NAB.MR.030.H

# NaveniBright™ on the BOND RX/RXᵐ staining instruments

NaveniBright™ BOND RX/RXᵐ is a flexible method for *in situ* proximity ligation assay on FFPE tissue or cell sections with a chromogenic 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 NaveniBright™ protocol has been optimized for use on the BOND RX/RXᵐ staining instruments, from Leica Biosystems. The stained slides can be imaged with any brightfield microscope. The NaveniBright™ BOND RX/RXᵐ kit contains reagents to stain 30 slides with dead volumes included, for a maximum of 4 individual runs.

**For research use only.**

## Materials

### Kit components

#### Box 1:

*Storage at +4 to +8°C. DO NOT FREEZE




| Material | Art.no | Amount |
|---|---|---|
| NaveniBright BOND Block NB (1x) | NA.1.030.13 | 6.6 mL |
| NaveniBright BOND Supplement 1 | NA.1.030.22 | 0.7 mL |
| NaveniBright BOND Supplement 2 | NA.1.030.23 | 1.3 mL |
| NaveniBright BOND Diluent 1 NB (1x) | NA.1.030.14 | 11.5 mL |
| NaveniBright BOND Diluent 2 NB (1x) | NA.1.030.15 | 11.5 mL |
| NaveniBright BOND Navenibody M1 NB (40x) | NA.1.030.16 | 300 µL |
| NaveniBright BOND Navenibody R2 NB (40x) | NA.1.030.17 | 300 µL |
| NaveniBright BOND HRP Diluent NB (1x) | NA.1.030.24 | 6.6 mL |
| NaveniBright BOND HRP Reagent (200x) | NA.1.030.25 | 30 µL |
| NaveniBright BOND HRP Substrate 1 | NA.1.030.26 | 180 µL |
| NaveniBright BOND HRP Substrate 2 | NA.1.030.27 | 120 µL |
| NaveniBright BOND HRP Substrate 3 | NA.1.030.28 | 120 µL |
| NaveniBright BOND HRP Substrate 4 | NA.1.030.29 | 180 µL |

#### Box 2:

*Storage at -25 to -15°C. FREEZE




| Material | Art.no | Amount |
|---|---|---|
| NaveniBright BOND Buffer 1 NB (5x) | NA.2.030.18 | 1.3 mL |
| NaveniBright BOND Enzyme 1 NB (20x) | NA.2.030.20 | 330 µL |
| NaveniBright BOND Buffer 2 NB (5x) | NA.2.030.19 | 2.3 mL |
| NaveniBright BOND Enzyme 2 NB (20x) | NA.2.030.21 | 580 µL |

* See COA for expiration date.

# Additional Materials and Reagents required to run NaveniBright BOND

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/RX$^m$ Materials** | **Leica, Cat #** |
|---|---|
| BOND Titration Kit (6ml) | OPT9049 |
| BOND Intense R Detection System* | DS9263 |
| BOND Universal covertiles (pack of 160) | S21.4611 |
| BOND Slide Tray | S21.4586.B |
| BOND Reagent Tray | S21.1003 |
| Slide Labels and Printer Ribbon | S21.4564 (Zebra) or S21.4604 (Cognitive) |
| BOND Dewax Solution | AR9222 |
| BOND Epitope Retrieval Solution1 | AR9961 |
| BOND Epitope Retrieval Solution 2 | AR9640 |
| BOND Wash solution 10X Concentrate | AR9590 |
| BOND Aspirating Probe Cleaning Kit | CS9100 |

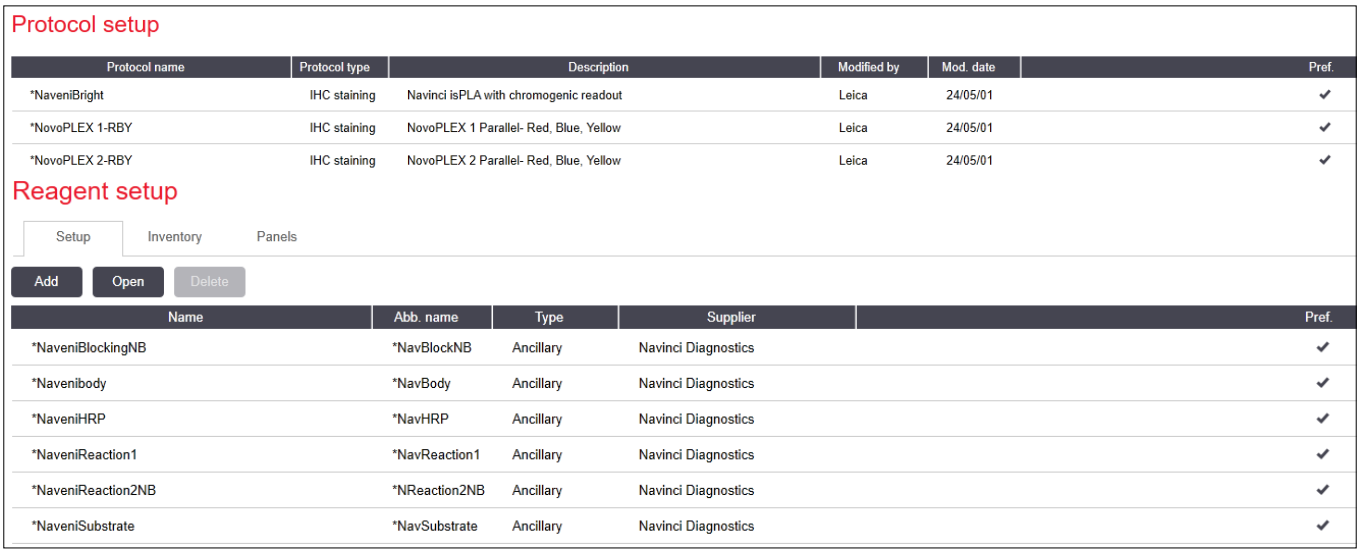
* Bond Polymer Refine Detection System (DS9800) can also be used.

| **Laboratory materials** | **Vendor, Cat #** |
|---|---|
| VectaMount® Express Mounting Medium* | Vector laboratories H-5700-60 |
| Glass coverslips | |
| 99.5% Isopropanol | |
| Deionized water | |
| Reagent Grade Alcohol | Refer to the BOND RX 7.0 User Manual |

*Other non-aqueous mounting mediums can be used.

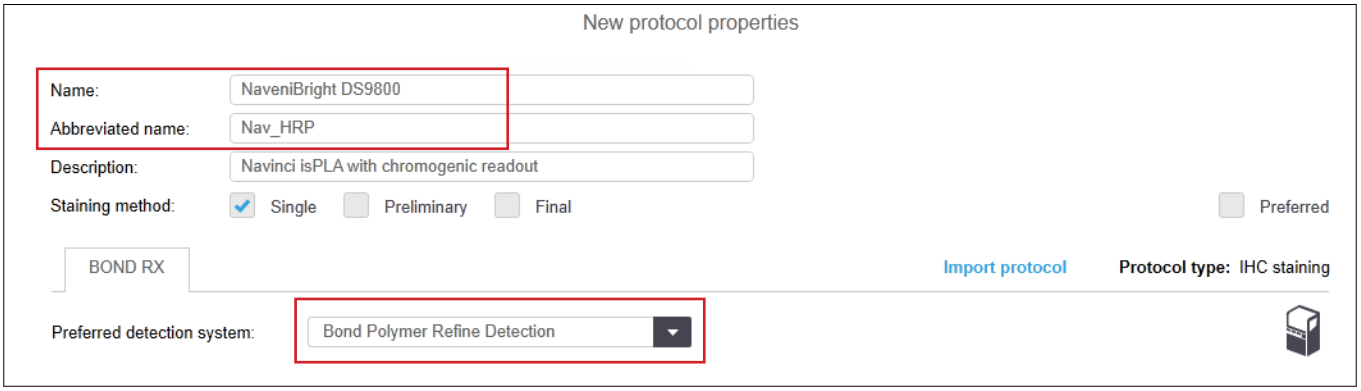
# Software requirements to run NaveniBright BOND

- BOND RX v7.0 software
- BXD
- Ensure that the *NaveniBright protocol and the NaveniBright reagents are “preferred” in the Protocol Setup and Reagent Setup screens. If not, open each one individually, check the ‘preferred’ box and save.



## General guidelines

- Use best practices when pipetting to minimize reagent consumption.
- Thoroughly defrost all buffer mixtures at room temperature. Vortex well and spin down before use.
- Gently mix and spin down 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.
- Any BOND Detection System that contains Peroxide Block and Hematoxylin can be used in combination with the NaveniBright protocol. In the *NaveniBright protocol, the Preferred Detection System is the *BOND Intense R Detection System*. If another detection system is chosen, the *NaveniBright protocol will need to be copied, renamed and the Preferred Detection System changed to the detection system to be used. The protocol can then be saved, ready for use.




- All NaveniBright BOND reagents are benchtop stable for at least 24 hours after preparation.
- The duration of the NaveniBright protocol is 9 hours on the BOND RX and BOND RX$^{m}$. This includes Preparation (Bake & Dewax) and Prestaining (Antigen Retrieval) and the Staining protocols.
- For best practice, we recommend using a delayed start of the BOND RX instrument. When the NaveniBright protocol is complete, there will be a continuous addition of DI Water until the slides are removed from the instrument. A delayed start will ensure that the time from the end of the run to mounting the slides is as short as possible, preserving the signal.
- The kit is validated with VectaMount® Express Mounting Medium from Vector Laboratories, but other non-aqueous mounting mediums can be used. Note that VectaMount® has a built-in clearing agent.
- Different mounting medium and clearing effects might have an influence on the signal intensity, such as prolonged incubation in ethanol.
- 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.
- Chromogens may be carcinogenic and should be handled with care.
- 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 on www.navinci.se/faq or contact us for any type of inquiries.*

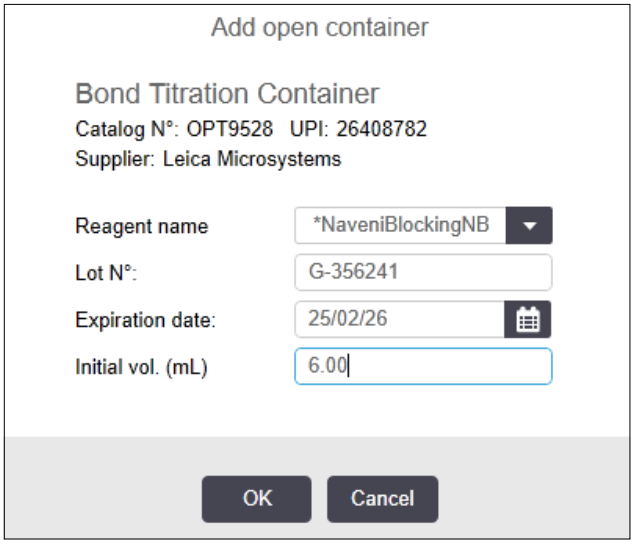
## Sample preparation

- The FFPE tissue should be sectioned at 4 µm and placed on the glass slide as recommended in the Leica BOND RX and RX$^m$ user instructions.
- 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.

## Assigning reagents to BOND Titration Containers

1. Assign each reagent to a new BOND Titration Container, as per **Table 1**.

   The Peroxide Block and Hematoxylin will be used from the preferred BOND Detection System.
2. Label each Titration Container and place it into a BOND Reagent Tray, ensuring a fresh 6 mL Titration Insert is placed in each container.

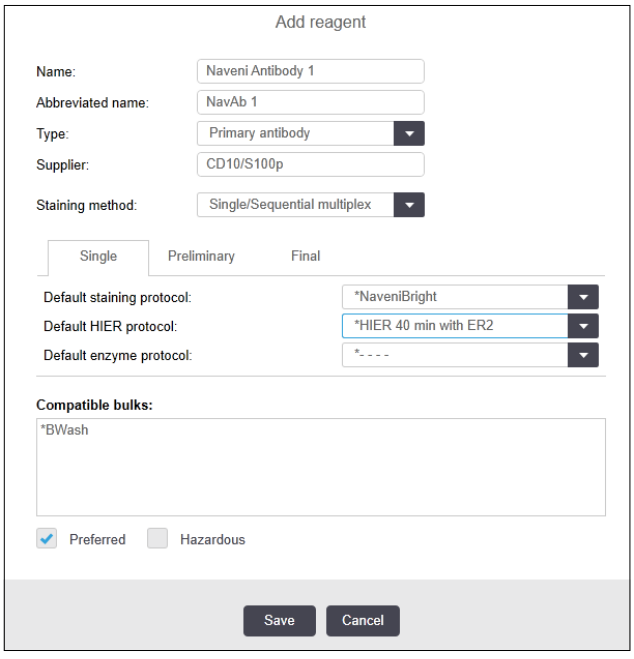



### Table 1: Assigning reagents to the BOND Titration containers.

| Reagent step | Container name/Reagent name in software |
|---|---|
| Blocking | NaveniBlockingNB |
| Primary antibody^ (*Marker) | User to define reagent name# |
| Navenibodies | Navenibody |
| Reaction 1 | NaveniReaction1 |
| Reaction 2 | NaveniReaction2NB |
| HRP Incubation | NaveniHRP |
| Substrate | NaveniSubstrate |

^Any primary mouse and rabbit antibodies of choice that bind to the protein target interaction or single protein target.

#The Primary Antibody pair will need to be added as a new reagent (in the Reagent Setup tab), in the software, before it can be assigned to a Titration Container. If more than one antibody pair is to be run simultaneously, each pair will need to be added to the software with a unique name. Assign the appropriate protocols to the Primary Antibody when adding the reagent. Controls such as a diluent or single antibody can also be added in this step. Refer to BOND RX/RX$^m$ Preparation, on page 6.



## 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 a 6 mL titration container**

or

**When using over 6 ml of reagent, use 2 pcs of 6 mL containers**

**Blocking –** single dispense (150 µL per slide)

1. Prepare **NaveniBlockingNB** by diluting **Supplement 1** **1:9** in **Block NB (1x)** in a titration insert, see below for example calculations.

| Kit component | Block NB (µL)<br>For 10 slides | Block NB (µL)<br>For 20 slides | Block NB (µL)<br>For 30 slides |
|---|---|---|---|
| Block NB | 1778 | 3111 | 4444 |
| Supplement 1 | 222 | 389 | 556 |
| **Total** | 2000 | 3500 | 5000 |

**2. Diluent 1, for Primary Antibodies** – single dispense (150 µL per slide).

2.1. Prepare the **Diluent 1** by diluting **Supplement 2** **1:9** in **Diluent 1 (1x)**, see below for example calculations.

| Kit component | Diluent 1 (µL)<br>For 10 slides | Diluent 1 (µL)<br>For 20 slides | Diluent 1 (µL)<br>For 30 slides |
|---|---|---|---|
| Diluent 1 NB (1x) | 1778 | 3111 | 4444 |
| Supplement 2 | 222 | 389 | 556 |
| **Total** | 2000 | 3500 | 5000 |

2.2. Use the prepared **Diluent 1** 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. When using additional antibodies or antibody pairs, each one will need to be added to the software with a unique name. Primary antibody concentrations need to be experimentally titrated for each antibody pair. Remember that more Diluent 1 needs to be prepared if multiple controls or antibody pairs are used.

**3. Navenibody** – double dispense (300 µL per slide)

3.1. Dilute **Navenibody M1 NB** and **Navenibody R2 NB** **1:40** each in **Diluent 2 NB (1x)**, see below for example calculations.

| Kit component | Navenibody (µL)<br>For 10 slides | Navenibody (µL)<br>For 20 slides- need 2 titration containers* | Navenibody (µL)<br>For 30 slides- need 2 titration containers* |
|---|---|---|---|
| Diluent 2 NB (1x) | 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 **Buffer 1 NB (5x)** **1:5** and **Enzyme 1 NB (20x)** **1:20** in Distilled Water, see below for example calculations.

   4.2. Add Enzyme 1 NB (20x) last and mix gently by pipetting.

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

5. **NaveniReaction2NB** – double dispense (300 µL per slide)

   5.1. Prepare **Reaction 2** by diluting **Buffer 2** **1:5** and **Enzyme 2** **1:20** in distilled water. Add Enzyme 2 last and mix gently by pipetting.

| Kit component | NaveniReaction2NB (µL) For 10 slides | NaveniReaction2NB (µL) For 20 slides- need 2 titration containers* | NaveniReaction2NB (µL) For 30 slides- need 2 titration containers* |
|---|---|---|---|
| Buffer 2 NB (5x) | 700 | 700 | 1000 |
| Enzyme 2 NB (20x) | 175 | 175 | 250 |
| Distilled Water | 2650 | 2625 | 3750 |
| **Total** | 3500 | 3500 | 5000 |

* Total volumes in table are for each titration container.

6. **NaveniHRP** – single dispense (150 µL per slide)

   6.1. Dilute the **HRP Reagent (200x)** reagent **1:200** in **HRP Diluent NB (1x)**, see below for example calculations.

| Kit component | NaveniHRP (µL) For 10 slides | NaveniHRP (µL) For 20 slides | NaveniHRP (µL) For 30 slides |
|---|---|---|---|
| HRP Reagent (200x) | 10 | 17.5 | 25 |
| HRP Diluent NB (1x) | 1990 | 3482.5 | 4975 |
| **Total** | 2000 | 3500 | 5000 |

7. **NaveniSubstrate** – double dispense (300 µL per slide)

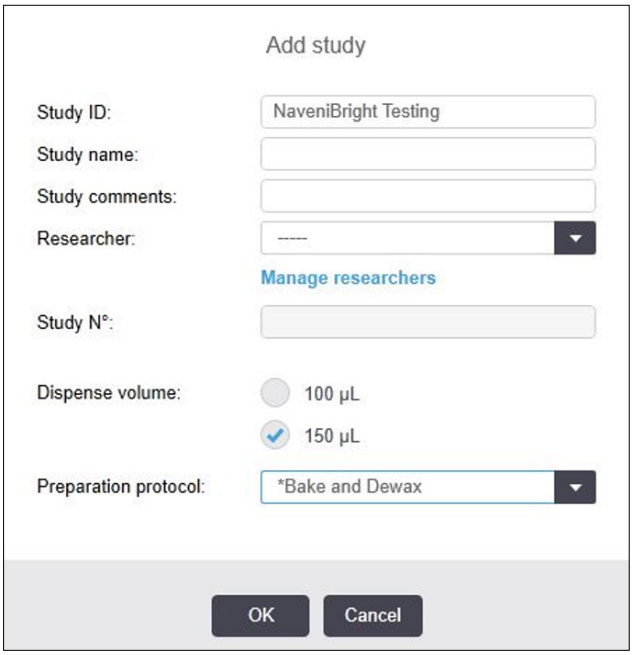
   7.1. Prepare the **NaveniSubstrate** solution by mixing **HRP Substrate 1** **(dilute 62.5x)**, **HRP Substrate 2** **(dilute 100x)**, **HRP Substrate 3** **(dilute 100x)** and **HRP Substrate 4** **(dilute 62.5x)** in Distilled Water, see example calculation below.

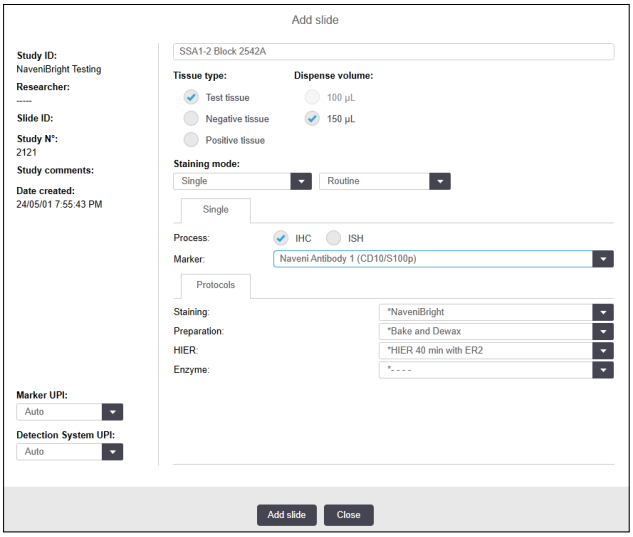
| Kit component | Navenibody (µL) For 10 slides | Navenibody (µL) For 20 slides- need 2 titration containers* | Navenibody (µL) For 30 slides- need 2 titration containers* |
|---|---|---|---|
| Distilled water | 3318 | 3318 | 4740 |
| HRP Substrate 1 | 56 | 56 | 80 |
| HRP Substrate 2 | 35 | 35 | 50 |
| HRP Substrate 3 | 35 | 35 | 50 |
| HRP Substrate 1 | 56 | 56 | 80 |
| **Total** | 3500 | 3500 | 5000 |

* Total volumes in table are for each titration container.

# Leica 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 software GUI to ensure all has been read correctly
4. Reagents:
   - 4.1. For the first use, register the BOND Intense R Detection System (or an alternative BOND Detection System).
   - 4.2. Load the Detection System and the BOND Reagent Tray, with the Naveni® reagents, onto the BOND RX/RX$^m$. Once scanned (and dip tested), check the software GUI for any notifications. Take action if needed.
5. Starting the Run:
   - 5.1. Once the start button is active for each run, right click on a slide in the first SSA and select "Delayed Start". Enter in the desired start time ***(estimated by calculating the desired end time minus run time (~hours for BOND RX and BOND RX$^m$). Click on OK.***
   - 5.2. Note the scheduled finish time - ***it is recommended to take out the slides from the BOND RX/RX$^m$ 1-2 hours after the run has been completed.***

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

## Dehydration and Mounting

6. Ending the Run:
   6.1. Remove the slide trays from the instrument.
   6.2. Remove the covertiles from the slide tray and put aside for cleaning.
   6.3. Remove the slides from the slide tray and place them in a slide rack, submerged in tap water**.
   ***Do not allow to stand in the tap water for longer than 5 minutes.***
7. Dehydrate and Coverslip:
   7.1. Dehydrate the slides using a 2 x 1 min washes in 99.5% Isopropanol.
   7.2. Remove excess isopropanol from the slides and mount directly with VectaMount® Express Mounting Medium in a fume hood. It is generally recommended to use this mounting medium, since it includes a clearing reagent. If any other mounting medium is used, refer to the guideline for that mounting medium.
   7.3. Apply coverslips and allow slides to dry flat at room temperature for 10 to 20 minutes.
8. Imaging and Storage:
   8.1. Image with a brightfield microscope.
   8.2. After imaging, store the slides at room temperature.

**Water quality may have an effect on color and hue. Other reagents such as Scott's tap water, ammonia water or other bluing solutions can also be used.

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