60009/60010

Instructions for use

NaveniFlex™ on the BOND RX/RX^{m} staining instruments

NaveniFlex BOND RX/RX^{m} is a flexible method for *in situ* proximity ligation assay on FFPE tissue or cell sections with a fluorescent readout. It is intended to be used together with two unlabeled primary antibodies, one each from mouse and rabbit hosts, targeting either different proteins in an interaction or multiple sites on the same protein.

The NaveniFlex protocol has been optimized for use on the BOND RX/RX^{m} staining instruments from Leica Biosystems. The stained slides can be imaged with any fluorescence microscope or scanner that supports the recommended filter set (DAPI, FITC, Cy3, TexasRed and Cy5). The NaveniFlex BOND RX/RX^{m} kit contains reagents to stain 30 slides for a maximum of 4 individual runs. There are two different fluorophores available to choose from for the readout.

For research use only.

Materials

Kit components

Box 1:

*Storage at +4 to +8°C. DO NOT FREEZE

Material	Art.no	Amount
Naveni Block	50014	6.6 mL
Naveni Diluent	50015	2x15 mL
Navenibody M1	50050	300 µL
Navenibody R2	50052	300 µL

Box 2:

*Storage at -25 to -15°C. FREEZE

Material	Art.no	Amount
Naveni Buffer 1 (5x)	50018	1.3 mL
Naveni Enzyme 1 (20x)	50035	300 µL
NaveniFlex™ Tissue Buffer 2 (5x)	50021	2.3 mL
Naveni Enzyme 2 (20x)	50036	600 µL
Naveni Post-block (40x)	50033	300 µL
Naveni Detection Mix Atto647N/Red (5x)**	50038/50040	1.3 mL

* See Certificate of Analysis for expiration date.
** The kit is supplied with the chosen fluorophore.

Additional Materials and Reagents required to run NaveniFlex BOND RX

These materials are not included in the kit and must be purchased separately.

Antibodies	
Mouse Antibody 1	Use an antibody of choice
Rabbit Antibody 2	Use an antibody of choice

BOND RX Materials	**Leica, Cat #**
BOND Titration Kit (6mL)	OPT9049
BOND Research Detection System (BOND Research Detection System 2)	DS9455 (DS9777)
BOND Universal Covertiles	S21.4611
Slide Tray	S21.0304
Reagent Tray	S21.1003
Slide Labels and Printer Ribbon	S21.4564
BOND Dewax Solution	AR9222
BOND Epitope Retrieval Solution 1	AR9961
BOND Epitope Retrieval Solution 2	AR9640
BOND Wash Solution 10X Concentrate	AR9590
BOND Aspirating Probe Cleaning Kit	CS9100

Laboratory materials	**Vendor, Cat #**
Mounting medium for fluorescent dyes	Sigma-Aldrich, F6182*
Coverslip for microscope slides	
DAPI (diluted in PBS to 1 µg/mL)**	Thermo Fisher Scientific, 62248
Deionized water	
Reagent Grade Alcohol	Refer to the BOND RX 7.0 User Manual
TBS-T (T=Tween)	

*Fluoroshield mounting medium or any mounting medium compatible with fluorescens dyes.
**It is possible to exclude the DAPI staining step in the instrument stain with DAPI off-board.

Software requirements to run NaveniFlex BOND RX

- BOND RX v7.0 software
- BXD 40

General guidelines

- Use best practices when pipetting to ensure accurate reagent consumption.
- Thoroughly defrost all buffer mixtures at room temperature. Vortex well and spin down before use.
- Gently mix and spin down Naveni Enzymes 1 and 2 before use.
- Keep the enzyme stocks on ice or in a frozen cold block during preparation.
- Prepare all reagents to the correct dilution in 6 mL or 30 mL BOND Titration Container Inserts.
- It is recommended to mix the reagents using a pipette. Remove any bubbles that may have formed.
- The dispense volume for all reagent steps is 150 µL. For a double dispense, the required volume for each slide is 300 µL. For a 6 mL Titration Insert, a dead volume of 500 µL is required in addition to the dispense volumes and this has been included in the example tables for reagent preparation. For a 30 mL Titration Insert, the dead volume is 2 mL.
- All NaveniFlex BOND reagents are benchtop stable for at least 24 hours after preparation.
- The duration of the NaveniFlex protocol is 11-12 hours on the BOND RX and BOND RX^{m}, depending on the number of slides and conditions. This includes Preparation (Dewax), and Prestaining (Antigen Retrieval), and the Staining protocols.
- When the NaveniFlex BOND staining protocol is complete, there will be a continuous addition of Bond Wash Solution until the slides are removed from the instrument.
- Fluoroshield Mounting Medium from Sigma is recommended for mounting the slides, but in practice any mounting medium for fluorescence can be used.
- Any DAPI can be used for the nuclear counterstain. If uneven DAPI stain occurs, the DAPI step can be deleted from the protocol and a mounting medium with DAPI can be used instead.
- As with any product derived from biological sources, proper handling procedures should be used.
- Wear appropriate personal protective equipment to avoid contact with eyes and skin.
- Unused solutions should be disposed of according to local regulations.
- Follow best practice and recommended maintenance of the BOND RX/RX^{m}, as per the BOND RX 7.0 User Manual.

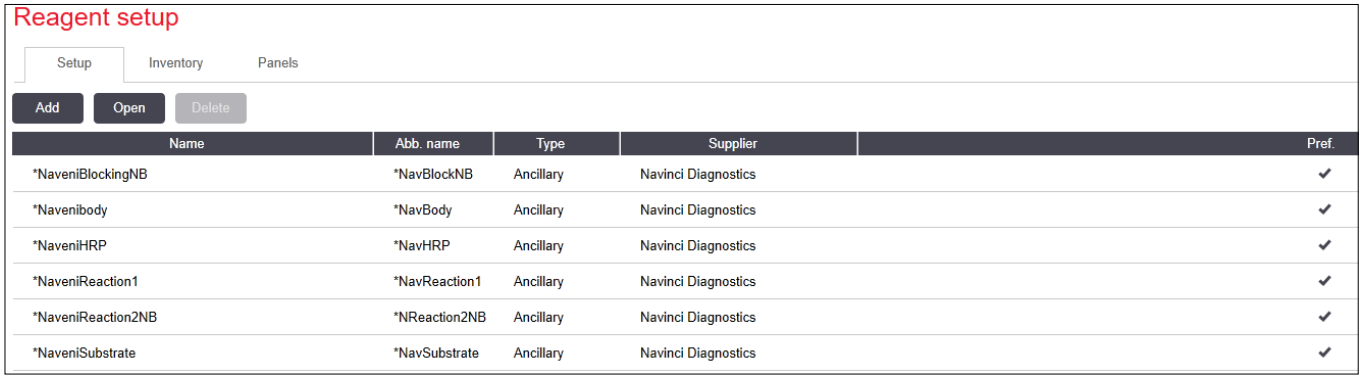
Visit the FAQs at www.navinci.se/faq or contact us at contact@navinci.se for any type of inquiries.

Sample preparation

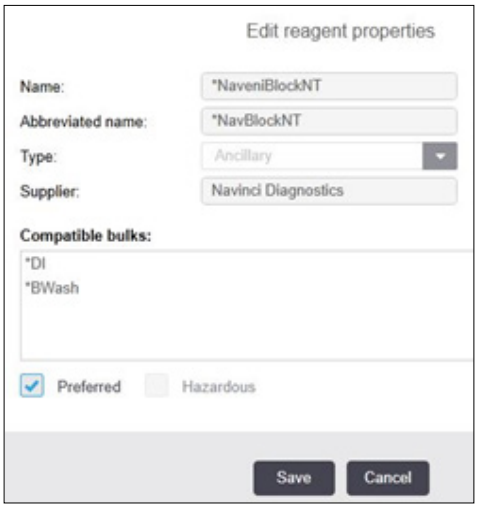
1. The FFPE tissue should be sectioned at 4 µm and a single section should be placed on the glass slide as recommended in the Leica BOND RX and RX^{m} user instructions.
2. Bake the slides for at least 45 min at 60°C in an oven or use the already established sample preparation protocol in the lab. Slides should then be loaded onto the BOND RX/RX^{m} for dewaxing and epitope retrieval. Baking can also be performed on the BOND RX/RX^{m}, using *Bake and Dewax.

Prepare the Naveni® reagents (one-time only)

1. Open the **"Reagent Setup"** screen in the BOND RX software and set the bottom right filter (Preferred Status) to **"All"**. Scroll through the reagents until the NaveniFlex reagents are visible.

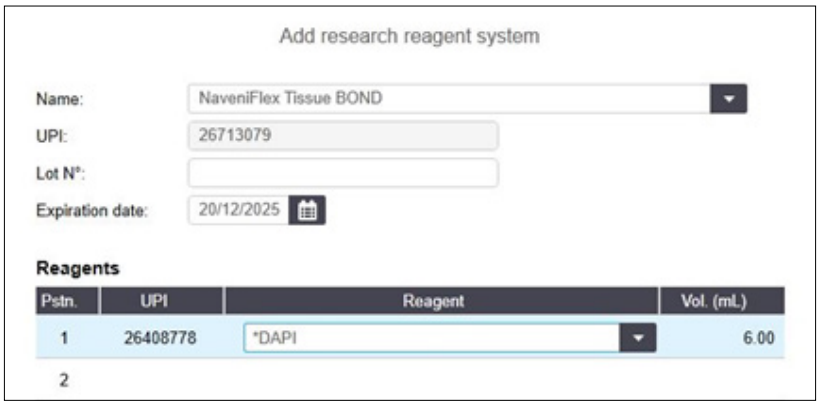


2. One at a time, open each reagent, mark as **"Preferred"** and **"Save"**. Refer to **Table 1** for a list of reagents.
3. Ensure you also prefer ***DAPI**.



Create a BOND Research Detection System

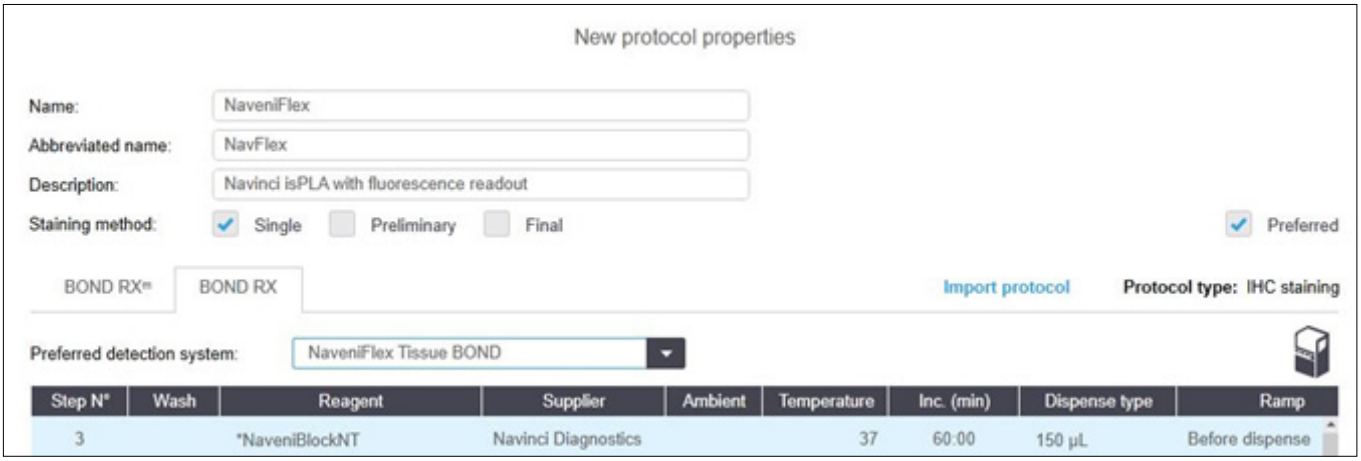
1. Scan the barcode on the side of the BOND Research Detection System
2. Give the **Detection System** a name (e.g., NaveniFlex BOND) and assign an **Expiry Date**.
3. Scan the barcode of a new Titration Container (the UPI will appear in Line 1) and select ***DAPI** from the drop-down menu.



4. Click on **"Add"**.
5. Label the **Titration Container** with DAPI and place it in position 1 of the detection tray.
6. This Detection System can be used for 200 tests.

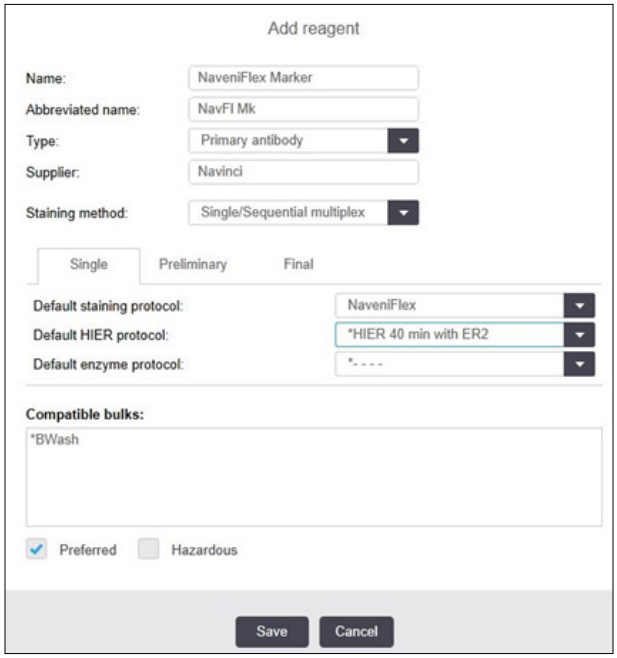
Create the User-defined Protocol

1. Open the **“Protocol Setup”** screen in the BOND RX software and set the bottom right filter (Preferred Status) to **“All”**. Scroll through the protocols until the *NaveniFlex protocol is visible in the Reagent Setup screen, highlight and click on **“Copy”**.
2. Give the protocol a new **Name**, **Abbreviated Name** and **Description**.
3. Select your Research Detection System, from above, as the **“Preferred Detection System”**
4. Tick the box for **“Preferred”** (top, right corner).
5. Click on **“Save”** and acknowledge the Validation notification.

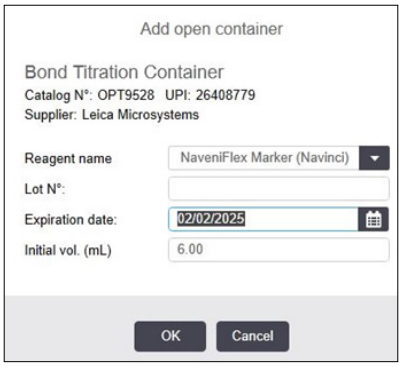


Create the User-defined Primary Antibody (*Marker)

1. In the **“Reagent Setup”** screen, click on **“Add”**.
2. Enter in a **Name** and **Abbreviated Name** for your Antibody.
3. Select **“Primary Antibody”** for the reagent type.
4. Enter in **Navinci** for the Supplier.
5. Select your **user-defined protocol**, created above, for the Staining Protocol.
6. Select **“HIER2 for 40 minutes with ER2”** for the HIER protocol.
7. Click on **“Add”**.



8. Scan the barcode of a new 6 mL Titration Container and select your antibody from above. Enter in an **Expiry Date** and click **"OK"**. Label the container with the antibody's name.



9. Label each of the remaining Titration Containers with the required reagent names, according to **Table 1** (excluding *DAPI and your Primary Antibody). Scan the barcode on the front of the container and select the corresponding name. Enter in an **Expiry Date** and click **"OK"**.
10. Add a new Titration Insert (tube) to each container.

Table 1: Titration Containers.

Reagent step	Container name/Reagent name in software	Reagent per slide	
		# Dispenses	µL per slide
Naveni Block	*NaveniBlockNT	1	150
Navenibodies M1 and R2	*Navenibody	2	300
Reaction 1	*NaveniReaction1	1	150
Reaction 2	*NaveniReaction2NT	2	300
Naveni Post-block	*NaveniPostBlock	2	300
Naveni Detection Mix	*NaveniDetection	1	150
Primary antibodies*	Interaction pair (Marker)	1	150
DAPI (or equivalent)	*DAPI	1	150

*Any primary mouse and rabbit antibodies of choice against the pair of interacting proteins, in Naveni Diluent.

Reagent preparation

Note: *Prepared reagents are stable at room temperature for at least 24 hours. It is advised to prepare reagents fresh for every run. Add the Naveni® reagents to their respective Titration Containers/Inserts.*

The general formula for calculating the volume of reagents for a run is:

(Number of slides × 150 µL × number of dispenses) + 500 µL = Total volume for one 6 mL Titration Container
Or
When using over 6 mL of reagent, use two 6 mL containers

1. **NaveniBlockingNB** – single dispense (150 µL per slide)
   1.1. Add the appropriate volume of **Naveni Block** to the Titration Insert e.g., for 10 slides, the total volume would be 2000 µL.
2. **Primary Antibodies** – single dispense (150 µL per slide)
   2.1. Use **Naveni Diluent** to dilute both the mouse and rabbit primary antibodies in the same container. Several antibodies or antibody pairs can be used during the same BOND RX/RX^{m} run. To use additional antibodies or antibody pairs, they will have to be **"Added"** into the Reagent Setup Screen with individual **Names** and **Abbreviated Names**. They will then be registered to individual 6 mL Titration Containers (as per above).
   2.2. Primary antibody concentrations need to be experimentally titrated for each antibody pair. When using pre-optimized antibodies from Navinci, the titration of antibodies has already been performed.
3. **NaveniBody** – double dispense (300 µL per slide)
   3.1. Dilute **Navenibody M1** and **Navenibody R2** **1:40** each in **Naveni Diluent**, see below for example calculations.

Kit component	Navenibody (µL) For 10 slides	Navenibody (µL) For 20 slides- need 2 titration containers*	Navenibody (µL) For 30 slides- need 2 titration containers*
Naveni Diluent	3325	3325	4750
Navenibody M1	87.5	87.5	125
Navenibody R2	87.5	87.5	125
Total	3500	3500	5000

* Total volumes in table are for each titration container.

4. **NaveniReaction1** – single dispense (150 µL per slide)
   4.1. Prepare **NaveniReaction1** by diluting **Naveni Buffer 1 (5x)** **1:5** and **Naveni Enzyme 1 (20x)** **1:20** in **Distilled Water**, see below for example calculations.
   4.2. Add **Naveni Enzyme 1 (20x)** last and mix gently by pipetting.

Kit component	NaveniReaction1 (µL) For 10 slides	NaveniReaction1 (µL) For 20 slides	NaveniReaction1 (µL) For 30 slides
Naveni Buffer 1 (5x)	400	700	1000
Naveni Enzyme 1 (20x)	100	175	250
Distilled Water	1500	2625	3750
Total	2000	3500	5000

* Total volumes in table are for each titration container.

5. **NaveniReaction2NT** – double dispense (300 µL per slide)

   5.1. Prepare **NaveniReaction2** by diluting **NaveniFlex™Tissue Buffer 2 (5x) 1:5** and **Naveni Enzyme 2 (20x) 1:20** in **Distilled Water**. Add **Naveni Enzyme 2 (20x)** last and mix gently by pipetting.

Kit component	NaveniReaction2NT (µL) For 10 slides	NaveniReaction2NT (µL) For 20 slides- need 2 titration containers*	NaveniReaction2NT (µL) For 30 slides- need 2 titration containers*
NaveniFlex™Tissue Buffer 2 (5x)	700	700	1000
Naveni Enzyme 2 (20x)	175	175	250
Distilled Water	2625	3000	5000
Total	3500	3500	5000

* Total volumes in table are for each titration container.

6. **NaveniPostBlock** – double dispense (300 µL per slide)

   6.1. Dilute the **Naveni Post-block 1:40** in **Naveni Diluent** to make a **Total Volume of 3500 µL, for 10 slides**. Mix gently by pipetting.

7. **NaveniDetection** – single dispense (150 µL per slide)

   7.1. Prepare the **NaveniDetection** working solution by diluting the **Naveni Detection Mix 1:5** in **Distilled Water** to make a **Total Volume of 2000 µL, for 10 slides**. Mix gently by pipetting.
   Note: In this step, optional immunofluorescence antibodies with a fluorophore, other than the ones used in the NaveniFlex kit, can be added for co-staining of additional proteins.

8. **Nuclear Stain**

   8.1. Prepare DAPI to 1 µg/mL, or use a nuclear stain of your choice, with a similar emission spectrum. Dilute it in PBS to a concentration recommended by the vendor. Place 2000 µL (for 10 slides) of this reagent in the Titration Insert for DAPI. If DAPI is stained offboard, add PBS, instead of DAPI, to the Titration Insert.

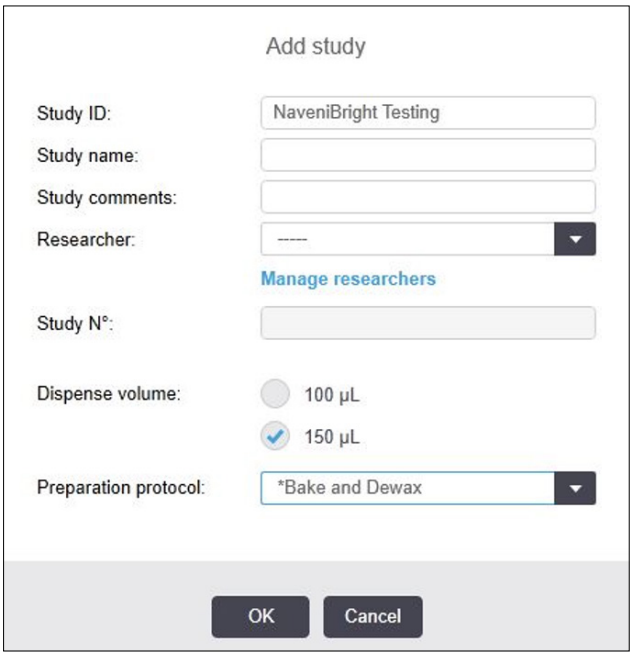
BOND RX/RX^{m} preparation

1. Follow all routines recommended by Leica Biosystems, as per the BOND RX v7.0 User Manual.
2. Turn on the BOND RX/RX^{m} instrument and open the BOND RX software- RX client.
3. Slides:

   3.1. Click on **"Add Study"** and fill in the appropriate fields. For the **Preparation Protocol** - if baking slides on-board, select *Bake & Dewax. If baking slides off-board, select *Dewax

   3.2. Click on **"Add Slides"** and fill in the appropriate fields. Select the desired primary antibody in the *Marker field and double-check the associated protocols are correct. If not change:

- Preparation = *Dewax or *Bake & Dewax
- HIER = *ER2 for 40 minutes (100 ° C) (unless the primary antibody has been optimised with an alternative protocol)
- Staining = *NaveniBright (or user-named protocol if a different detection system is to be used)



3.3. Click on **"Add"**, and repeat for subsequent slides (changing the *Marker selection if required).

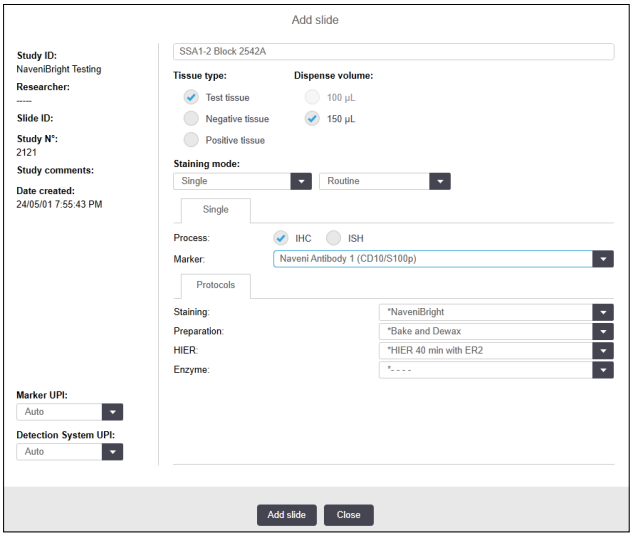
3.4. Once all slides have been added, click on **"Close"**.

3.5. Click on **"Print labels"** and select the middle option. Click **"Print"**.

3.6. Place the labels at the top of the slides. Avoid overhanging or positioning the label too low.

3.7. Place the slides onto the slide tray and cover with a BOND Covertile. Ensure the Covertile has been placed in the correct orientation.

3.8. Load the slide tray onto the BOND RX/RX^{m} and press the button to lock/lower the tray.



3.9. Once slides have been scanned, check the “System Status” screen in the BOND RX software to ensure all has been read correctly.

4. Reagents:

4.1. Load the Detection System Tray with the Naveni® reagents onto the BOND RX/RX^{m}. Once scanned (and dip tested), check the software UI for any notifications. Take action if needed.

4.2. The run can be started immediately or with a delayed start. The slides are stable in the instrument for up to 72 hours after staining has finished, and the 1X reagents are stable in the instrument for up to 24 hours.

For more guidelines using the BOND RX/RX^{m}, refer to the BOND RX v7.0 User Manual or visit the FAQs at www.navinci.se/faq or contact us directly for any type of inquiries.

Mounting

1. Unload the slides from the instrument and remove the Covertile (for cleaning). Remove the slides and soak in TBS-T for 5-15 minutes.
2. Remove slides and apply mounting medium followed by coverslips.
3. Dry slides either with centrifugation or by laying them flat.
4. Image with a fluorescent microscope, scanner or a confocal microscope.
5. Filter sets corresponding to your chosen nuclear stain and detection. Fluorophores ´excitation/emission spectra are required for imaging, see **Table 4**.
6. After imaging, store the slides in the fridge for optimal fluorophore stability.

Table 4. Filter sets for imaging.

Filter set	Detecting	λ Excitation	λ Emission
DAPI	Nuclei		
FITC	Immunofluorescence (Optional)	480-490 nm	525-535 nm
Cy3	Immunofluorescence (Optional)	545-555 nm	575-585 nm
Texas Red	Proximity signal detected by Detection Reagent NT Red	585-595 nm	615-625 nm
Cy5	Proximity signal detected by Detection Reagent NT Atto647	635-645 nm	665-675 nm