5 LS Rinse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• RNAscope 2.5 Bluing Reagent</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Prepare the instrument

1. Fill the large containers located in the bottom of the instrument with the Leica BOND RX bulk reagents. 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.

2. Use clean, dry covertiles for every run. Clean used covertiles with water, bleach, and ethanol. Air dry before reuse. See Leica documentation for details.

3. Before starting a run, empty bulk waste containers. Discard waste according to all local, state/provincial, and/or national regulations.

Prepare the instrument reagents

1. If using the mock probe workflow from Chapter 5, obtain one empty 30 mL BOND Open container and label it as **Mock Probe**.

2. Fill the **Mock Probe** container with Leica Biosystems 1X BOND Wash.

3. Carefully transfer all the RNAscope LS reagents into the empty 30 mL BOND Open containers.

4. Determine the volume of probe reagent needed for the run.

   **Note:** If using other open containers to deliver your probe, you must account for the dead volume required in each container. Suggested volumes can be found in the following table.
5. Using the Barcode Scanner, scan the front barcode on the 30 mL BOND Open container. A window will appear.
6. From the drop-down menu, select the corresponding name of the reagent as shown in the following table under **Container Name**:

**Note:** The table is a summary of the container names needed based on the different software workflows. If you are unsure, open the protocols you are using and confirm the reagents names the protocol calls for then register your containers to match.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Version 6.0/7.0 Container Names</th>
<th>Version 5.2 Container Names</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNAscope 2.5 LS Hydrogen Peroxide</td>
<td>*RNAscope 2.5 LSx Hydrogen Peroxide</td>
<td>*Open 0 Haz</td>
</tr>
<tr>
<td>RNAscope 2.5 LS Protease III or LS PretreatPro</td>
<td>*RNAscope 2.5 LSx Enzyme</td>
<td>*ACD Enzyme</td>
</tr>
<tr>
<td>RNAscope 2.5 LS AMP 1</td>
<td>*ACD Amp 1</td>
<td>*ACD Amp 1</td>
</tr>
<tr>
<td>RNAscope 2.5 LS AMP 2</td>
<td>*ACD Amp 2</td>
<td>*ACD Amp 2</td>
</tr>
<tr>
<td>RNAscope 2.5 LS AMP 3</td>
<td>*ACD Amp 3</td>
<td>*ACD Amp 3</td>
</tr>
<tr>
<td>RNAscope 2.5 LS AMP 4 – RED</td>
<td>*ACD Amp 4</td>
<td>*ACD Amp 4</td>
</tr>
<tr>
<td>RNAscope 2.5 LS AMP 5 – RED</td>
<td>*ACD Amp 5 Red</td>
<td>*ACD Amp 5 Red</td>
</tr>
<tr>
<td>RNAscope 2.5 LS AMP Pro</td>
<td>ACD Amp Pro</td>
<td>ACD Amp Pro</td>
</tr>
<tr>
<td>RNAscope 2.5 LS AMP 6 – RED</td>
<td>*ACD Amp 6 Red</td>
<td>*ACD Amp 6 Red</td>
</tr>
<tr>
<td>RNAscope 2.5 LS Rinse</td>
<td>*LS Rinse</td>
<td>*LS Rinse</td>
</tr>
<tr>
<td>Hematoxylin</td>
<td>N/A -- in BOND Polymer Refine Detection</td>
<td>N/A -- in BOND Polymer Refine Detection</td>
</tr>
<tr>
<td>RNAscope 2.5 LS Bluing Reagent†</td>
<td>*ACD Blue</td>
<td>*ACD Blue</td>
</tr>
<tr>
<td>RNAscope 2.5 LS Target Probe</td>
<td>Appropriate Probe RNA name</td>
<td>Appropriate Ancillary probe name</td>
</tr>
<tr>
<td>1X BOND Wash</td>
<td>N/A</td>
<td>Mock Probe</td>
</tr>
</tbody>
</table>

* Indicates reagent is hard coded in software by Leica Biosystems.
† Bluing is optional

**Note:** Leica BOND Polymer Refine Detection comes in a pre-filled Leica BOND RX container.

7. Enter the RNAscope 2.5 LS Reagent Kit lot number and the expiration date in their respective fields. Select **OK**.
IMPORTANT!  Do not introduce bubbles into the solutions by shaking the containers. To mix reagents, gently invert the containers several times. If bubbles are present, leave the containers out at room temperature until the bubbles dissipate.

Start the run

1. Attach the barcode labels to the slides and add the slides to the slide tray with the label sides facing up.

   **Note:** Add a coverticle on top of each slide. The rectangular-shaped neck of the coverticle should fit into the groove of the slide tray. Verify placement and seating of coverticle.

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.

IMPORTANT!  Before leaving the instrument unattended, ensure that the instrument is running successfully. In the event of a problem, please contact support.acd@bio-techne.com or your Field Application Scientist.

Complete the run

1. After the run is complete, press the button on the front of the instrument to unload the slides.

2. Place the slides onto the Tissue-Tek Slide Rack and move the rack into a staining dish containing distilled water.

3. Flush the slides with flowing tap water for a few minutes.

4. (Optional) Perform offline bluing of choice or use the RNAscope 2.5 LS Bluing Reagent included in the kit by pipetting a small amount onto each slide for **1–2 MIN** followed by rinsing.

Dry and mount the samples

1. Remove the slide rack from the staining dish and dry slides in a **60°C** dry oven for **30 MIN**.

   **IMPORTANT!**  The RED substrate is alcohol sensitive. Do not dehydrate the slides in alcohol.

2. Cool the slides for **5 MIN** at **RT**.

3. Briefly dip one slide into fresh pure xylene and immediately place 1–2 drops of EcoMount or Vectamount on the slide before the xylene dries.

   **IMPORTANT!**  Use EcoMount (Biocare Medical) or Vectamount (VectaLabs) mounting media.
4. Carefully place 24 mm x 50 mm coverslip over the tissue section. Avoid trapping air bubbles.
5. Repeat steps 2 and 3 for each slide.
6. Air dry slides for 5 MIN.
7. Proceed to Chapter 8. Evaluate the Results.
Chapter 8. Evaluate the Results

Examine tissue sections under a standard bright field microscope at 20–40X magnification:

- Assess tissue and cell morphology.
- Assess positive control signal strength. Positive control signal should be visible as punctate dots within the cell at 20–40X magnification.
- Assess negative control background. One dot to every 10 cells displaying background staining per 20X microscope field is acceptable.
- Evaluate target probe signal using the scoring guidelines in the next section.

Scoring guidelines

The RNAscope 2.5 LS Assay enables a semi-quantitative 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 RNAscope staining intensity is presented below for a gene with expression level varying between 1 to >10 copies per cell.

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

Categorize staining into five grades: 0, 1+, 2+, 3+, and 4+ according to the following table:

<table>
<thead>
<tr>
<th>Staining Score</th>
<th>Microscope Objective Scoring*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No staining or less than 1 dot for every 10 cells (40X magnification)</td>
</tr>
<tr>
<td>1</td>
<td>1–3 dots/cell (visible at 20–40X magnification)</td>
</tr>
<tr>
<td>2</td>
<td>4–9 dots/cell. No or very few dot clusters (visible at 20–40X magnification)</td>
</tr>
<tr>
<td>3</td>
<td>10–15 dots/cell and/or &lt; 10% positive cells have dot in clusters (visible at 20X magnification)</td>
</tr>
<tr>
<td>4</td>
<td>&gt;15 dots/cell and/or &gt;10% positive cells have dot in clusters (visible at 20X magnification)</td>
</tr>
</tbody>
</table>

* Discount cells with artificially high nuclear background staining.
Control example

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

Figure 2. RNAscope 2.5 Assay detection of PPBP mRNA in HeLa cells

Troubleshooting

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

- If you observe the presence of background staining, increase the Epitope Retrieval 2 (ER2) in increments of five minutes and/or increase the protease time in increments of ten minutes. (see Appendix B and C for instructions on editing protocols).
- Use the above process for over-fixed tissues.
- The RNAscope 2.5 LS RED and LS BROWN assays utilize Leica Biosystems’ BOND Polymer Refine Detection and BOND Polymer Refine Red Detection kits, respectively. Do not use 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.
- **Do not alter the staining protocol in any way except for the hematoxylin incubation time.** The parameters in the staining protocol have been optimized to run the RNAscope assay on the instrument.

For troubleshooting information, please contact technical support at support.acd@biotechne.com.
Appendix A. 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.

<table>
<thead>
<tr>
<th>Species</th>
<th>Tissue Type</th>
<th>Pathology</th>
<th>Pretreatment Condition</th>
<th>Species</th>
<th>Tissue Type</th>
<th>Pathology</th>
<th>Pretreatment Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse/Rat</td>
<td>Intestine</td>
<td>Normal</td>
<td>Standard</td>
<td>Human</td>
<td>Head</td>
<td>Cancer</td>
<td>Standard</td>
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<tr>
<td></td>
<td>Intestine</td>
<td>Tumor</td>
<td>Standard</td>
<td></td>
<td>Neck</td>
<td>Cancer</td>
<td>Standard</td>
</tr>
<tr>
<td></td>
<td>Embryo</td>
<td>Normal</td>
<td>Standard</td>
<td></td>
<td>Liver</td>
<td>Cancer</td>
<td>Standard</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>Normal</td>
<td>Standard</td>
<td></td>
<td>Liver</td>
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<td>Standard</td>
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<tr>
<td></td>
<td>Spleen</td>
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<td>Standard</td>
<td></td>
<td>Heart</td>
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<td></td>
<td>Eye/Retina</td>
<td>Normal</td>
<td>Extended</td>
<td></td>
<td>GI tract</td>
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<td></td>
<td>Liver</td>
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<td>Standard</td>
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<td>Kidney</td>
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<td>Lymphoma</td>
<td>Cancer</td>
<td>Standard</td>
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<td>Thymus</td>
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<td>Mild/Standard</td>
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<td>Melanoma</td>
<td>Tumor</td>
<td>Standard</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Nevus</td>
<td>Benign</td>
<td>Standard</td>
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<td>Placenta</td>
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<td>Nevus (TMA)</td>
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<td></td>
<td>Stomach (TMA)</td>
<td>Normal</td>
<td>Standard</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Stomach (TMA)</td>
<td>Tumor</td>
<td>Standard</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cell pellets</td>
<td>Fixed with 10% NBF</td>
<td>Mild</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HeLa cells</td>
<td>Fixed with 10% Formaldehyde (\text{PBS/ACD Control})</td>
<td>Mild</td>
</tr>
<tr>
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<td></td>
<td></td>
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<th>Pathology</th>
<th>Pretreatment Condition</th>
</tr>
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*Tissue Microarray
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<th>Pathology</th>
<th>Pretreatment Condition</th>
<th>Species</th>
<th>Tissue Type</th>
<th>Pathology</th>
<th>Pretreatment Condition</th>
</tr>
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<td>Mild</td>
<td></td>
<td>Lymph Node</td>
<td>Mild</td>
<td>Mild</td>
</tr>
<tr>
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<td>Normal</td>
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<td></td>
<td>Tonsil</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
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<td>Mild</td>
<td></td>
<td>Thymus</td>
<td>Mild</td>
<td>Mild</td>
</tr>
<tr>
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<td>Mild</td>
<td></td>
<td>Retina</td>
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<td>Mild</td>
</tr>
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<td></td>
<td>Prostate Gland</td>
<td>Mild</td>
<td>Mild</td>
</tr>
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<td>Mild</td>
<td>Mild</td>
</tr>
<tr>
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<td>Testis</td>
<td>Normal</td>
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<td>Mild/Standard</td>
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<td>Mild/Standard</td>
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</tr>
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<td>Normal</td>
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<tr>
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<td>Mild</td>
</tr>
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<td>Mild/Standard</td>
<td></td>
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<td>Normal</td>
<td>Standard/Mild</td>
</tr>
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<td>Mild</td>
<td>Mild</td>
</tr>
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<td></td>
<td>Tonsil</td>
<td>Normal</td>
<td>N.A.</td>
<td></td>
<td>Tonsil</td>
<td>N.A.</td>
<td>N.A.</td>
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<td>Prostate Gland</td>
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<td>Testis</td>
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<td>Standard</td>
<td></td>
<td>Ovary</td>
<td>Standard</td>
<td>Standard</td>
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<tr>
<td></td>
<td>Duodenum</td>
<td>Normal</td>
<td>Standard/Mild</td>
<td></td>
<td>Duodenum</td>
<td>Normal</td>
<td>Standard/Mild</td>
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For Research Use Only. Not for use in diagnostic procedures.
<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Standard</th>
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</thead>
<tbody>
<tr>
<td>Jejunum</td>
<td>Normal</td>
<td>Standard</td>
</tr>
<tr>
<td>Colon</td>
<td>Normal</td>
<td>Standard</td>
</tr>
<tr>
<td>Adrenal Gland</td>
<td>Normal</td>
<td>N.A</td>
</tr>
</tbody>
</table>
Appendix B. Edit the Epitope Retrieval Protocol

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

Create a prestaining protocol

1. Open the Leica BOND software and click on the Protocol setup icon as shown.
2. Select **Prestaining** under the Protocol group menu and **Heat pretreatment** under the Protocol type menu to access the heat pretreatment protocols.

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

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

4. Rename the protocol as **ACD HIER 15 min with ER2 (88)**.

5. Rename the Abbreviated name as **ER2-88**.

6. Rename the Description to **ACD RNAscope heat pretreatment 88**.

7. Highlight the third **BOND ER Solution 2** step (see above) and change temperature to **88°C**.
8. For RNAscope, ER 2 temperature varies between 95°C and 88°C depending on the tissue type used. Please see Appendix A for a list of tissues.
9. Select Save to create a protocol for ER2 pretreatment at 88°C.
10. If needed, repeat Steps 1–8 to create new heating protocols for different incubation times (for example, ACD 25minER2).
Appendix C. Edit the Protease Protocol

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

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

3. Rename the protocol to ACD 25min Protease.
4. Rename the Abbreviated name to 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).
3. **Select Save.**

4. 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. How to use LS PretreatPro**

**Prepare the instrument reagents**

In this workflow, two new reagents are used in place of traditional reagents.

<table>
<thead>
<tr>
<th>Traditional protease workflow</th>
<th>New protease free workflow</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS Protease III</td>
<td>LS PretreatPro</td>
</tr>
<tr>
<td>ACD Amp5 - Red</td>
<td>LS Amp Pro</td>
</tr>
</tbody>
</table>

1. Place RNAscope 2.5 LS PretreatPro into a BOND Open container and register it as *ACD Enzyme.*

**Note:** Visually identify the containers as LS PretreatPro to avoid unintended use.

2. Register RNAscope 2.5 LS AMP Pro as a new reagent (for example, ACD Amp Pro) in the BOND database. See Register the reagents in Chapter 5.

3. Place the ACD Amp Pro into a BOND Open container and register it as reagent created.

**Note:** Place only one *ACD Enzyme reagent on the instrument at a time.

4. When registering containers select the corresponding name of the reagent from the drop-down menu as showing in the following table under **Container Name**:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Container Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNAscope 2.5 LS Hydrogen Peroxide</td>
<td>*Open 0 Haz</td>
</tr>
<tr>
<td>RNAscope 2.5 LS PretreatPro</td>
<td>*ACD Enzyme</td>
</tr>
<tr>
<td>RNAscope 2.5 LS AMP 1</td>
<td>*ACD Amp 1</td>
</tr>
<tr>
<td>RNAscope 2.5 LS AMP 2</td>
<td>*ACD Amp 2</td>
</tr>
<tr>
<td>RNAscope 2.5 LS AMP 3</td>
<td>*ACD Amp 3</td>
</tr>
<tr>
<td>RNAscope 2.5 LS AMP 4 - RED</td>
<td>*ACD Amp 4</td>
</tr>
<tr>
<td>RNAscope 2.5 LS AMP Pro</td>
<td>ACD Amp Pro</td>
</tr>
<tr>
<td>RNAscope 2.5 LS AMP 6 – RED</td>
<td>*ACD Amp 6 Red</td>
</tr>
<tr>
<td>RNAscope 2.5 LS Rinse</td>
<td>*LS Rinse</td>
</tr>
<tr>
<td>RNAscope 2.5 LS Bluing Reagent</td>
<td>*ACD Blue</td>
</tr>
<tr>
<td>RNAscope 2.5 LS Target Probe</td>
<td>Variable (probe 1 2.5)</td>
</tr>
<tr>
<td>1X BOND Wash</td>
<td>Mock Probe</td>
</tr>
</tbody>
</table>

* Indicates reagent is hard coded in software by Leica Biosystems.
**Note:** When paired with correlated Enzyme treatment protocol, container names *Open 0 Haz and *RNAscope 2.5 LSx Hydrogen Peroxide are equivalent and *ACD Enzyme and *RNAscope 2.5 LSx Enzyme are equivalent.

5. Enter the RNAscope 2.5 LS Reagent Kit lot number and the expiration date in their respective fields. Select OK.

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

---

**Create the protocol changes needed**

1. Filter **Protocol Group** by **Prestaining**, and filter for **Protocol type** by **Enzyme Pretreatment**. Find existing protocol *ACD 15min Protease* that includes the two reagents *ACD Enzyme and *Open 0 Haz.

2. Copy and create a new protocol:
   a. Change the *ACD Enzyme incubation time to 15 MIN per step for two steps and the temperature to 40°C.
   b. Keep *Open 0 Haz incubation time at 10 MIN and temperature at Ambient. Save the protocol with a new name such as ACD 15min PretreatPro.

3. Refer to **Appendix C** for detailed instructions on editing an Enzyme treatment protocol.

4. Filter **Protocol Group** by **Staining**.

5. Copy the standard LS 2.5 Red staining protocol used and replace the Amp 5 reagents steps with the newly created ACD Amp Pro reagent using the same incubations conditions.

6. Run all the other parts of the assay as usual and change only these two reagents.

7. The final Slide setup for an FFPE sample should look like the following considering proper HIER condition choice.
   a. Staining: Choose an edited staining protocol with ACD Amp Pro incorporation depending on your 5.2 or 6.0 workflow.
   b. Preparation: Select *Bake and Dewax.
   c. HIER: Choose *ACD HIER 15 min with ER2 (95).
   d. Enzyme: Choose the protocol created in step 2.
   e. Probe Application: Select *RNAscope 2.5 LSx Probe Application.
   f. Denaturation: Select *....
   g. Hybridization: Select ACD 1min Hybridization or *RNAscope 2.5 LSx Hybridization depending on your 5.2 or 6.0 workflow.
   h. Probe Removal: Select *RNAscope 2.5 LSx Probe Removal.
   i. Select Add slide.

8. After adding all the slides to the study, select **Close** to return to the Slide setup screen.

9. Proceed to Error! Reference source not found..

**Optimization Note:** For all protease-free workflows using LS PretreatPro and AMP Pro involving a low RNA expressor and/or a dense tissue like liver or spleen, we recommend boosting the ISH signal by increasing the antigen retrieval strength [duration, temperature, or volume].
Appendix E. Slide Setup for Additional Tissue Types

Alternatively prepared samples can be stained on the BOND RX using the following slide setup parameters.

Note: Choose appropriate staining and hybridization related protocols depending on whether you are using the mock probe workflow (Chapter 5) or the standard probe workflow (Chapter 6).

Fixed-frozen tissues
As described in Chapter 4, these tissues need a gentle target retrieval step.

1. In Slide setup, select the following:
   a. Staining: Choose the appropriate protocol for the chemistry and workflow you are using.
   b. Preparation: Select *----.
   c. HIER: Choose *ACD HIER 5 min with ER2 (95). See Appendix B to create this protocol.
   d. Enzyme: Select the appropriate protocol for the chemistry and workflow you are using; *ACD 15min Protease or RNAscope LSx Enzyme.
   e. Probe Application: Select *RNAscope 2.5 LSx Probe Application.
   f. Denaturation: Choose *....
   g. Hybridization: Choose the appropriate protocol for the chemistry and workflow you are using; ACD 1 min Hybridization or *RNAscope 2.5 LSx Hybridization.
   h. Probe Removal: Select *RNAscope 2.5 LSx Probe Removal but start with 15 minutes as that works for most tissues.

Note: When the run is complete, the BOND RX rinses the slides every 10 minutes which can impact the counterstain. Set up the instrument as late in the day as possible. Rinsing does not affect the RNAscope signal and counterstaining can be repeated offline in the morning if needed.

Fresh-frozen tissues
As described in Chapter 4, these tissues do NOT need a target retrieval. Instead, permeabilize the tissue at ambient temperature with a stronger protease; RNAscope LS Protease IV Cat. No. 322140

1. In Slide setup, please skip the following steps: 1) Bake or Bake and Dewax 2) Heat retrieval. Choose the following instead:
   a. Staining: Select the appropriate protocol for the chemistry and workflow you are using.
b. Preparation: Choose *----.
c. HIER: Choose *----.
d. Enzyme: Select **ACD 30min RT with LS Protease IV** (see **Appendix C. Edit the Protease Protocol** to edit the protease protocol).
e. Probe Application: Select **RNAscope 2.5 LSx Probe Application**.
f. Denaturation: Select *....
g. Hybridization: Choose the appropriate protocol for the chemistry and workflow you are using: **ACD 1 min Hybridization** or **RNAscope 2.5 LSx Hybridization**.
h. Probe Removal: Select **RNAscope 2.5 LSx Probe Removal**.

**Note:** Start your run immediately after setting it up. Do not use a delayed start. This causes poor protease spreadability and negatively impacts results.
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) before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see [http://www.acdbio.com/technical-support/user-manuals](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](http://www.cdc.gov/biosafety)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR §1910.1030), found at: [www.access.gpo.gov/nara/cfr/waisidx_01/%2029cfr1910a_01.html](http://www.access.gpo.gov/nara/cfr/waisidx_01/%2029cfr1910a_01.html)
• 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:


• Information about the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) can be found at: http://echa.europa.eu/regulations/reach
Documentation and Support

Obtaining SDSs
Safety Data Sheets (SDSs) are available at: http://www.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: http://www.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
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: order.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 at http://www.acdbio.com/store/terms. If you have any questions, please contact Advanced Cell Diagnostics at http://www.acdbio.com/about/contact.