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User Manual

ViewRNA™ eZ Assay

For Leica BOND RX Instrument

P/N 15891 Rev. C 140910

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When describing a procedure for publication using this product, please refer to it as the ViewRNA eZ Assay.

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Introduction

About This Manual

This manual provides basic instructions for automating the *in situ* detection of RNA in formalin-fixed paraffin-embedded (FFPE) samples using the ViewRNA™ eZ Assay and the Leica BOND RX instrument. It also covers guidelines on sample preparation, pretreatment optimization, and assay protocol setup.

Please refer to the Leica BOND RX User Manual (Rev. A01 issued August 2011) for detailed instructions on operating the Leica BOND RX instrument and using the Leica BOND RX software.

Assay Overview

In situ hybridization (ISH) techniques are often used to visualize DNA or localize RNAs within cells and tissues. However, these assays are usually cumbersome as well as time-consuming, and when performed manually, are not amenable to high-throughput processing and are prone to inconsistencies due to operational errors.

The ViewRNA eZ Assay—an ISH assay based on highly specific third generation branched DNA signal amplification technology and automated on the Leica BOND RX instrument—provides improved slide-to-slide and run-to-run reproducibility while offering higher throughput capability with the convenience of walk away automation.

General Assay Workflow

Figure 1.1 illustrates the assay workflow and Table 1.1 explains the assay steps.

Figure 1.1 ViewRNA eZ Assay Workflow

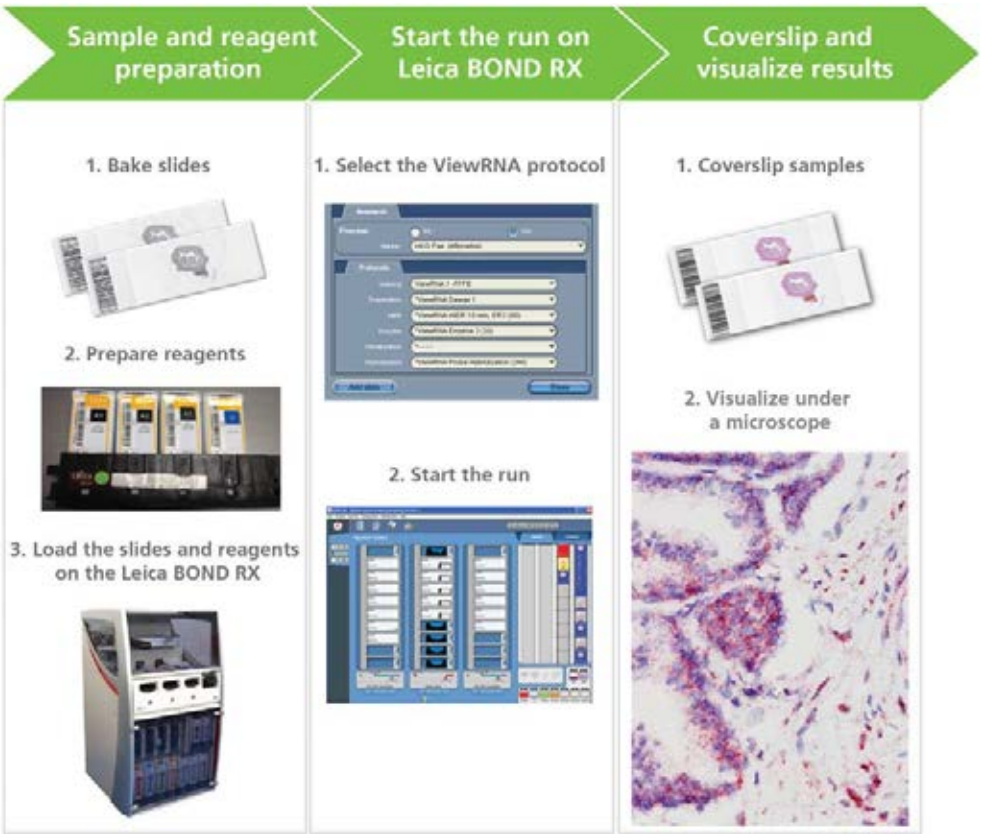


Table 1.1 ViewRNA eZ Assay Workflow

Assay Step	Typical Schedule	Description
Prepare reagents and samples.	Start at 1:00 PM Day 1	▪ Bake FFPE tissue sections at 60 °C to increase adhesion to glass slide. ▪ Pre-warm. ViewRNA eZ Detection reagents. ▪ Check Leica BOND RX bulk reagents.
Start overnight run on BOND RX.	Start at 4:00 PM Day 1	▪ Prepare freshly made probes, proteinase K, and AMP 4. ▪ Create or select saved user-specific ViewRNA eZ protocol. ▪ Insert ViewRNA eZ Detection reagents. ▪ Select delay start time and note when the run will end. ▪ Start run.
End of run on BOND RX.	8:00 AM Day 2	▪ Run finished. ▪ Remove samples within 30 minutes of run end. ▪ Rinse slides with water and air dry to 30 minutes at room temperature.
Coverslip tissue sections.	9:00 AM Day 2	Coverslip slides using the recommended organic or aqueous mounting solution.
View samples.	10:00 AM Day 2	View and analyze samples at 10 – 40x on a standard pathology grade microscope.

Table 1.2 Performance Highlights

Specification	Description
Sample Types	▪ Formalin-fixed paraffin-embedded (FFPE) sections. ▪ 19 x 46 mm assay area on a standard 25 x 75 mm glass slide. ▫ FFPE tissue thickness: 5 ± 1 µM ▫ FFPE tissue microarray (TMA): > 1 mm diameter and 5 ± 1 µM thickness
Sensitivity	Single RNA molecule per dot
RNA Stain	Red dot
Nuclear Stain	Hematoxylin
Instruments	▪ Leica BOND RX instrument ▪ Bright-field microscope or scanner.

Safety

General Precautions

ViewRNA eZ Detection Kit is for research use only. Some components required for this assay may pose significant health risks. Follow prudent laboratory practices when handling and disposing of carcinogens and toxins. Refer to the manufacturer's Material Safety Data Sheet for additional information.

Wear appropriate personal protective equipment when performing this assay. At a minimum, wear safety glasses and chemical resistant gloves. Perform all procedural steps in a well-ventilated area at room temperature unless otherwise noted. Discard all reagents in accordance with local, state, and federal laws.

CAUTION:

- **Formaldehyde is a poison and an irritant. Avoid contact with skin and mucous membranes. Use in a fume hood.**
- **ViewRNA eZ Probe Diluent and Amp 1– Amp 3 contain formamide, a teratogen, irritant, and possible carcinogen. Avoid contact with mucous membranes.**
- **ViewRNA eZ DAPI is a possible mutagen. Avoid contact with skin and mucous membranes.**

Operating Leica BOND RX

Refer to the *Leica BOND RX User Manual* for information about chemical and mechanical hazards, electrical hazards, and additional cautionary information.

Reagents, Consumables, and Equipment Required

Affymetrix Reagents Required
Leica Biosystems Reagents and Consumables Required on page 6
Additional Reagents and Consumables Required on page 7
Equipment Required on page 7

The ViewRNA™ eZ Assay is intended for use on the Leica BOND RX instrument ONLY. The assay protocol has been optimized and validated using the reagents, consumables, and equipment listed in this chapter. For optimal results, we strongly recommend that you adhere to the described protocol without any deviations, and when specified, do not substitute materials or suppliers.

Affymetrix Reagents Required

The ViewRNA eZ Detection Kit–1-Plex is compatible with TYPE 1 Probe Sets (sold separately) and is configured for processing 90 slides in five experimental runs. If the kit is used for more than five experimental runs, its capacity will be less than 90 slides. Table 3.1 lists the kit components and their recommended storage conditions.

Refer to the Package Insert for quantities of individual components supplied. The kit is shipped in one box and has a shelf life of six months from the date of delivery when stored as recommended.

Table 3.1 Affymetrix Reagents Required

Item	Storage	Part Number
ViewRNA eZ Detection Kit–1-Plex (90 slides)		QVR0001
▫ Amp 1	2 – 8 °C	
▫ Amp 2	2 – 8 °C	
▫ Amp 3	2 – 8 °C	
▫ Amp 4, 500X	2 – 8 °C	
▫ Amp 4 Diluent	2 – 8 °C	
▫ Red 1	2 – 8 °C	
▫ Red 2	2 – 8 °C	
▫ Rinse	2 – 8 °C	
▫ Probe Diluent	2 – 8 °C	
▫ Color-coded Labels		
ViewRNA TYPE 1 Probe	–20 °C	Various*
ViewRNA eZ Probes	–20 °C	Various*
ViewRNA eZ DAPI (optional for fluorescence detection only)	2 – 8 °C	QVR0502
ViewRNA eZ Control Kit (optional)	See product insert.	QVR0503
ViewRNA eZ Check (optional)	–20 °C	Various*

**Visit our website at www.affymetrix.com to view available probes or to order probes custom-made to your target.*

Leica Biosystems Reagents and Consumables Required

Table 3.2 Leica Biosystems Reagents and Consumables Required

Item	Storage	Part Number
BOND Dewax Solution	2 – 8 °C	AR9222
BOND Epitope Retrieval Solution 1 (ER1)	2 – 8 °C	AR9961
BOND Epitope Retrieval Solution 2 (ER2)	2 – 8 °C	AR9640
BOND Enzyme Pretreatment Kit	2 – 8 °C	AR9551
BOND Wash Solution 10X Concentrate	2 – 8 °C	AR9590
Novocastra Hematoxylin (two bottles)	2 – 8 °C	RE7107-CE
BOND Aspirating Probe Cleaning Kit	2 – 8 °C	CS9100
BOND Universal Covertiles	RT	S21.2001
BOND Slide Trays	RT	S21.0304
BOND Reagent Trays	RT	S21.1003
BOND Open Containers – 30 ml	RT	OPT309700
BOND Mixing Stations	RT	S21.1971
BOND Research Detection 2 ▫ Six standard 30 mL BOND Open Containers in reagent tray ▫ Barcode	RT	DS9777
BOND Slide Labels and Printer Ribbon	RT	S21.4564
White X-tra® Slides Case, 1mm	RT	3800200 (U.S., Canada, and Asia Pacific) 3800210 (Europe)

Additional Reagents and Consumables Required

Table 3.3 Additional Reagent and Consumables Required

Item	Supplier	Part Number
10% Neutral Buffered Formalin (NBF)	Fisher Scientific	SF98-4
100% Ethanol	VWR	89125-188
Xylene	Major Laboratory Supplier	
Mounting Media		
▫ HistoMount Mounting Solution	Life Technologies	00-8030
▫ Fluoromount/Plus™	Diagnostic BioSystems	K048
▫ ADVANTAGE Mounting Media	Innovex Biosciences	NB300
Cover Glass, 24 x 55 mm	Affymetrix	QVC05001
	VWR	48382-138
Double-Distilled Water (dd$H_2$0)	Major Laboratory Supplier	

Equipment Required

Table 3.4 Equipment Required

Equipment	Supplier	Part Number
Leica BOND RX Instrument	Leica Biosystems	
▫ Processing Module(s)		21.2701
▫ BOND RX Controller with BDZ 6.0 Software		S21.4574
▫ Handheld ID Scanner		
▫ BOND Sys Cont Add Kit–US		21.1997.F
▫ BOND Sys Cont Add Kit–AUS		21.1995.G
▫ BOND Sys Cont Add Kit–UK		21.1996.G
▫ BOND Sys Cont Add Kit–EUR		21.1998.G
▫ Slide Labeler		S21.2012
Fume Hood	Major Laboratory Supplier	
Dry incubator or oven capable of maintaining 60 °C (for baking slides)	Affymetrix or equivalent	QS0704 (120V) QS0714 (220V)
Water Bath, capable of maintaining 40 °C (for pre-warming reagents)	Major Laboratory Supplier	
Microscope and Imaging Equipment	See Appendix D, *Microscopy and Imaging Equipment Guidelines* on page 51.	

Best Practices

Sample Preparation
Experiment Design on page 10
Heat Treatment and Proteinase Digestion Optimization on page 10
Pretreatment Lookup Table on page 10
Leica BOND RX Cleaning and Maintenance on page 12

Sample Preparation

The following are critical guidelines for preparation of FFPE tissue blocks, FFPE tissue slides, and TMA slides for use with the ViewRNA eZ Assay. Samples prepared outside of these guidelines may not produce optimal results.

FFPE Tissue Block Preparation

- Immediately place freshly dissected tissues in ≥ 20 volumes of fresh 10% Neutral Buffered Formalin (NBF) or 4% paraformaldehyde (PFA) for 16-24 hours at room temperature. Trim larger specimens to ≤ 3 mm thickness to ensure faster diffusion of the fixative into the tissue.

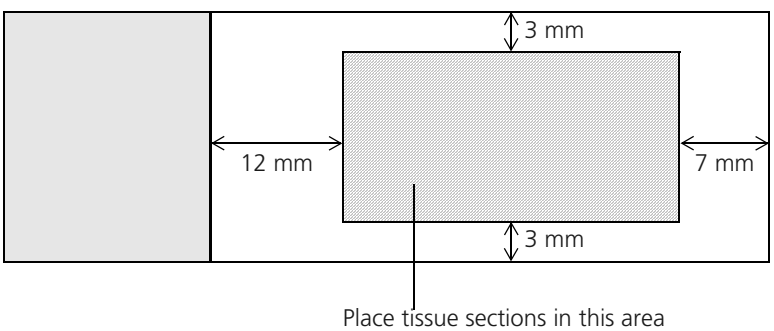
NOTE: Fix dissected tissue immediately to prevent RNA degradation. If this is not possible, it is critical that the tissue be placed on dry ice or in liquid nitrogen until fixation.

- Rinse, dehydrate, and embed in paraffin block.
- Store FFPE tissue blocks at room temperature.

FFPE Tissue Slide Preparation

- Section FFPE tissue to a thickness of 5 ± 1 µm.
- If working with TMAs, core size should be > 1.0 mm diameter.
- Position tissue sections within the area of the slide shown in Figure 4.1 to ensure even heating during hybridization and proper reagent coverage under the Covertile during processing on the Leica BOND RX.

Figure 4.1 Correct Tissue Section Placement on Glass Slide



- Mount sections on the recommended positively-charged glass slides: Leica White X-tra® Slides, P/N 3800200 (U.S., Canada, and Asia Pacific regions) or P/N 3800210 (Europe).

- Air dry freshly mounted sections at room temperature overnight or at 37 °C for five hours.
- Bake slides at 60 °C for one hour to immobilize tissue sections.
- Short-term storage – Store sections in a slide box at room temperature for up to two weeks.
- Long term storage – Store sections in a slide box at -20 °C for up to one year.
- Slides can be shipped at the same temperature as their storage temperature.

Experiment Design

Assay Controls

In addition to using biological controls during assay implementation, we recommend running positive and negative assay control slides , based on your sample type, in every ViewRNA eZ Assay (Table 4.1). This will allow you to qualify and interpret your results with greater confidence.

Table 4.1 Recommended Assay Controls

Assay Control	Uses	Recommended Action
Negative Control	▪ Assess assay background (typically, less than 1 dot per 3 cells).	Choose one of the following negative control options: ▪ Run bacterial gene dapB. ▪ Run no probe. ▪ Run probe set known NOT to be present in your tissue samples.
Positive Control 1	▪ Check RNA integrity. ▪ Check for proper unmasking within a run. ▪ Optimize tissue pre-treatment, particularly when expression of the gene of interest is not known.	Run ViewRNA eZ Check. These products, available separately from Affymetrix, provide a pooled panel of probes for housekeeping genes (encoding GAPDH, cyclophilin B, and beta-actin) and are available for human, mouse or rat. Follow the instructions in the package insert.
Positive Control 2	▪ Check Leica BOND RX performance for the ViewRNA eZ Assay. ▪ Check operator proficiency at running the ViewRNA eZ Assay on Leica BOND RX. ▪ Check ViewRNA eZ Detection Kit during a run.	Run ViewRNA eZ Control Kit. This product, available separately from Affymetrix, provides certified FFPE rat kidney tissue sections on microscope slides and ViewRNA TYPE 1 probes for rat Spp1, rat Arbp, and *B subtilis* dapB. The package insert includes instructions for use and example expected results.

Heat Treatment and Proteinase Digestion Optimization

The pretreatment of tissue sections is critical for the success of all *in situ* assays. For the ViewRNA eZ Assay, pretreatment consists of heat treatment and proteinase digestion. These pretreatment steps help unmask the RNA targets, allowing for better probe accessibility and thereby increasing assay signal. However, excessive pretreatment can have a negative effect on tissue morphology. Thus, we recommend using either the tissue- or time-preserving approach below, to optimize the pretreatment conditions on the Leica BOND RX when:

- Changing from the manual ViewRNA ISH Tissue Assay to the automated ViewRNA eZ Assay.
- Performing the ViewRNA eZ Assay with a new tissue type.

Once optimal pretreatment conditions are determined for a particular tissue, they can generally be used for most targets within that tissue. If the transcript is very rare or expressed at an extremely low level, the optimal pretreatment condition may need to be one that favors signal over morphology.

Pretreatment Lookup Table

Please visit our website and refer to the View RNA eZ Assay Pretreatment Lookup Table for an updated list of heat treatment and proteinase conditions. These conditions are optimal for several tissue types prepared according to the recommended guidelines in this manual using 10% NBF.

Time-Preserving Pretreatment Optimization Procedure

Use this optimization procedure if tissue sample is abundant and time is a limiting factor.

1. Obtain 12 FFPE tissue sections. See *Sample Preparation* on page 9 for guidelines.
2. Process slides using the ViewRNA eZ Assay on the Leica BOND RX with the Heat and Proteinase Treatments shown in Table 4.2. See Appendix C on page 49 for instructions on creating a custom Enzyme protocol.

   For each condition, run both a negative and a positive control. See Chapter 5, *ViewRNA eZ Assay Protocol* on page 13 for processing instructions.
3. Evaluate results and determine the pretreatment conditions that yield optimal assay signal and tissue morphology (as assessed by good hematoxylin staining) with minimal background or artifacts (see Table 5.5 on page 32).

Table 4.2 Recommended Pretreatment Optimization Grid for Time-Preserving Approach

Heat Treatment (10 minutes)	**Proteinase Treatment (20 minutes)**		
	ViewRNA Enzyme 1 (1:500 dilution recommended)	**ViewRNA Enzyme 2 (1:1000 dilution recommended)**	**ViewRNA Enzyme 3 (1:2000 dilution recommended)**
ViewRNA HIER[1] at 95 °C with ER1[2]	Slide 1 – No Probe	Slide 3 – No Probe	Slide 5 – No Probe
	Slide 2 – HKG Mix[4]	Slide 4 – HKG Mix	Slide 6 – HKG Mix
ViewRNA HIER at 90 °C with ER2[3]	Slide 7 – No Probe	Slide 9 – No Probe	Slide 11 – No Probe
	Slide 8 – HKG Mix	Slide 10 – HKG Mix	Slide 12 – HKG Mix

[1]HIER – Heat-induced epitope retrieval
[2]ER1 – BOND Epitope Retrieval Solution 1
[3]ER2 – BOND Epitope Retrieval Solution 2
[4]HKG Mix – ViewRNA eZ Check for human, mouse, or rat samples, pooled housekeeping gene probes for other species.

Tissue-Preserving Pretreatment Optimization Procedure

Use this optimization procedure if tissue sample is a limiting factor.

1. Obtain four FFPE tissue sections. See *Sample Preparation* on page 9 for guidelines.
2. Process slides using the ViewRNA eZ Assay on the Leica BOND RX with the Heat and Proteinase Treatments shown in Table 4.3. See Appendix C on page 49 for instructions on creating a custom Enzyme protocol.

   For each condition, run both a negative and a positive control. See Chapter 5, *ViewRNA eZ Assay Protocol* on page 13 for processing instructions.
3. Evaluate results and determine the pretreatment conditions that yield optimal assay signal and tissue morphology (as assessed by good hematoxylin staining) with minimal background or artifacts.

Table 4.3 Recommended Pretreatment Optimization Grid for Tissue-Preserving Approach

Heat Treatment (10 minutes)	Proteinase Treatment (10 minutes, 1:1000 dilution recommended)
ViewRNA HIER[1] at 95 °C with ER1[2]	Slide 1 – No Probe
	Slide 2 – HKG Mix[4]
ViewRNA HIER at 90 °C with ER2[3]	Slide 3 – No Probe
	Slide 4 – HKG Mix

[1]HIER – Heat-induced epitope retrieval
[2]ER1 – BOND Epitope Retrieval Solution 1
[3]ER2 – BOND Epitope Retrieval Solution 2
[4]HKG Mix – ViewRNA eZ Check for human, mouse, or rat samples, pooled housekeeping gene probes for other species.

NOTE: See **Appendix A on page 41** for additional optimization strategies.

Leica BOND RX Cleaning and Maintenance

Perform cleaning and maintenance tasks, including refilling and emptying bulk containers, as instructed in *11.1 Cleaning and Maintenance Schedule* in the *Leica BOND RX User Manual*.

When performing ViewRNA eZ Assay on Leica BOND RX:

- Clean the aspirating probe using the BOND Aspirating Probe Cleaning System after processing 100 slides. See *11.6.1 Cleaning the Aspirating Probe* in the *Leica BOND RX User Manual* for instructions.
- Replace the aspirating probe every 1000 slides. See *11.6.2 Replacing the Aspirating Probe* in the *Leica BOND RX User Manual* for instructions.
- Replace the mixing station every six months. See the *11.7 Wash Block and Mixing Station* in the *Leica BOND RX User Manual* for instructions.

ViewRNA eZ Assay Protocol

This chapter provides instructions for automating the ViewRNA™ eZ Assay on the Leica BOND RX. The workflow steps include:

Step 1: Getting Started

Step 2: Create a Study and Add Slides

Step 3: Prepare and Load Reagents and BOND Research Detection 2 System on page 21

Step 4: Label and Process Slides on Leica BOND RX on page 26

Step 5: Mount and View Slides on page 30

Step 1: Getting Started

1. Bake slides at 60 °C for one hour.
2. Pre-warm Probe Diluent , Amp 1, Amp 2, Amp 3, and Amp 4 Diluent from the ViewRNA eZ Detection Kit to 40 °C.
3. Confirm Leica BOND RX cleaning and maintenance tasks have been performed, including refilling and emptying bulk containers.
4. Turn on the Leica BOND RX processing module (instrument), log onto the computer, and initialize the Leica BOND RX software.
5. Ensure that the Slider Labeler has labels.
6. Ensure that the new ViewRNA eZ Detection Kit has been configured and registered into inventory before proceeding to Step 2. See Appendix B, *Registering a ViewRNA eZ Detection Kit on page 43* for instructions.

Step 2: Create a Study and Add Slides

Creating a Study

See *6.3 Working With Studies* in the *Leica BOND RX User Manual* for additional information about creating and managing studies.

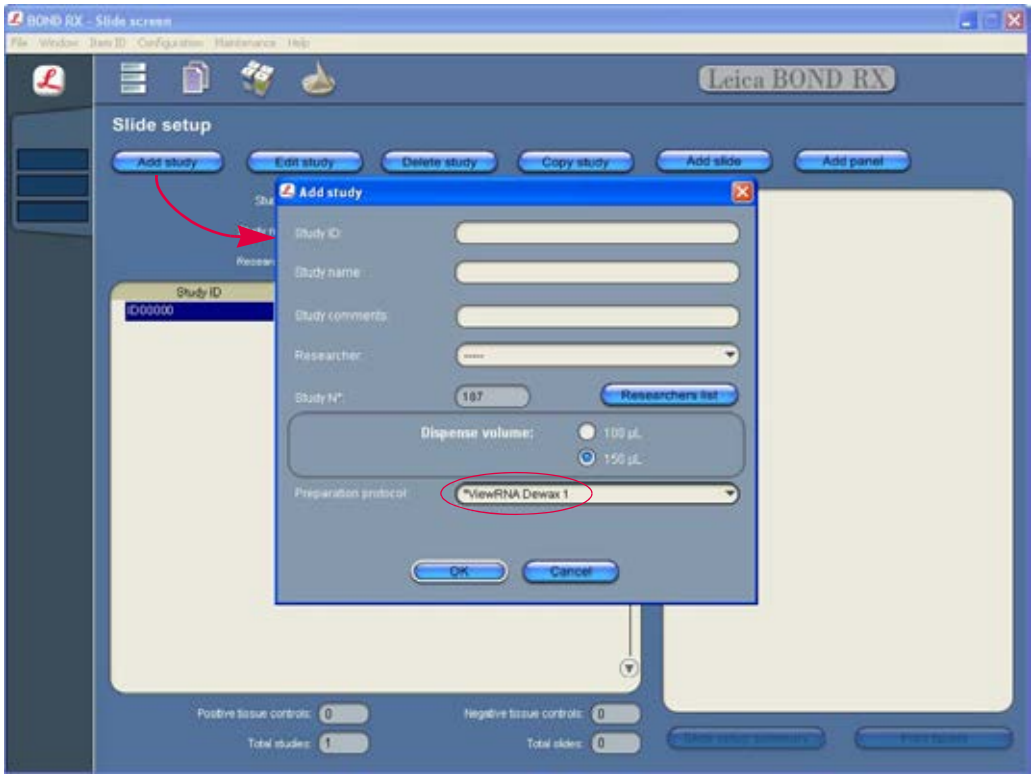
1. Start the Leica BOND RX software.
2. Click the  icon to open the "Slide setup" window (Figure 5.1).
3. Click **Add study** and enter information relevant to the experiment (Figure 5.1):
   - Study ID – required.
   - Study name – optional.
   - Study comments – optional.
   - Select a researcher name – optional.

To add a new name to the Researcher list:

A. Click **Researchers list** and select **New**.

B. Enter the new researcher *Name* in the "Edit Researcher" window that appears. Click **Save** and **OK**.

Figure 5.1 Add a Study



4. Verify that the **150 µL** dispense volume is selected.
5. For preparation protocol, confirm that ***ViewRNA Dewax 1** is selected.
6. Click **OK** to add the new study to the "Slide setup" window.

Adding Slides to a Study

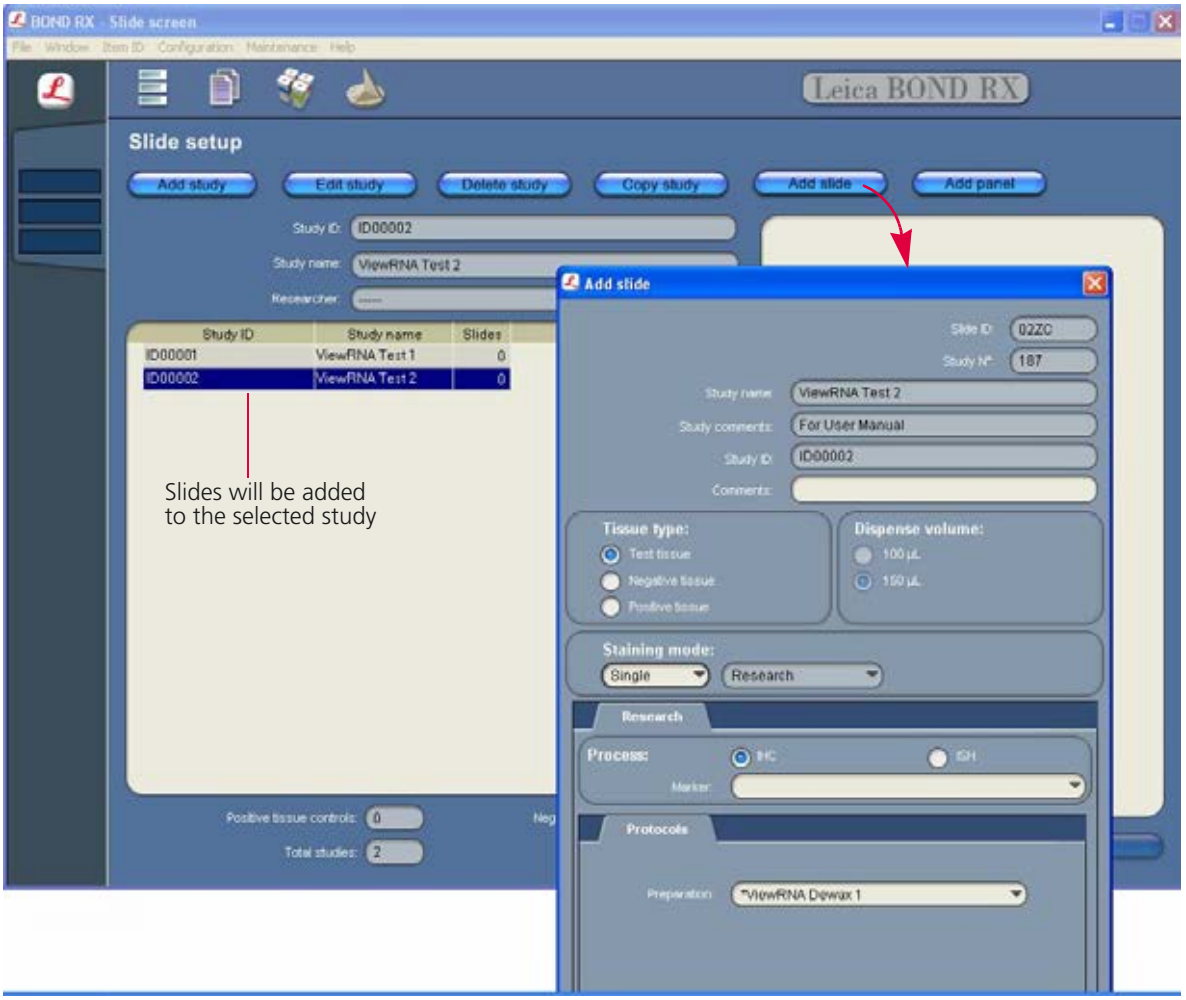
1. Confirm that the correct study ID is highlighted in the "Slide setup" window (Figure 5.2).

   If the study ID was just added, it should be at the bottom of the study ID list and highlighted by default.
2. Click **Add slide**.

   The "Add slide" dialog box appears (Figure 5.2).
3. Select a tissue type.
   - Test tissue – Test samples.
   - Negative tissue – Negative control samples such as a:
     - No probe control.
     - Probe designed against a target not present in the sample (for example, bacterial gene dapB) or the sense strand of the target.
   - Positive tissue – Positive control sample known to express the target.
4. Enter any additional information about the sample on this slide under *Comments*.
5. Confirm that **150 µL** dispense volume is selected.

   If the dispense volume is incorrect, close the "Add slide" dialog box. Choose the study name from the Study ID list and click **Edit Study**. Select the **150 µL dispense** volume in the dialog box that appears. Restart at Step 2.

Figure 5.2 Slide Setup Window



6. For *Staining mode*, select **Single** from the drop-down list (Figure 5.4).
7. For *Process*, choose **ISH** and select a **Probe (Affymetrix)** to be applied on this slide from the Marker drop-down list (for example, HKG Pan shown in Figure 5.4).

To add a custom probe name to the Marker List:

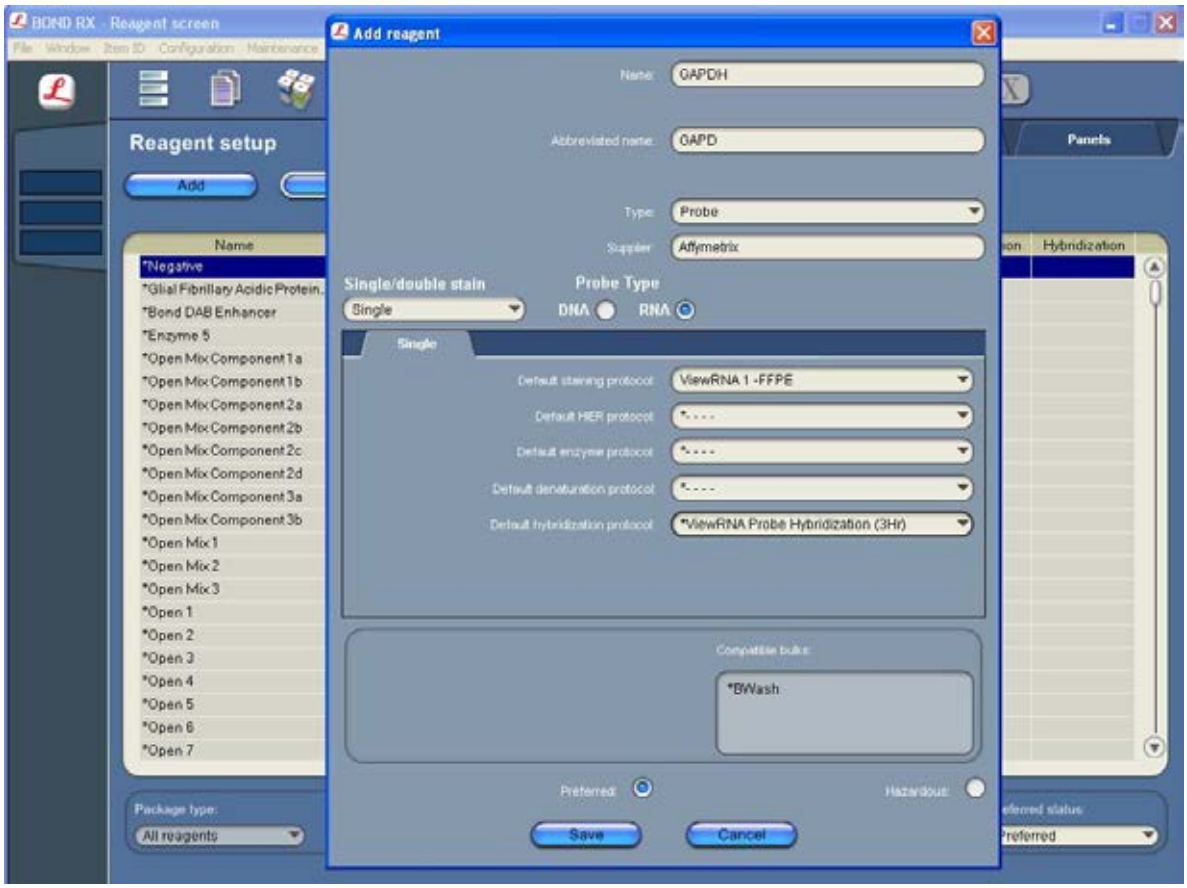
A. Click the icon to open the "Reagent setup" window (Figure 5.3).

B. Click **Add** to open the "Add reagent" window. Enter the *Name* of the probe and enter "Affymetrix" for *Supplier*. Choose **RNA** for *Probe Type*.

C. Choose the *Staining and Hybridization* protocols to be associated with the probe. See Table 5.1 on page 18 for options and descriptions. Click **Save**.

NOTE: Once entered and saved, the protocols associated with a specific probe are the defaults that appear during the slide setup process, unless changed.

Figure 5.3 Adding a Custom Probe Name to the Marker List



8. Select the appropriate protocols for *Staining*, *Preparation*, *HIER* (Heat-induced epitope retrieval), *Enzyme* (proteinase), and *Hybridization* (Figure 5.4). See Table 5.1 on page 18 for information about the protocols. See Appendix Con page 49 for instructions on creating a custom Enzyme protocol.

Figure 5.4 Select Probe (Affymetrix) Protocol

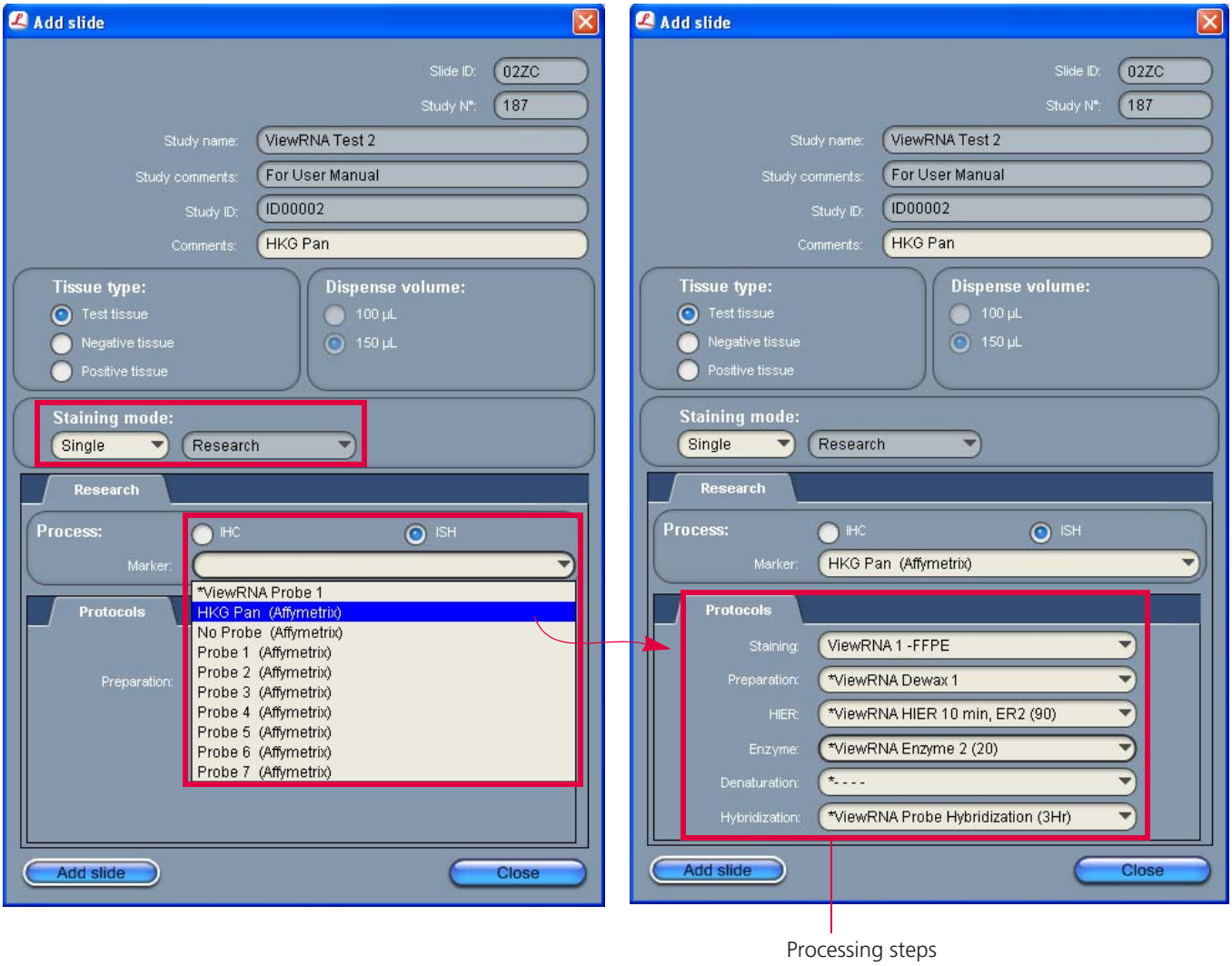


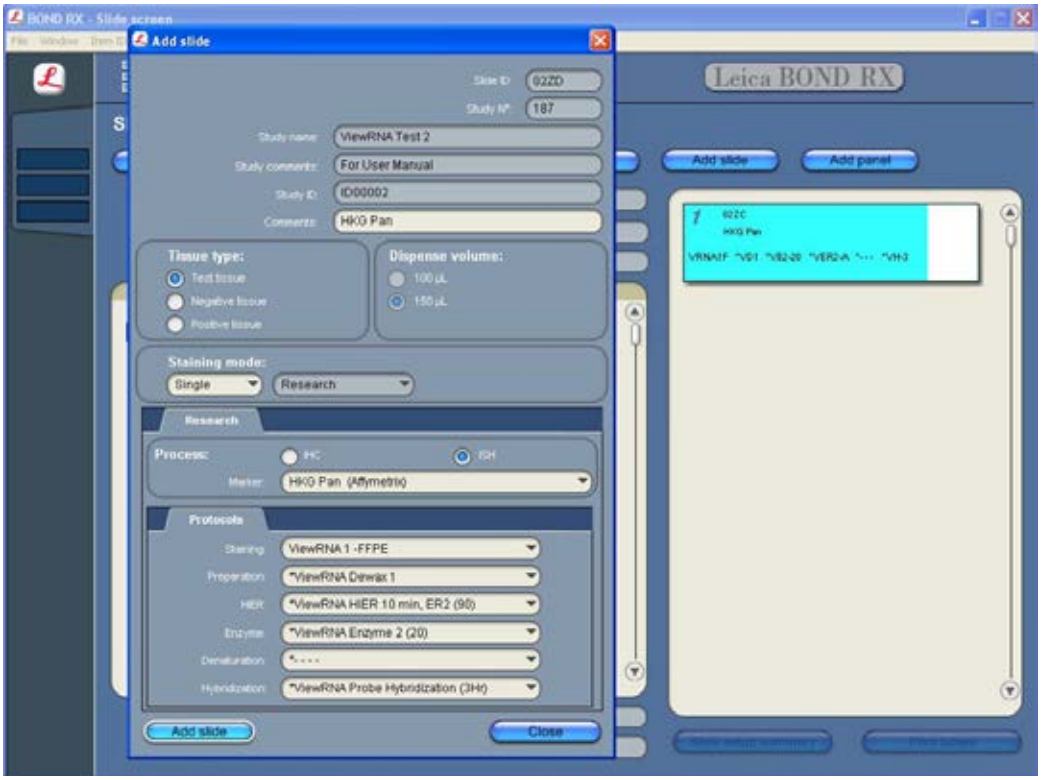
Table 5.1 ViewRNA eZ Processing Step Protocols on Leica BOND RX

Processing Step	Select	Description
Staining	*ViewRNA 1 - FFPE	Affymetrix® ViewRNA eZ Assay, FFPE protocol 1.[a]
	*ViewRNA 2	Affymetrix® ViewRNA eZ Assay, Alternate 2.[b]
	*ViewRNA 3	Affymetrix® ViewRNA eZ Assay, Alternate 3.[b]
Preparation	*ViewRNA Dewax 1	Dewax protocol for Affymetrix ViewRNA eZ Assay (15 min drying). This is the default and recommended protocol for FFPE sections.
	*ViewRNA Dewax 2	Dewax protocol for Affymetrix ViewRNA eZ Assay (5 min drying); for TMA or small FFPE sections.
	*ViewRNA Dewax 3	Dewax protocol for Affymetrix ViewRNA eZ Assay (20 min drying); for larger sections requiring longer drying time.
HIER (heat-induced epitope retrieval)	*ViewRNA 10 min, ER1 (95)	10 minute heat treatment using ER1 at 95 °C for Affymetrix ViewRNA eZ Assay.
	*ViewRNA 10 min with ER2 (90)	10 minute heat treatment using ER2 at 90 °C for Affymetrix ViewRNA eZ Assay.
Enzyme	*ViewRNA enzyme 1 (20)	20 minute proteinase pretreatment for Affymetrix ViewRNA eZ Assay.
	*ViewRNA enzyme 2 (20)	20 minute proteinase pretreatment for Affymetrix ViewRNA eZ Assay.
	*ViewRNA enzyme 3 (20)	20 minute proteinase pretreatment for Affymetrix ViewRNA eZ Assay.
Denaturation	*_ _ _ _	The Denaturation step is never used in the ViewRNA eZ Assay.
Hybridization	*ViewRNA Probe Hybridization (3hr)	3 hour probe hybridization.

[a]The default protocol for FFPE sections that is detailed in this manual.
[b]The use of this protocol will be addressed in an Application Note.

9. Verify once more that the correct processing protocols have been selected, based on your sample type.
10. Click **Add slide**.
    The slide appears in the "Slide setup" window (Figure 5.5).

Figure 5.5 Slide Added to the Selected Study



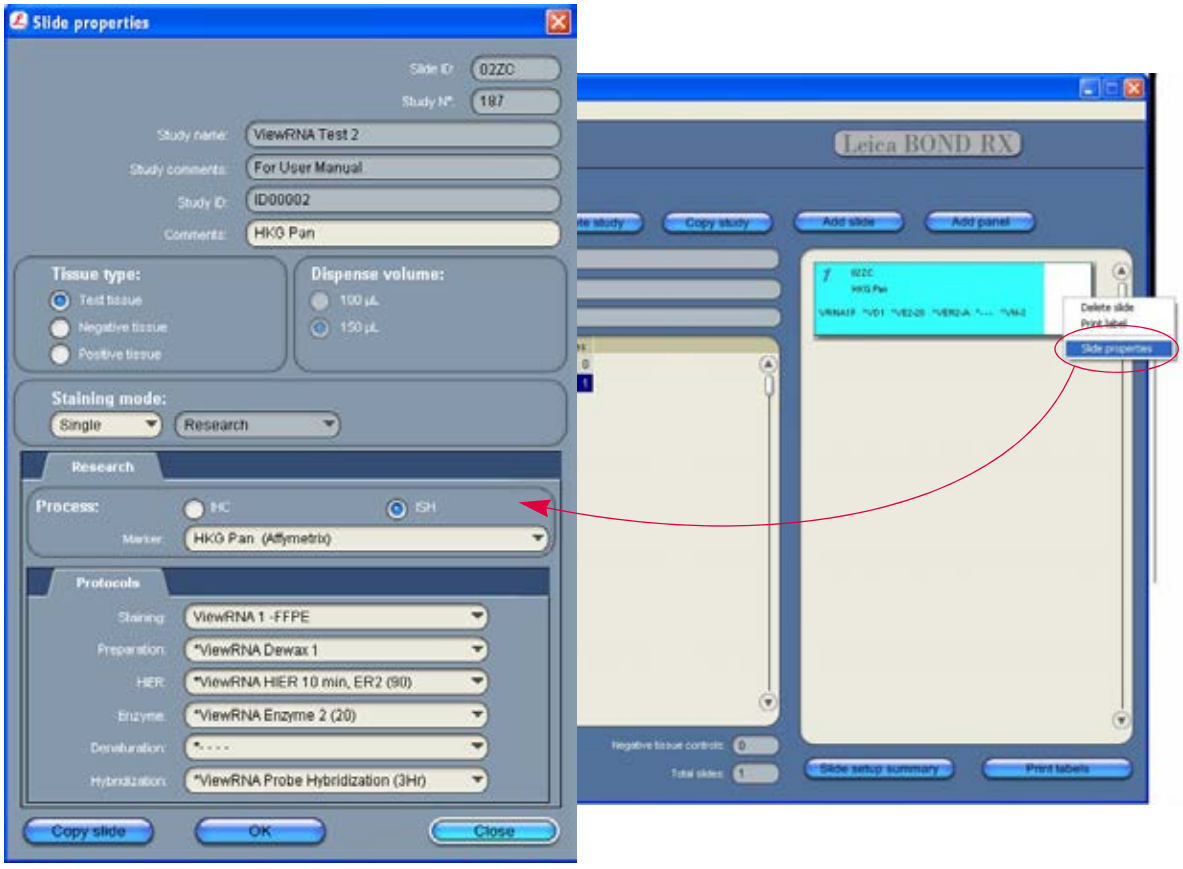
11. To add a replicate slide or copy an existing slide:

A. Right-click the slide and select **Slide properties** on the shortcut menu (Figure 5.6).

B. Click **Copy slide** in the dialog box that appears.

C. Click **Add slide**.

Figure 5.6 Copy a Slide



12. Add another slide by doing either of the following:

- Copy and edit an existing slide:

  1) Copy and add a slide (see Step 11A and Step 11B above).

  2) Edit the slide properties in the "Add slide" dialog box that appears and click **Add slide.**

OR

- Repeat Step 2 on page 14 to Step 10 on page 18.

13. To delete a slide, right-click the slide and select **Delete slide** on the shortcut menu. Click **Yes** to delete the slide.

14. Click **Close** when you are done adding slides to the study.

15. Print the slide labels:

A. Click **Print labels**.

The "Print slide labels" dialog box appears.

B. Choose an option in the dialog box (in most cases, it is **Current study**), and click **Print**.

Reserve the slide labels for *Step 4: Label and Process Slides on Leica BOND RX* on page 26 and proceed to reagent preparation.

Step 3: Prepare and Load Reagents and BOND Research Detection 2 System

NOTE: Do not mix and match kit components from different lots.

BOND Research Detection 2 System

1. Prepare reagents for the BOND Research Detection 2 System as instructed in Table 5.2. If this is the first use of the registered kit, transfer the indicated volume for each ready-to-use, one-time transfer reagent to its designated and labeled 30 mL Open container.
2. Place the open containers in the Research Detection 2 Reagent Tray in the order shown in Figure 5.7 on page 22. Also see *8.5 Loading Reagents* in the *Leica BOND RX User Manual* for instructions on loading a reagent tray.

Table 5.2 Preparing Reagents for BOND Research Detection 2 Reagent Tray

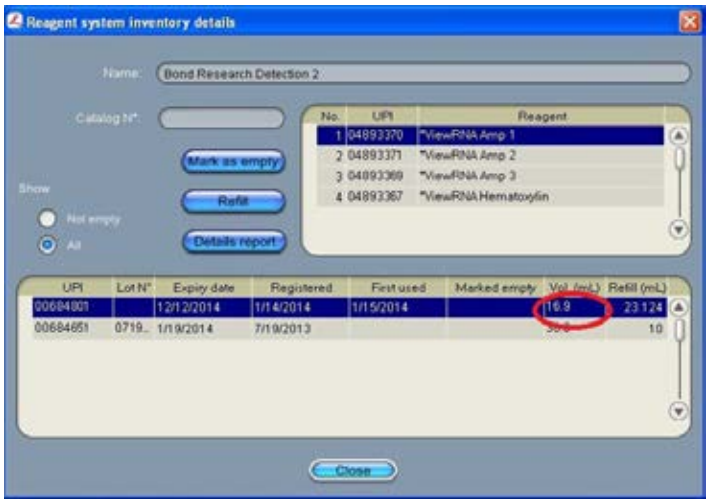
Reagent Name (Abbreviation) in BOND RX Software	Reagent Name (ViewRNA eZ Detection Kit Includes the Highlighted Reagents)	Label Name and Color	Position in BOND Research Detection 2 Tray	Preparation /Usage	Reagent Volume to Transfer to 30 mL Open Container
*ViewRNA Amp 1 (*VA1)	Amp 1	A1 – Black	1	▪ Ready to use, one-time transfer. ▪ Pre-warm to 40 °C for one hour, invert gently to mix before transfer. ▪ Store capped at 4 °C.	Entire content
*ViewRNA Amp 2 (*VA2)	Amp 2	A2 – Black	2	▪ Ready to use, one-time transfer. ▪ Pre-warm to 40 °C for one hour, invert gently to mix before transfer. ▪ Store capped at 4 °C.	Entire content
*ViewRNA Amp 3 (*VA3)	Amp 3	A3 – Black	3	▪ Ready to use, one-time transfer. ▪ Pre-warm to 40 °C for one hour, invert gently to mix before transfer. ▪ Store capped at 4 °C.	Entire content
*ViewRNA Hematoxylin (*VHx)	Hematoxylin	H – Blue	4	▪ Ready to use, one-time transfer. ▪ Store capped at 4 °C.	30 mL

Figure 5.7 Research Detection 2 Tray – ViewRNA eZ Kit Reagents

3. Scan the bar code on the reagent tray.

NOTE: Do not click "Refill" or "Mark as empty" in the dialog box that appears (Figure 5.8)

Figure 5.8 Reagent System Inventory Details

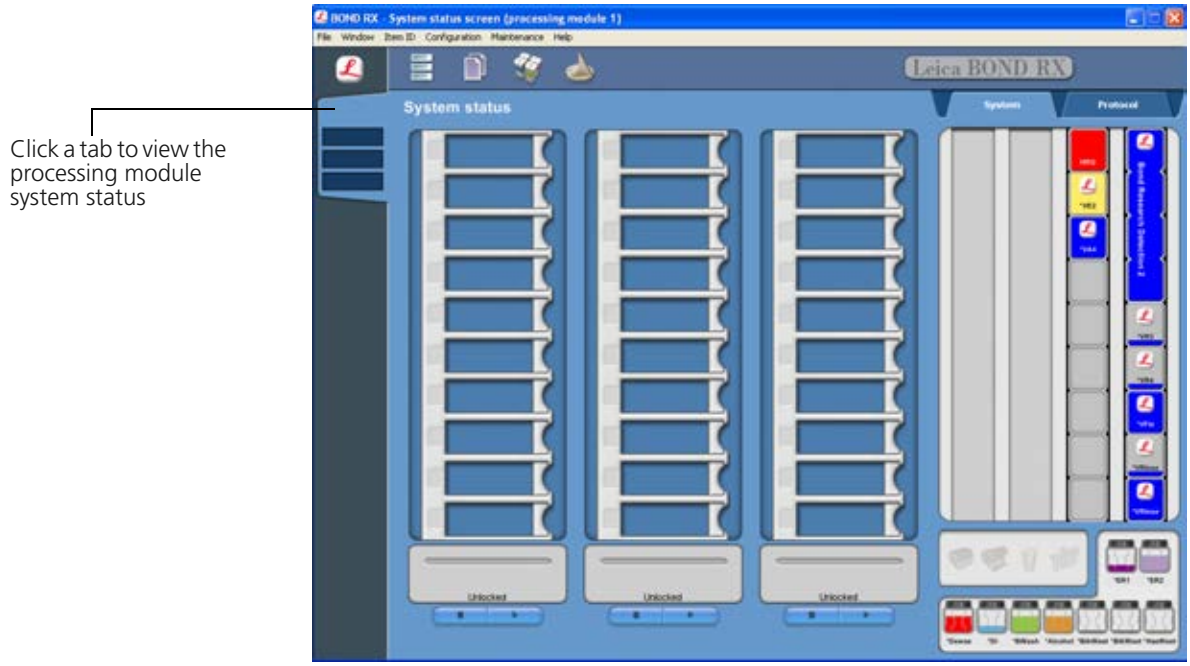


4. Verify that the remaining or available volume ("Vol (ml)" value in Figure 5.8) for the BOND Research Detection 2 reagents is sufficient for the number of slides being processed. (See the lookup table in Appendix E, *Reagent Preparation Volumes on page 53*.) Click **Close**.

NOTE: If the volume of the Research Detection 2 system is low or insufficient, you will need to either reduce the number of slides being processed or register a new ViewRNA eZ Detection Kit. Do not refill and/or mix components from different kits.

5. Open all containers, making sure that the lids are snapped back securely and the barcodes near the openings are tightly adhered to the containers.
6. Insert the reagent tray into the deck for the system to verify the volumes. See *8.3 Reagent Inventory Screen and 8.5 Loading Reagents* in the *Leica BOND RX User Manual* for instructions.
7. View the System status screen to confirm that there are no problems with the reagents (Figure 5.9). See *5.1.3 Reagent Status* in the *Leica BOND RX User Manual* for more information on the System status screen and correcting reagent problems.

Figure 5.9 System Status Screen

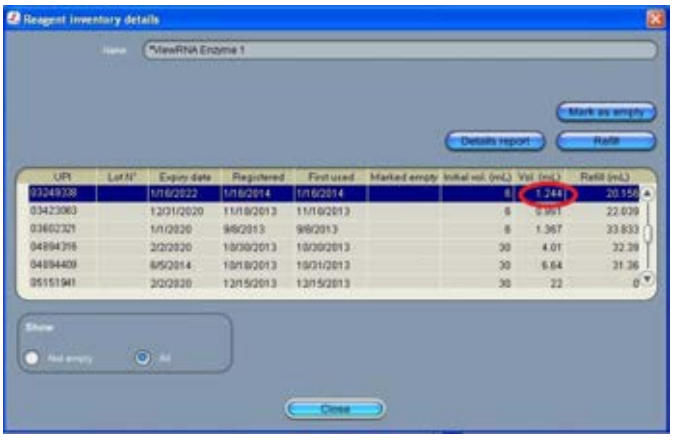


Ancillary Reagents

1. For reagents prepared fresh just before the start of each run (ViewRNA Amp4, Enzyme 1, Enzyme 2, Enzyme 3, and Probe):
   - **A.** Scan the bar code of a container designated for an ancillary reagent.
   - **B.** Click **Refill** in the dialog box that appears.
   - **C.** Verify that the remaining or available volume ("Vol (ml)" value in Figure 5.10) for the reagent is sufficient for the number of slides being processed. (See the lookup table in Appendix E, *Reagent Preparation Volumes on page 53*.)

NOTE: If the volume is low or insufficient, you will need to either reduce the number of slides being processed or register a new kit. Do not refill and/or mix components from different kits.

Figure 5.10 Reagent Inventory Details



D. Repeat Step A to Step C for each reagent prepared fresh before each run. Click **Close** in the dialog box after all of the reagent volumes are verified.

2. For ready-to-use, one-time transfer reagents (ViewRNA Fix, Rinse, Red 1, Red 2, and DAPI):

   A. Scan the bar code of a container designated for an ancillary reagent.

   B. Do not click **Mark as empty** or **Refill** in the dialog box that appears (Figure 5.10).

   C. Verify that the remaining or available volume ("Vol (ml)" value in Figure 5.10) for the reagent is sufficient for the number of slides being processed. (See the lookup table in Appendix E, *Reagent Preparation Volumes on page 53*.)

   NOTE: If the volume is low or insufficient, you will need to either reduce the number of slides being processed or register a new kit. Do not refill and/or mix components from different kits.

   D. Repeat Step A to Step C for each ready-to-use, one-time transfer reagent. Click **Close** in the dialog box after all of the reagent volumes are verified.

3. Prepare and transfer the reagents to BOND containers following the instructions in Table 5.3 on page 25. If this is the first use of the registered kit, transfer the indicated volume for each ready-to-use, one-time transfer reagent to its designated and labeled 30 mL Open container. See the lookup table in Appendix E, *Reagent Preparation Volumes on page 53* for the required volumes of of proteinase, Amp 4, and probe, based on the number of slides being processed.

Table 5.3 Preparing Ancillary Reagents for Open Reagent Tray

Reagent or Container Name (Abbreviation) in BOND RX Software	Reagent Name (ViewRNA eZ Detection Kit includes the Highlighted Reagents)	Label Name and Color	Preparation/Usage	Reagent Volume to Transfer to a Container
*ViewRNA Enzyme 1, 2, or 3 (*VA1, *VA2 or *VA3)	Enzyme	E – Red	▪ **Prepare fresh just before the start of each run.** ▪ BOND Enzyme Concentrate in BOND Enzyme Diluent (typically at 1:500, 1:1000, and 1:2000). ▪ Discard unused reagent after run.	See Appendix E on page 53.
*ViewRNA Fix (*VFix)	Fix	F – Blue	▪ Ready to use 10% NBF (not included in the ViewRNA eZ Detection Kit). One-time transfer. ▪ Use within six months. ▪ Store capped at 4 °C.	20 mL to a 30 mL Open container.
*ViewRNA Probe 1 (*VP1)	ViewRNA Probe	P – Green	▪ Dilute 1:20 in pre-warmed ViewRNA eZ Probe Diluent. ▪ Pre-warm Probe Diluent to 40 °C for one hour before use. ▪ Prepare working dilution on day of use and avoid using leftover.	See Appendix E on page 53.
*ViewRNA Rinse (*VRinse)	Rinse	R – Blue	▪ Ready to use, one-time transfer. ▪ Use within six months. ▪ Store capped at 4 °C.	Entire content (fill three 30 mL Open containers).
*ViewRNA Amp 4 (*VA4)	Amp 4, 500X and Amp 4 Diluent	A4 – Red	▪ **Prepare fresh just before the start of each run.** ▪ Dilute Amp 4, 500X stock 1:500 in pre-warmed Amp 4 Diluent. ▪ Discard unused reagent after run.	See Appendix E on page 53.
*ViewRNA Red 1 (*VR1)	Red 1	R1 – Blue	▪ Ready to use, one-time transfer. ▪ Use within six months. ▪ Store capped at 4 °C.	Entire content to a 30 mL Open container.
*ViewRNA Red 2 (*VR2)	Red 2	R2 – Blue	▪ Ready to use, one-time transfer. ▪ Use within six months. ▪ Store capped at 4 °C.	Entire content to a 30 mL Open container.
*ViewRNA DAPI (*DAPI)	DAPI (optional)	D – Blue	▪ Ready to use, one-time transfer. ▪ Use within six months. ▪ Store capped at 4 °C.	Entire content to a 30 mL Open container.

4. Place all of the ancillary containers in the Open Reagent Tray, in any order. More than one Open Reagent Tray may be necessary, depending on the number of probes and proteinase dilutions being used.
5. Open all containers, making sure that the lids are snapped back securely and the barcodes near the openings are tightly adhered to the containers. Insert the Open Reagent Tray into the deck for the system to verify the volumes. See *8.3 Reagent Inventory Screen and 8.5 Loading Reagents* in the *Leica BOND RX User Manual* for instructions.
6. View the System status screen to confirm that there are no problems with the reagents (Figure 5.9 on page 23). See *5.1.3 Reagent Status* in the *Leica BOND RX User Manual* for more information on the System status screen and correcting reagent problems.

Step 4: Label and Process Slides on Leica BOND RX

The default ViewRNA eZ Assay protocols require 9 – 10 hours for FFPE samples. For FFPE samples, there is an optional "system delay start" that conveniently allows you to set up the assay at the end of a workday, run the assay overnight, and retrieve the slides the next morning.

Start the run immediately or use the “System Delay Start” feature to ensure that processed slides can be removed within 30 minutes of assay completion. Please schedule the run so that the:

- Run delay is no longer than six hours.
- Processed slides can be removed within 30 minutes after assay completion to avoid deterioration of the hematoxylin signal and tissue morphology.

Label and Load Slides

1. Apply the printed labels to the frosted area of the slides. See *6.5 Slide Labeling* in the *Leica BOND RX User Manual* for instructions.
2. Place the slides in a slide tray and ensure that they are correctly oriented in the tray. Apply a clean, unscratched Covertile over each slide and ensure that the Covertile is correctly positioned on the slide (Figure 5.11). See *6.6 Loading and Unloading Slides* in the *Leica BOND RX User Manual* for instructions.

Figure 5.11 Correct Placement of Covertile Over Slide

3. Load the slide tray(s) into the processing module and press the Load/Unload button below the tray opening (Figure 5.12).

   This locks the tray in place and initiates scanning of the slide labels.

Figure 5.12 Processing Module – Load/Unload Button

4. Confirm that all of the slides have been identified in the System status screen. If necessary, manually identify slides that failed to be identified automatically. See *Manual Slide Identification* under *5.1.4 Slide Identification* in the *Leica BOND RX User Manual* for instructions.
5. Address and correct any notification that appears in the "System status" screen (Figure 5.14).
6. Start the run immediately or delay the start for up to 6 hours:

To delay the run start:

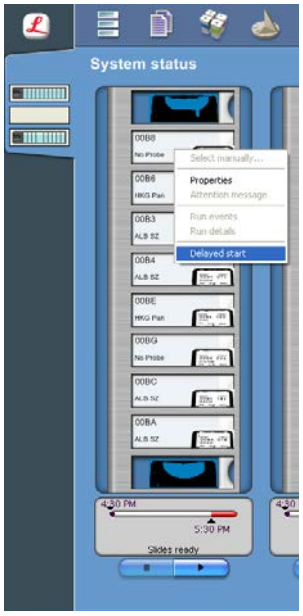
A. Right-click any slide on the screen and select **Delay start** on the shortcut menu (Figure 5.13).
B. Enter the start time for the run and change the date if necessary.
C. Wait until the system provides an END time for the run before walking away.
D. If the END times does not enable the slides to be removed within 30 minutes of assay completion, adjust the Delay start.
   1) To change the start time which was set as Delay start, push the Load/Unload button below the tray to reset the Delay start time.

      When the tray is uplifted, the set time for Delay start will be canceled. Do not remove the tray from the instrument.
   2) Reload the tray by pushing the Load/Unload button below the tray. After the system recognizes the tray, go to Step A above.

IMPORTANT: Do not click the on-screen Stop button under the loaded slide tray to cancel the set time for Delay start. This cancels the run and program linked to the slide labels. If the Stop button is clicked, new slide labels are required for the run. To obtain a new slide label, open the "Slide setup" window, right-click the slide to be run, and click "Print Label".

TIP: Schedule the starting time for each SSA 10 – 15 minutes apart to avoid scheduling difficulty.

Figure 5.13 System Status Screen – Delayed Start

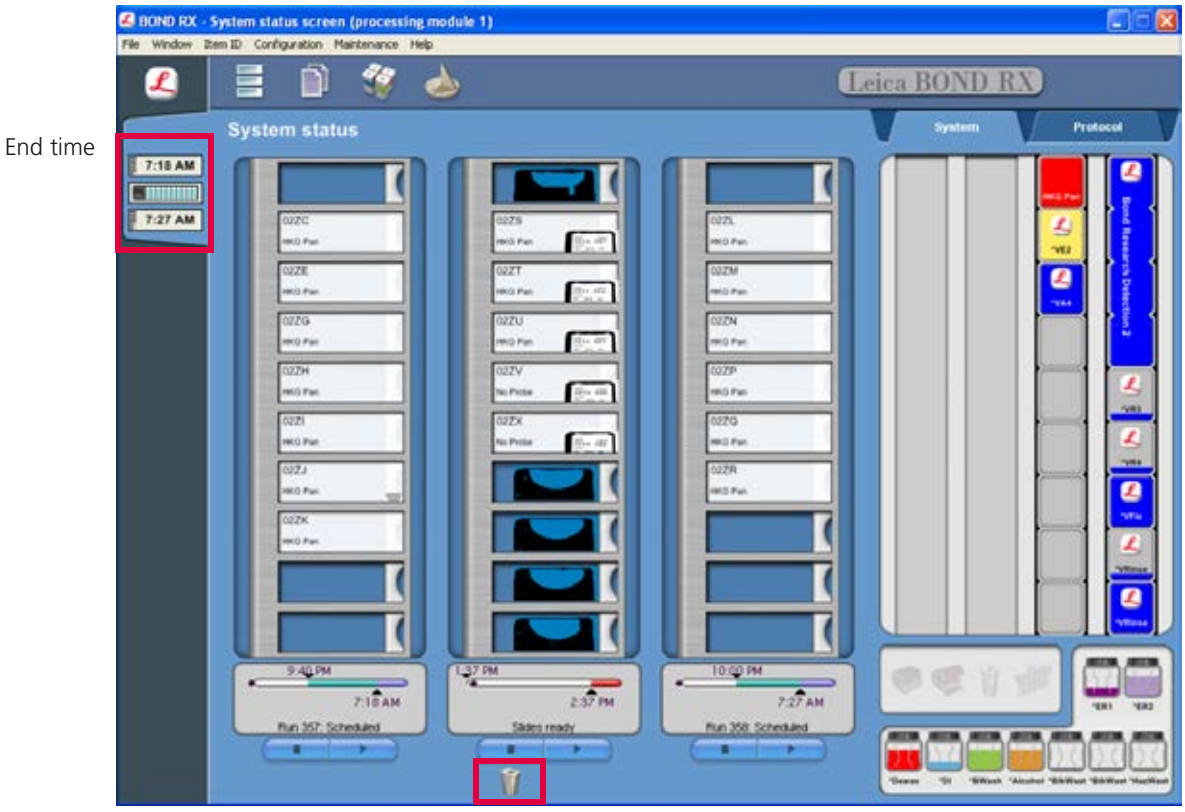


To start the run immediately (optional):

A. Click  under each loaded slide tray in the System status screen (Figure 5.14).

B. Wait until the system provides an END time for the run before walking away (Figure 5.14).

Figure 5.14 System Status Screen – Start Run Immediately



Notification icon – Right-click to get more information.

Unload Slides and Reagents

1. Remove the Open Reagent and Research Detection 2 trays after the run is complete. Store or discard the reagents as specified in Table 5.2 on page 21 and Table 5.3 on page 25, according to the color-coded label on the Open containers.
2. Press the Load/Unload button on the processing module and remove the slide tray.
3. Remove and clean the Covertiles. See *11.3 Covertiles* in the *Leica BOND RX User Manual* for instructions.
4. Rinse slides in ddH_2O, air dry for 30 minutes at room temperature, then cover slip with aqueous or organic mounting medium as outlined in *Step 5: Mount and View Slides*.

Step 5: Mount and View Slides

NOTE: Slides must be removed from the instrument within 30 minutes of the end of the run.

Table 5.4 lists the recommended mounting media. Choose the mounting medium that is most appropriate for your application, based on type of signal (chromogenic or fluorescent) you will be viewing.

Table 5.4 Recommended Mounting Media

Mounting Medium	Type	Application	Disadvantages
Histomount Mounting Solution	Organic	Chromogenic	Incompatible with DAPI.
Fluoromount/Plus	Aqueous	▪ Chromogenic ▪ Fluorescent	▪ Bubble formation at room temperature. ▪ 4 °C long term storage required.
ADVANTAGE Mounting Media	Aqueous	Chromogenic	▪ Autofluorescence.

Mounting Slides

If Using HistoMount Mounting Solution

NOTE: Perform Step 1 to Step 4 under a fume hood. Red signal is soluble in ethanol, therefore, we do not recommend incorporating the alcohol dehydration steps before the xylene clearing.

1. Dip slides in xylene at room temperature.
2. Pipet ~75 µL of HistoMount solution onto each tissue section without creating any bubbles, and immediately place a cover glass on the mounting medium.
3. Allow the slides to air dry for 15 minutes at room temperature.
4. Mounted slides can be viewed and imaged at this point, or stored at room temperature.

If Using Using Fluoromount/Plus

1. Dab the first 2 – 3 drops of medium on a paper towel to remove bubbles.
2. Add a minimum of 2 – 4 drops of Ultramount to the tissue section without creating any bubbles. If necessary, use a pipette tip to draw out any air bubbles in the drops.
3. Slowly place the cover glass on the specimen at an angle.
4. Dab the edges of the slide on a laboratory wipe to remove any excess mounting medium. Allow the slides to air dry for 5 minutes at room temperature.
5. Mounted slides can be viewed and imaged at this point, or stored at 4 °C to prevent bubble formation over time.

If Using ADVANTAGE Mounting Media

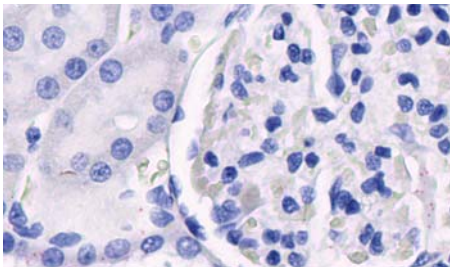
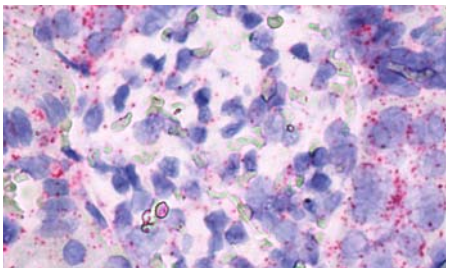
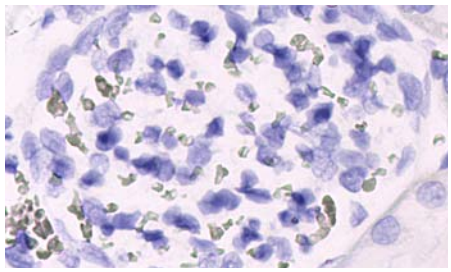
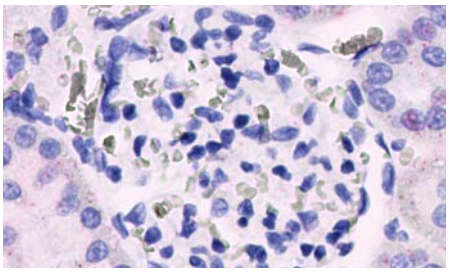
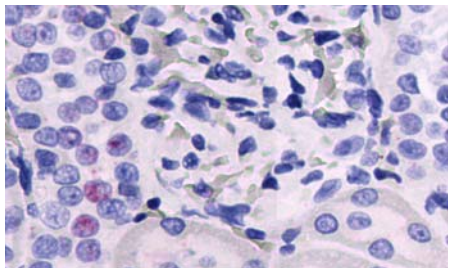
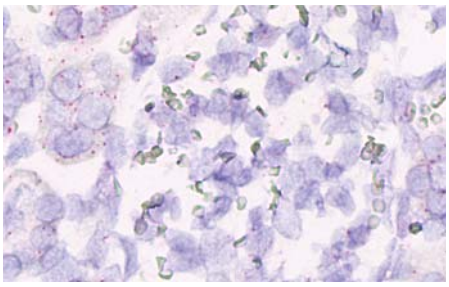
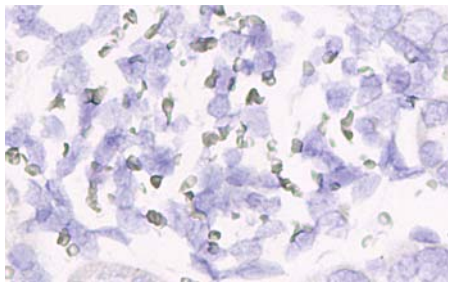
1. Place a cover glass on a clean, flat surface.
2. Dab the first 2 – 3 drops of mounting medium on a paper towel to remove bubbles.
3. Add 2 – 3 drops of mounting medium directly onto the middle of the cover glass. Use a pipette tip to draw out any air bubbles in the drops.
4. Invert the specimen slide and slowly place it on the mounting medium at an angle.
5. Flip the slide over and dab the edges of the slide on a laboratory wipe to remove any excess mounting medium.

6. Allow slides to air dry for 15 minutes at room temperature.
7. Mounted slides can be viewed and imaged at this point, or stored at room temperature.

Assessing Pretreatment Conditions

Table 5.5 shows example images obtained from the ViewRNA eZ Assay performed on rat kidney tissue. The assay results illustrate the effects of optimal and suboptimal pretreatment conditions on Arbp signal strength versus morphology, and demonstrate how to evaluate data from the *in situ* assay to qualitatively determine target expression.

Table 5.5 Example ViewRNA eZ Assay Results – Arbp Expression in Rat Kidney Tissue

Example Results	Description
Untreated Reference Morphology Slide 	▪ Good morphology ▪ Intact cellular structures ▪ Good hematoxylin counterstaining of nuclei ▪ Little or no probe accessibility ▪ Little to no Arbp signals ▪ Little to no dots observed
Optimal Pretreatment and Sample Preparation  (+) Arbp  (–) Arbp	▪ Good morphology ▪ Cellular structures and boundaries are retained and still identifiable ▪ Good hematoxylin counterstaining of nuclei ▪ Optimal accessibility ▪ Strong, punctated, and ubiquitous signals in (+) Arbp sample and clean background in (–) probe sample
Insufficient Pretreatment or Over Fixation of Tissue  (+) Arbp  (–) Arbp	▪ Good morphology ▪ Intact cellular structures ▪ Strong hematoxylin counterstaining of nuclei ▪ Poor probe accessibility ▪ Weak, diffused, and non-ubiquitous Arbp signals ▪ Nuclear staining in (–) probe control
Over Pretreatment or Under Fixation  (+) Arbp  (–) Arbp	▪ Poor morphology ▪ Loss of cellular structures and boundaries due to excessive heat treatment and proteinase digestion ▪ Poor hematoxylin counterstaining of nuclei ▪ Loss of RNA target due to over digestion ▪ Weak Arbp signals and fewer number of dots

Analyzing Target Expression

Each observable dot represents a single RNA molecule within the cell that the ViewRNA eZ Assay is able to detect, assuming the RNA target is intact and properly unmasked for probe access.

The dots are typically uniform in size, but smaller than average size dots can also be present. This usually indicates that the transcript is not properly unmasked, resulting in the binding of only one or a few pairs of oligonucleotides from the probe set. Conversely, a larger than average size dot can occur when multiple targets are found clustered in the same physical area. All things being equal, an RNA target with low expression will yield fewer dots than a target with high expression.

It is important to consider the pattern and number of dots in the negative control (e.g. bacterial dapB or sense strand of the target) to confidently differentiate between low expressing targets and non-specific background dots when assessing RNA target expression. The average background of the ViewRNA eZ Assay is usually less than 1 dot/3 cells. Consequently, as long as your target consistently shows an expression level above the negative control threshold, the detection is reliable and real, even if the RNA target expression is extremely low, such as 2 dots/ cell.

Troubleshooting

Contacting Technical Support
Weak or No Signals
Poor Cell Morphology/Weak Hematoxylin Staining on page 37
Tissue Detachment From Slide on page 37
Areas of Tissue Devoid of Red or Hematoxylin Staining on page 38
Diffused Signals on page 38
High Background or Nuclear Staining on page 39
High Non-Specific Binding on Glass Slide on page 40
Hematoxylin and/or Red Stain on Covertile on page 40

Contacting Technical Support

For technical support, contact the appropriate resource provided below based on your geographical location. Visit our website at www.affymetrix.com/panomics for an updated list of FAQs and product support literature.

Table 6.1 Technical Support Contact Information

Location	Affymetrix	Leica
North America	Tel: 1.877.726.6642 option 1, then option 3 E-mail: pqbhelp@affymetrix.com	1700 Leider Lane Buffalo Grove, IL 60089 USA Tel: 1.800.248.0123 or 1.847.405.0123 Fax: 1.847.405.0164
Europe	Tel: +44 1628-552550 E-mail: techsupport_europe@affymetrix.com	Visit Leica website and select your country.
Asia	Tel: +81 3 6430 430 E-mail: techsupport_asia@affymetrix.com	Visit Leica website and select your country.

Weak or No Signals

Table 6.2 Troubleshooting Weak or No Signals

Probable Cause	Recommended Action
Incorrect pretreatment conditions. ▪ Under-pretreatment yields good morphology but poor signal due to insufficient unmasking of target. ▪ Over-pretreatment yields poor morphology and loss of signal resulting from release of mRNA from the sample due to heat over-treatment or proteinase over-digestion.	▪ Repeat pretreatment assay optimization procedure to determine optimal heat treatment and proteinase digestion that will strike a balance between morphology and signal. ▪ Use ViewRNA eZ Check to optimize pretreatment.
Probe not added.	Check to make sure that the correct probe was selected during slide setup.

Table 6.2 Troubleshooting Weak or No Signals (Continued)

Probable Cause	Recommended Action
Reagents applied in the wrong sequence.	Be sure to transfer the correct reagents to the assigned BOND Open containers.
Target of interest is not expressed.	▪ Verify expression using other tissue lysate methods such as QuantiGene® 2.0, QuantiGene Plex Assay, or Affymetrix® Array. ▪ Run the same probe set on known samples that have been validated to express the target of interest.
Incorrect storage or preparation of reagents.	▪ Store the components at the recommended storage conditions. ▪ Prepare reagents according to the recommended protocol, including temperature and dilution.
Reagents not freshly prepared.	▪ Be sure that the ancillary reagents (Enzyme, Amp 4) are freshly prepared just before the start of the run. ▪ Verify that the delayed start is not set for longer than six hours from the time the reagents are loaded onto the Leica BOND RX.
RNA in tissue is degraded.	Verify tissue fixation: ▪ Ensure that the tissue is freshly harvested, but to a thickness of 3 – 5 mm, and immediately fixed in 10% neutral buffered formalin or 4% paraformaldehyde for 16-24 hours. ▪ If fixation cannot be performed immediately, be sure that the tissue is placed on dry ice or in liquid nitrogen to prevent RNA degradation. ▪ Use ViewRNA eZ Check to assess RNA integrity.
Sample preparation.	▪ Ensure that freshly-dissected tissues are fixed in 10% neutral buffered formalin or 4% paraformaldehyde for the recommended 16 – 24 hours. ▪ Ensure that the tissue section falls within the recommended area on the glass slide (see Figure 4.1 on page 9).
Over-fixation of tissue after proteinase digestion.	Make sure the tissue sections are not fixed more than 5 minutes in ViewRNA Fix after proteinase digestion.
Slides were exposed to alcohol during processing.	Avoid: ▫ Mounting medium containing alcohol. ▫ Alcohol dehydration when using organic mounting medium.
Tissue dries up during processing.	▪ Ensure that Covertiles are correctly placed on the glass slide. ▪ Ensure that the tissue section falls within the recommended area on the glass slide.
Small targets, splice variants, or RNA fusions.	Increasing ViewRNA eZ Probe concentration by diluting target probe 1:10 instead of 1:20 and hybridizing for 3 hours may increase sensitivity, but note that there is always a general trade-off between specificity and sensitivity. Do not use View RNA eZ Probe at a dilution higher than 1:10.
Samples not promptly removed after run.	Remove samples from Leica BOND RX within 30 minutes of assay completion to prevent deterioration of morphology and hematoxylin stain.
BOND RX errors in run event log.	Contact your Leica Microsystems representative (contact information is available at www.leica-microsystems.com).
BOND RX probe is misaligned.	Verify instrument function by running the ViewRNA eZ Control Kit.
BOND Dewax Solution, BOND Wash Solution, Alcohol, ER1, ER2 or Enzyme (proteinase K) is expired.	Replace with new BOND Dewax Solution, BOND Wash Solution, Alcohol, ER1, ER2 or Enzyme (proteinase K).

Poor Cell Morphology/Weak Hematoxylin Staining

Table 6.3 Troubleshooting Cell Morphology/Hematoxylin Staining

Probable Cause	Recommended Action
Incorrect pretreatment conditions.	Perform full pretreatment optimization procedure to determine optimal heat treatment and proteinase digestion time.
Tissue sample not fixed properly.	Make sure that freshly dissected tissues are fixed in 10% buffered formalin or 4% paraformaldehyde for 16 – 24 hours.
Section thickness is variable or not optimal.	Make sure the microtome is calibrated and tissue are sectioned at 5 ± 1 µm.
Samples not promptly removed after run.	Remove samples from Leica BOND RX within 30 minutes of assay completion to prevent deterioration of morphology and hematoxylin stain.
BOND RX errors in run event log.	Contact your Leica Microsystems representative (contact information is available at www.leica-microsystems.com).
BOND RX probe is misaligned.	Verify instrument function by running the ViewRNA eZ Control Kit.
BOND Dewax Solution, BOND Wash Solution, Alcohol, ER1, ER2, Enzyme (proteinase K), or Novocastra Hematoxylin is expired.	Replace with new BOND Dewax Solution, BOND Wash Solution, Alcohol, ER1, ER2, Enzyme (proteinase K), or Novocastra Hematoxylin.

Tissue Detachment From Slide

Table 6.4 Troubleshooting Tissue Detachment From Slide

Probable Cause	Recommended Action
Improper tissue preparation.	Make sure that the sample preparation is as recommended in Chapter 4, *Best Practices* on page 9, including: ▪ Fixation time and reagent ▪ Section thickness ▪ Brand of positively charged glass slide ▪ Placement of section on glass slide ▪ Baking of the sections at 60 °C for one hour before storing at –20 °C.
Insufficient baking of slides.	Verify that the baking step 60 °C for 60 minutes at was performed prior to storage of slides at –20 °C and again just before the deparaffinization step to ensure adhesion of tissue to slide.
Incorrect pretreatment conditions.	Perform full pretreatment optimization procedure to determine optimal heat treatment and proteinase digestion time.
Temperature of pretreatment condition too high.	Use the *ViewRNA HIER 10 min, ER2 (90 °C) heat treatment condition when samples are delicate such as colon or breast tissues.
Proteinase treatment is too long or at too high of a concentration.	Reduce proteinase concentration and/or incubation time.
BOND RX errors in run event log.	Contact your Leica Microsystems representative (contact information is available at www.leica-microsystems.com).
BOND RX probe is misaligned.	Verify instrument function by running the ViewRNA eZ Control Kit.
BOND Dewax Solution, BOND Wash Solution, Alcohol, ER1, ER2, Enzyme (proteinase K), or Novocastra Hematoxylin is expired.	Replace with new BOND Dewax Solution, BOND Wash Solution, Alcohol, ER1, ER2, Enzyme (proteinase K), or Novocastra Hematoxylin.

Areas of Tissue Devoid of Red or Hematoxylin Staining

Table 6.5 Troubleshooting Staining

Probable Cause	Recommended Action
Bubbles in assay reagents.	Do not vigorously mix or vortex the reagents as many of them contain detergent. Gently invert to mix well.
Incomplete drying of tissue section after the alcohol rinse in the dewax step.	Use ViewRNA Dewax 3 (20 minute drying) instead of ViewRNA Dewax 1.
Tissue section mounted outside the recommended area on glass slide.	Mount tissue section within the recommended area on the glass slide to ensure full reagent coverage under the Covertile during processing (see Figure 4.1 on page 9).
BOND RX errors in run event log.	Contact your Leica Microsystems representative (contact information is available at www.leica-microsystems.com).
BOND RX probe is misaligned.	Verify instrument function by running the ViewRNA eZ Control Kit.

Diffused Signals

Table 6.6 Troubleshooting Diffused Signals

Probable Cause	Recommended Action
Suboptimal pretreatment conditions.	Perform the pretreatment optimization procedure to determine the optimal heat treatment and proteinase digestion time.
Tissue dries up during processing.	▪ Ensure that Covertiles are correctly placed on the glass slide. See *6.6 Loading and Unloading Slides* in the Leica BOND RX User Manual. ▪ Ensure that the tissue section falls within the recommended area on the glass slide (see Figure 4.1 on page 9).
Reagents not freshly prepared.	▪ Be sure that the ancillary reagents (Enzyme and ViewRNA Amp 4) are freshly prepared. ▪ Verify that the delayed start is not set for longer than 6 hours from the time the reagents are loaded onto the Leica BOND RX.
Mounting solution contained alcohol.	Avoid any mounting medium containing alcohol or any cover slipping method requiring alcohol dehydration.
BOND RX errors in run event log.	Contact your Leica Microsystems representative (contact information is available at www.leica-microsystems.com).
BOND RX probe is misaligned.	Verify instrument function by running the ViewRNA eZ Control Kit.
BOND Dewax Solution, BOND Wash Solution, Alcohol, ER1, ER2, Enzyme (proteinase K), or Novocastra Hematoxylin is expired.	Replace with new BOND Dewax Solution, BOND Wash Solution, Alcohol, ER1, ER2, Enzyme (proteinase K), or Novocastra Hematoxylin.

High Background or Nuclear Staining

Table 6.7 Troubleshooting High Background

Probable Cause	Recommended Action
Suboptimal pretreatment conditions.	▪ Perform the pretreatment optimization procedure to determine the optimal heat treatment and proteinase digestion time. ▪ Increase proteinase digestion time or concentration to improve background from nuclear staining.
Concentration of Amp 4 too high.	Double-check calculations and ensure that Amp 4 is diluted 1:500.
Endogenous alkaline phosphatase activity.	▪ Verify by manually incubating a dewaxed FFPE sample with the Red Substrate (three parts Red 1 and one part Red 2) at room temperature for 40 minutes. If endogenous alkaline phosphatase activity is present, diffused signals (which can be weak or strong) will appear. ▪ Manually inactivate endogenous alkaline phosphatase activity in a dewaxed FFPE sample with 0.2 M HCl/300 mM NaCl for 15 minutes at room temperature. Wash samples twice with 1X PBS before proceeding with the pretreatment on the Leica BOND RX.
Tissue dries up during processing.	▪ Ensure that Covertiles are correctly placed on the glass slide. See *6.6 Loading and Unloading Slides* in the Leica BOND RX User Manual. ▪ Ensure that the tissue section is within the recommended area on the glass slide (see Figure 4.1 on page 9).
Bubbles in reagents.	Do not vigorously shake or vortex reagents; instead, invert gently to mix.
Failure to properly clean and maintain BOND RX.	▪ Perform cleaning and maintenance tasks, including refilling and emptying bulk containers, as instructed in the *Leica BOND RX User Manual*. See *11 Cleaning and Maintenance* in the *Leica BOND RX User Manual*. ▪ Clean the aspirating probe. ▪ Replace the mixing station every six months. ▪ Replace the aspirating probe after 1000 slides. ▪ Clean the Covertiles as instructed in the *Leica BOND RX User Manual* and discard any scratched ones. See *11.3 Covertiles* in the *Leica BOND RX User Manual*.
Incomplete removal of paraffin.	Manually dewax FFPE sample with xylene and process sample on Leica BOND RX starting with the heat treatment step.
BOND RX errors in run event log.	Contact your Leica service representative.
BOND RX probe is misaligned.	Verify instrument function by running the ViewRNA eZ Control Kit.
BOND Dewax Solution, BOND Wash Solution, alcohol, ER1, ER2, Enzyme (proteinase K), or Novocastra Hematoxylin is expired.	Replace with new BOND Dewax Solution, BOND Wash Solution, Alcohol, ER1, ER2, Enzyme (proteinase K), or Novocastra Hematoxylin.
BOND RX errors in run event log.	Contact your Leica Microsystems representative (contact information is available at www.leica-microsystems.com).
BOND RX probe is misaligned.	Verify instrument function by running the ViewRNA eZ Control Kit.
BOND Dewax Solution, BOND Wash Solution, Alcohol, ER1, ER2, Enzyme (proteinase K), or Novocastra Hematoxylin is expired.	Replace with new BOND Dewax Solution, BOND Wash Solution, Alcohol, ER1, ER2, Enzyme (proteinase K), or Novocastra Hematoxylin.

High Non-Specific Binding on Glass Slide

Table 6.8 Troubleshooting Non-specific Binding on Glass Slide

Probable Cause	Recommended Action
Incompatible glass slide.	▪ Use Leica Non-Clipped X-tra Slide, 1 mm White P/N 3800200 or 3800210 ▪ Pre-validate each new batch of slides by running the entire assay, including probe set, on slides without tissue to determine if the slides are suitable for the assay.
High concentration of probe set.	Check and make sure that the probe set dilution is correct. A dilution of 1:40 may be sufficient for very high expressing targets .
Over pretreatment of tissue (with very high expressing target).	Reduce proteinase treatment to avoid over-unmasking of high expressing targets and their subsequent non-specific binding to glass slides.
Incomplete removal of paraffin.	Manually dewax FFPE sample with xylene and process sample on Leica BOND RX starting with the heat treatment step.
Polymerization of poor quality paraffin.	Do not bake the slides at temperatures higher than 60 °C
BOND RX errors in run event log.	Contact your Leica Microsystems representative (contact information is available at www.leica-microsystems.com).
BOND RX probe is misaligned.	Verify instrument function by running the ViewRNA eZ Control Kit.
BOND Dewax Solution, BOND Wash Solution, Alcohol, ER1, ER2, Enzyme (proteinase K), or Novocastra Hematoxylin is expired.	Replace with new BOND Dewax Solution, BOND Wash Solution, Alcohol, ER1, ER2, Enzyme (proteinase K), or Novocastra Hematoxylin.

Hematoxylin and/or Red Stain on Covertile

Table 6.9 Troubleshooting Hematoxylin and/or Red Staining

Probable Cause	Recommended Action
BOND RX errors in run event log.	Contact your Leica Microsystems representative (contact information is available at www.leica-microsystems.com).
BOND RX probe is misaligned.	Verify instrument function by running the ViewRNA eZ Control Kit.

Additional Optimization Strategies

If none of the conditions for the ViewRNA eZ Assay in Table 4.2 on page 11 or Table 4.3 on page 12 yield acceptable results on the first run, you may need to go through an iterative process, varying the heat treatment and/or proteinase digestion to find the optimal pretreatment conditions. As a general guideline, the factors to consider in order of importance are: assay signal, morphology, and nuclear background.

Table A.1 shows possible results and further optimization actions. Table A.2 outlines the expected trends in assay signal, morphology, and nuclear background as a function of increasing or decreasing proteinase.

Table A.1 Possible Results and Further Optimization

Results Assessment	Action
One heat treatment condition yields signal and the other does not.	Choose the heat treatment condition that gives signal.
Both heat treatment conditions give comparable signal.	▪ First check morphologies and choose the heat treatment condition that gives better morphology. ▪ If morphologies for the two heat treatment conditions are comparable, choose the heat treatment condition that gives a lower nuclear background.
Neither heat treatment condition gives signal.	Look at the morphology for both heat treatment conditions: ▪ If morphology is good for both ER1 and ER2, choose ER1 and increase concentration/time of proteinase treatment. ▪ If morphology is good with ER1 and bad with ER2, choose ER1 and increase concentration/time of proteinase treatment. ▪ If morphology is bad with ER1 and good with ER2, choose ER2 and increase concentration/time of proteinase treatment. ▪ If morphology is bad with both ER1 and ER2, choose ER2 and decrease concentration/time of proteinase treatment.

Table A.2 Expected Trends in Assay Signal, Morphology, and Nuclear Background With Increasing or Decreasing Enzyme

Factor	Increasing Proteinase (Time/Concentration)	Decreasing Proteinase (Time/Concentration)
Assay Signal	For under-digested sample, assay signal will gradually increase with more proteinase treatment before decreasing due to over-digestion.	For over-digested sample, assay signal will increase with less proteinase treatment before decreasing due to insufficient unmasking.
Morphology	Poorer morphology.	Better morphology.
Nuclear Background	Lower nuclear background.	Higher nuclear background.

Registering a ViewRNA eZ Detection Kit

ViewRNA eZ Assay Reagents
Registering BOND Research Detection 2 Reagents
Registering Ancillary Reagents on page 46
Pairing a Staining Protocol With BOND Research Detection 2 on page 47

ViewRNA eZ Assay Reagents

Prior to use, the components of each new ViewRNA™ eZ Detection Kit need to be registered and configured in the Leica BOND RX software, some as the BOND Research Detection 2 reagents and others as ancillary reagents (see Table B.1).

Table B.1 BOND RX Research Detection 2 and Ancillary Reagents

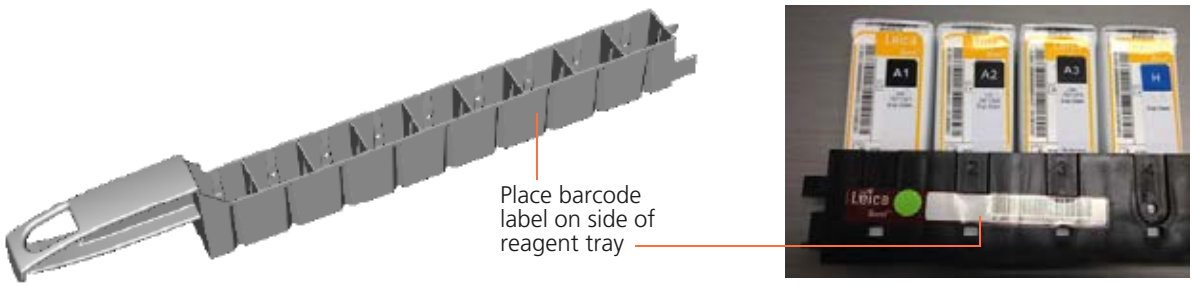
Type of Reagent	Reagent or Container Name (Abbreviation) in BOND RX Software	Reagent Name (ViewRNA eZ Detection Kit Includes the Highlighted Items)	Label Name and Color
Research Detection 2 Reagents These reagents are collectively assigned to a unique barcode label and configured as the BOND Research Detection 2.	*ViewRNA Amp 1 (*VA1)	Amp 1	A1 – Black
	*ViewRNA Amp 2 (*VA2)	Amp 2	A2 – Black
	*ViewRNA Amp 3 (*VA3)	Amp 3	A3 – Black
	*ViewRNA Hematoxylin (VHx)	Hematoxylin	H – Blue
Ancillary Reagents Stand-alone reagents, each with its own barcode label.	*ViewRNA Enzyme 1, 2, or 3 (*VE1, *VE2 or *VE3)	Enzyme	E – Red
	*ViewRNA Fix (*VFix)	Fix	F – Blue
	*ViewRNA Probe 1 (*VP1)	ViewRNA Probe	P – Green
	*ViewRNA Rinse (*VRinse)	Rinse	R – Blue
	*ViewRNA Amp 4 (*VA4)	Amp 4 Diluent and Amp 4, 500X	A4 – Red
	*ViewRNA Red 1 (*VR1)	Red 1	R1 – Blue
	*ViewRNA Red 2 (*VR2)	Red 2	R2 – Blue
	*ViewRNA DAPI (*VDAPI)	ViewRNA eZ DAPI (optional)	D – Blue

Registering BOND Research Detection 2 Reagents

1. Apply a new barcode label to a reagent tray as shown in Figure B.1. The new label may be placed over an old barcode label if necessary.

NOTE: A barcode number is unique and it can only be associated with one ViewRNA eZ Detection Kit.

Figure B.1 BOND Research Detection 2 Reagent Tray

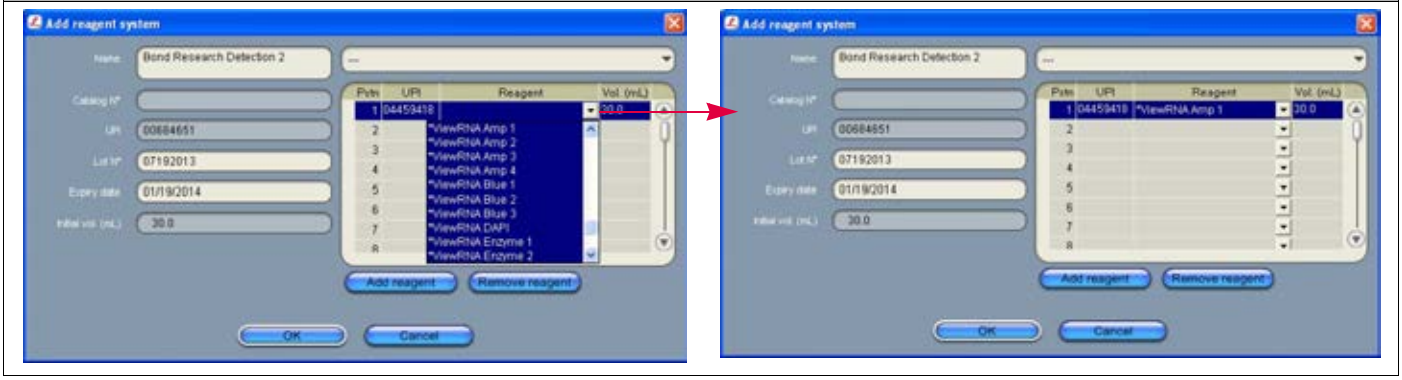


2. Scan the barcode on the reagent tray. In the "Add reagent system" dialog box that appears (Figure B.2), fill in:
   - *Name* – Name of interest, for example, "BOND Research Detection 2".
   - *Lot N°* – the ViewRNA eZ Detection Kit lot number
   - *Expiry date* – the ViewRNA eZ Detection Kit expiration date.

Figure B.2 Add Reagent System Dialog Box

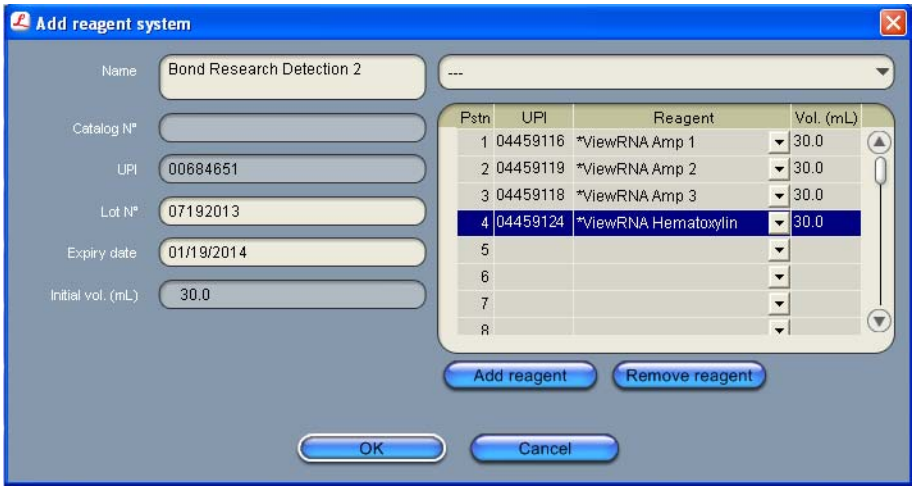
3. Scan the barcode on the side of a new 30 mL Open container and select **Amp 1** from the Reagent drop-down list for Position (Pstn) 1 (Figure B.3). Apply the appropriate color-coded label (supplied in the ViewRNA eZ Detection Kit) to the assigned container. See Table B.1 on page 43.

Figure B.3 Registering and Assigning Kit Reagents to Open Containers in BOND Research Detection2



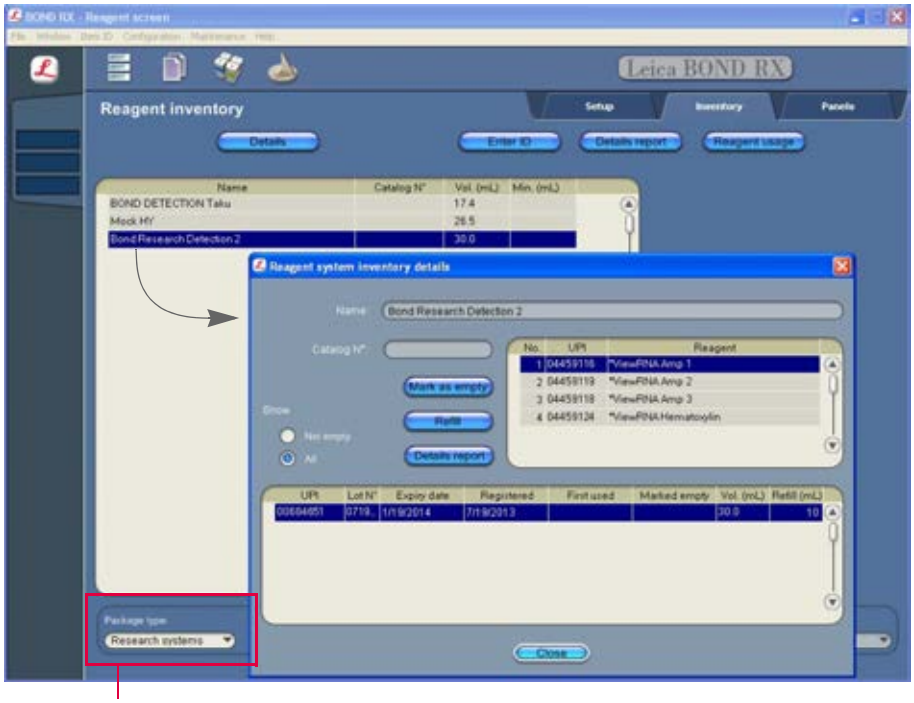
4. Repeat Step 3 to register and assign "Amp 2", "Amp 3", "ViewRNA Hematoxylin" to positions 2 – 4, respectively (Figure B.4). Apply the appropriate color-coded label (supplied in the in the ViewRNA eZ Detection Kit) to the assigned Open containers (see Table B.1 on page 43). Click **OK**. This completes the registration process.

Figure B.4 Add Reagent System Dialog Box



5. Check the Reagent inventory screen to confirm successful registration:
   A. Click the  icon, then click the Inventory tab (Figure B.5). Sort *Package type* by **Research systems**.
   B. Double-click "BOND Research Detection 2" in the inventory list. Alternatively, select "BOND Research Detection 2" and click **Details**.

Figure B.5 Reagent Inventory



Package type – Choose Research Systems

Registering Ancillary Reagents

NOTE: For the life of the kit, three 30 mL open containers are needed for the Rinse ancillary reagent. Also, depending on the number of probes and/or proteinase dilutions being used, more than one Open container may be required for these ancillary reagents.

1. Scan the barcode on the side of a new 30 mL Open container.
2. In the dialog box that appears (Figure B.6), make a selection from the drop-down lists to assign the following to the barcode:
   - *Reagent name*
   - *Lot N°*
   - *Expiry date*

Figure B.6 Adding a BOND 30 mL Open Container

3. Apply the appropriate color-coded label (supplied in the in the ViewRNA eZ Detection Kit) to the assigned containers (see Table B.1 on page 43). Click **OK**.
4. Repeat Step 2 to Step 3 to register the other ancillary reagents.

NOTE: See *8.2.1 Adding and Editing a Reagent* in the Leica BOND RX User Manual for instructions on managing reagents in the Reagent Setup screen (for example, add new reagents, edit reagent details, delete reagents).

Pairing a Staining Protocol With BOND Research Detection 2

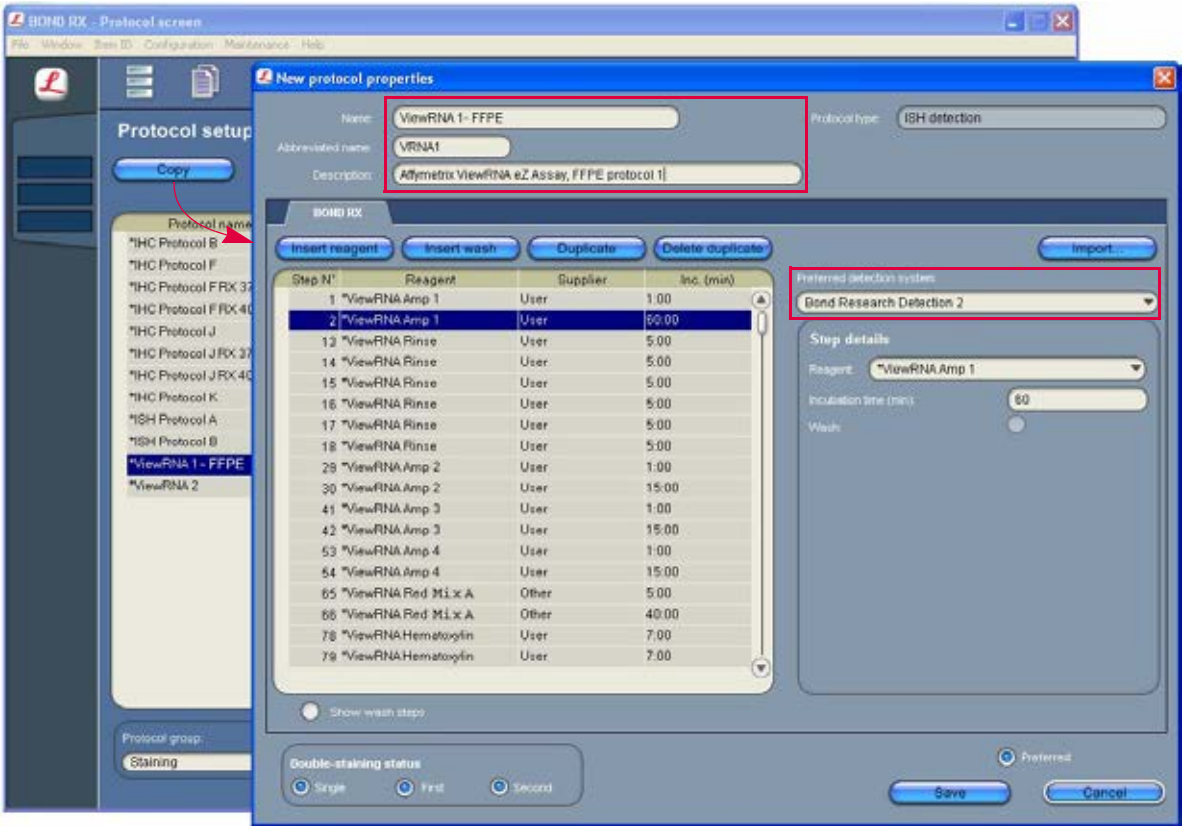
After reagent containers have been registered, the default protocol will need to be copied and paired with the BOND Research Detection 2 before proceeding to slide setup (*Step 2: Create a Study and Add Slides on page 13*).

To prepare the default staining protocol and pair it with BOND Research Detection 2:

1. Click the  icon to open the "Protocol setup" window.
2. Click once to highlight the "*ViewRNA 1 - FFPE". Select **Copy** (Figure B.7).
3. In the "New protocol properties" window that opens, remove the "*" from the *Name* and *Abbreviated name*. If necessary, give the protocol a new name and a new abbreviated name (Figure B.7).
4. Select from the drop-down menu the "Preferred detection system" that you want to use or the one that you just registered (i.e. "BOND Research Detection 2" unless a different name was used) (Figure B.7).
5. If not using DAPI staining, go to Step 7.
6. To insert the DAPI counterstaining steps (optional):
   A. Click [Show wash steps] to expand the protocol. Scroll down and highlight the last step of the protocol which is "Deionized water".
   B. Click **Insert reagent** and **Above**, then select "ViewRNA eZ DAPI" from the Reagent drop-down menu to insert the DAPI counterstaining step. Enter [1:00] minute for *Incubation time (min)*.
   C. To insert deionized water wash after the DAPI step, click **Insert wash**, select **Deionized water** for *Reagent*, and **[0:00]** for *Incubation time (min)*. Repeat this step twice to add a total of three washes.
7. Click **Save protocol**.

   The system will display the message: "The protocol you are saving has not been validated by Leica Microsystems. Do you want to continue?" Click **OK** to save the modified protocol.
8. Verify each step in the protocol:
   A. Highlight the saved protocol and click **Report**.
   B. Review the steps, including the reagents, incubation time, and temperature.

Figure B.7 Pairing a Staining Protocol With BOND Research Detection 2



Creating a New Enzyme Protocol

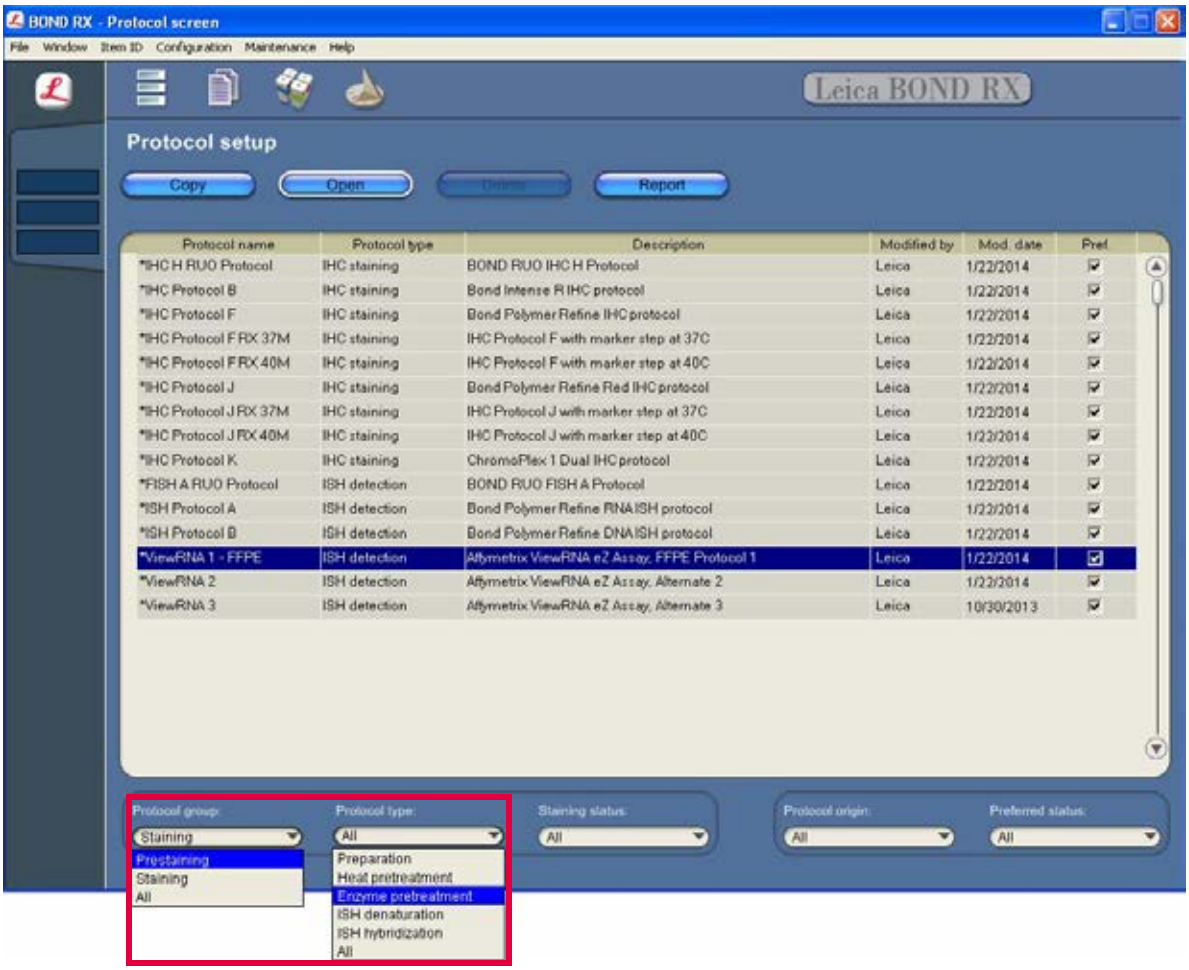
The Enzyme protocol specifies the proteinase incubation parameters. If the Enzyme protocols available on Leica BOND RX do not include the optimal proteinase digestion time for your tissue, you will need to create a new protocol.

Factory-loaded protocols, denoted by an asterisk (*), cannot be modified. Instead, copy a protocol, edit the copy, and save it to a new name.

To Create a New Enzyme Protocol:

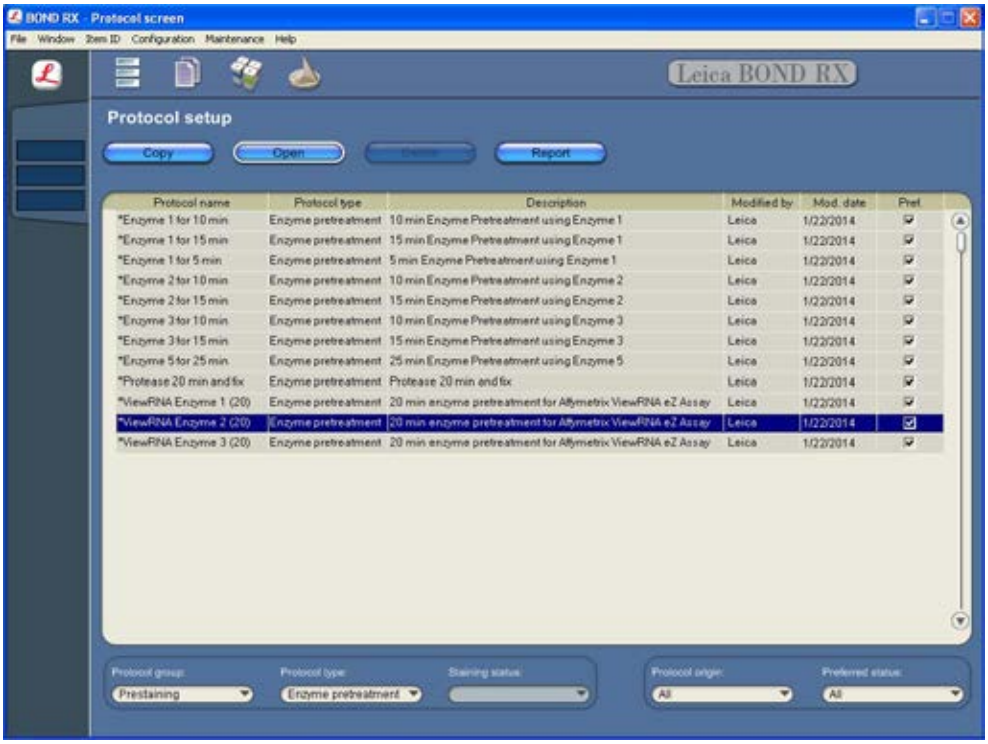
1. Go to the Protocol setup window (click the  icon).
2. Select *Prestaining* for Protocol group and *Enzyme pretreatment* for Procotol type (Figure C.1).

Figure C.1 Select Protocol Group and Protocol Type



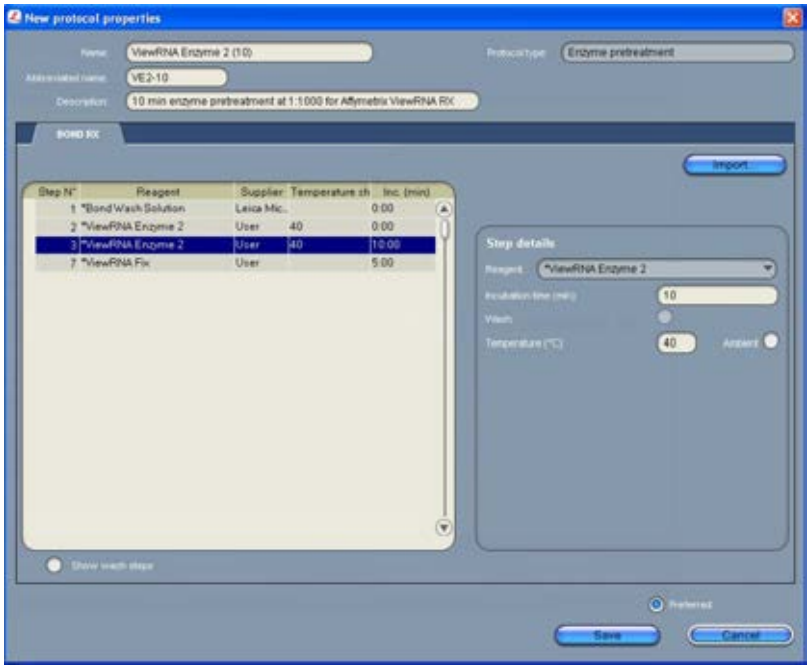
3. Select a protocol to modify: *ViewRNA enzyme 1, *ViewRNA enzyme 2, or *ViewRNA enzyme 3. Click **Copy** (Figure C.2).

Figure C.2 Select a Protocol to Modify



4. In the dialog box that appears, enter:
   - A new name and abbreviated name. Do not include an asterisk (*) in the name (Figure C.3).
   - Edit the description to match the new enzyme protocol.

Figure C.3 Enter New Protocol Name and Description



5. Select Step 3 of the enzyme protocol and change the Incubation time (min). Click **Save**.

Microscopy and Imaging Equipment Guidelines

Table D.1 ViewRNA eZ Assay Imaging Options

Viewing and Digital Capturing Options	Microscope Type	Recommended Microscope/System	Optics Required	Recommended Filter
Bright field viewing	Standard pathology grade bright field microscope	▪ Leica DM Series ▪ Nikon E Series ▪ Olympus BX Series ▪ Zeiss Axio Lab/ Scope/Imager ▪ Or equivalent	20 and 40X objectives	Requires neutral density filters and/or color filters for white balancing.
Fluorescence viewing and image capture	Microscope with camera and fluorescence options. Verify that the camera does not have infrared blocking filter.	▪ Leica DM Series ▪ Nikon E Series ▪ Olympus BX Series ▪ Zeiss Axio Lab/ Scope/Imager ▪ Or equivalent	▪ 20 and 40X objectives ▪ Numerical Aperture (NA) > 0.5	For Fast Red Substrate, use Cy3/TRITC filter set: Excitation 530 ± 20 nm Emission 590 ± 20 nm Dichroic: 562 nm For DAPI filter set: Excitation: 387/11 nm Emission: 447/60 nm
Automated image capture in bright field and/or fluorescence modes	Digital pathology scanner system	▪ Aperio ScanScope AT/XT/CS, use FL version for fluorescence ▪ Leica SCN400-F ▪ Olympus Nanozoomer RS ▪ Or equivalent	Scanning at 40X is recommended when expression is low.	Compatible with the above.

Reagent Preparation Volumes

Table E.2 on page 54 is a lookup table that provides a quick way to determine the volume of each reagent needed for a run, based on the number of slides being processed. The volumes in Table E.2 do not include the dead volume for BOND containers.

Take into account the dead volume when:

- Determining the total volume of Enzyme, Amp 4, or probe to prepare.
- Choosing a container (see Table E.1).

Table E.1 Dead Volumes for BOND Containers

Type of Container	Dead Volume	Combined Usable and Dead Volume
BOND Open Container, 7 mL	2 mL	7 mL
BOND Open Container, 30 mL	2.5 mL	30 mL
BOND Titration Container	550 µL	6 mL

Table E.2 Lookup Table – Volume of Each Reagent Needed for a Run

Number of Slides	Research Detection 2 (µL)	Ancillary Reagents (µL)							
		Fix	Rinse	Probe	Red 1	Red 2	DAPI	Amp 4	Enzyme
5	**1500**	**750**	**4500**	**1100**	**1125**	**375**	**750**	**1500**	**1000**
6	1800	900	5400	1320	1350	450	900	1800	1200
7	2100	1050	6300	1540	1575	525	1050	2100	1400
8	2400	1200	7200	1760	1800	600	1200	2400	1600
9	2700	1350	8100	1980	2025	675	1350	2700	1800
10	**3000**	**1500**	**9000**	**2200**	**2250**	**750**	**1500**	**3000**	**2000**
11	3300	1650	9900	2420	2475	825	1650	3300	2200
12	3600	1800	10800	2640	2700	900	1800	3600	2400
13	3900	1950	11700	2680	2925	975	1950	3900	2600
14	4200	2100	12600	3080	3150	1050	2100	4200	2800
15	**4500**	**2250**	**13500**	**3300**	**3375**	**1125**	**2250**	**4500**	**3000**
16	4800	2400	14400	3520	3600	1200	2400	4800	3200
17	5100	2550	15300	3740	3825	1275	2550	5100	3400
18	5400	2700	16200	3960	4050	1350	2700	5400	3600
19	5700	2850	17100	4180	4275	1425	2850	5700	3800
20	**6000**	**3000**	**18000**	**4400**	**4950**	**1500**	**3000**	**6000**	**4000**
21	6300	3150	18900	4620	5175	1575	3150	6300	4200
22	6600	3300	19800	4840	5400	1650	3300	6600	4400
23	6900	3450	20700	5060	5625	1725	3450	6900	4600
24	7200	3600	21600	5280	5850	1800	3600	7200	4800
25	**7500**	**3750**	**22500**	**5500**	**6075**	**1875**	**3750**	**7500**	**5000**
26	7800	3900	23400	5720	6300	1950	3900	7800	5200
27	8100	4050	24300	5940	6525	2025	4050	8100	5400
28	8400	4200	25200	6160	6750	2100	4200	8400	5600
29	8700	4350	26100	6380	6975	2175	4350	8700	5800
30	**9000**	**4500**	**27000**	**6600**	**7200**	**2250**	**4500**	**9000**	**6000**

NOTE: Add a dead volume to the volumes in Table E.2 as follows:

- **2.5 mL dead volume when using a BOND 30 mL Open container.**
- **2 mL dead volume when using a BOND 7 mL Open container.**
- **550 µL when using a BOND Titration container,**