6197 Cornerstone Court E, Ste 102
San Diego, CA 92121

UltraPlex mxIF
Multiplex Immunofluorescence Staining Protocol – suggested for BOND RX

For use with ***UltraPlex mxIF*** "A" or "B" panels. "A" panels are labeled with 490, 550, 650 and 750 nm fluors. Please ensure that your imaging scanner or fluorescent microscope can detect these wavelengths. Alternatively, you may want to use a "B" kit labeled with 490, 550, 594 and 650 nm fluors. Please contact us if you have any questions about which kits to select. "B" panels are commonly used with spectral imaging scanners.

"A & B" panels are optimized for use on FFPE tissue sections.

Materials Provided – Store all components at 2-8ºC

1. Vial 1: Antibody Diluent Solution
2. Vial 2: Protein Block Solution
3. Vials 3-6: Individual Primary Antibody-Hapten Conjugates (suggested dilution for use is 1/100)
4. Vials 7-10: Individual Secondary Anti-Hapten Fluor Conjugates (suggested dilution for use is 1/100)

Required Reagents/Equipment from Leica:

Leica Cat #	Description
AR9640	BOND Epitope Retrieval Solution 2- 1L (RTU)
AR9222	BOND Dewax Solution- 1 L (RTU)
AR9590	BOND Wash Solution 10X Concentrate – 1 L
S21.2001	BOND Universal Covertiles 100 pack
OPT9049	BOND Titration Kit (includes 6 mL Titration containers and inserts)
DS9455	BOND Research Detection System
S21.1003.D	Reagent Tray
	BOND RX/RX^{m} Fully Automated Research Stainer

User-Supplied material

Description
Cover Glass 24 x 50mm
Deionized Water
Reagent-grade Alcohol
Suggested mounting medium, Fluoroshield plus DAPI (ImmunoBioSciences, Inc, Cat # AR- 6501-01)

Tissue Preparation:

Formalin fixed paraffin embedded (FFPE) sections should be cut to 3 -- 5 µm thickness and evenly spaced across slide surface. All tissue should be mounted on positively charged slides for enhanced adherence. Dry/bake the slides as per your routine IHC/IF processes for BOND RX/RX^{m}.

BOND RX/RX^{m} Protocols to use

Preparation: BOND RX *Dewax
HIER: *HIER 20 minutes with ER2
Note: For HI-1A and HI-1B panels it is recommended to use *HIER 10 minutes with ER2
Staining: Custom Staining protocol (assigned as Cell IDx mxIF staining protocol in this document)

SUGGESTED BOND RX PROTOCOL

BOND RX dewax protocol *D
Step Reagent
1 *Bond Dewax Solution
Supplier: Leica Microsystems
Step type: Reagent Inc. (min): 0:30 Temperature: 72 Dispense type: 150 µL

Step Reagent
2 *Bond Dewax Solution
Supplier: Leica Microsystems
Step type: Reagent Inc. (min): 0:00 Temperature: 72 Dispense type: 150 µL

Step Reagent
3 *Bond Dewax Solution
Supplier: Leica Microsystems
Step type: Reagent Inc. (min): 0:00 Temperature: Ambient Dispense type: 150 µL

Step Reagent
4 *Alcohol
Supplier: Not applicable
Step type: Reagent Inc. (min): 0:00 Temperature: Ambient Dispense type: 150 µL

Step Reagent
5 *Alcohol
Supplier: Not applicable
Step type: Wash Inc. (min): 0:00 Temperature: Ambient Dispense type: 150 µL

Step Reagent
6 *Alcohol
Supplier: Not applicable
Step type: Wash Inc. (min): 0:00 Temperature: Ambient Dispense type: 150 µL

Step Reagent
7 *Bond Wash Solution
Supplier: Leica Microsystems
Step type: Wash Inc. (min): 0:00 Temperature: Ambient Dispense type: 150 µL

Step Reagent
8 *Bond Wash Solution
Supplier: Leica Microsystems
Step type: Wash Inc. (min): 0:00 Temperature: Ambient Dispense type: 150 µL

Step Reagent
9 *Bond Wash Solution
Supplier: Leica Microsystems
Step type: Wash Inc. (min): 0:00 Temperature: Ambient Dispense type: 150 µL

Pre-treatment Antigen Retrieval: *HIER 20 minutes with ER2 (or 10 minutes with HI-1 panels)

Step Reagent
1 *Bond ER Solution 2
Supplier: Leica Microsystems
Step type: Reagent Inc. (min): 0:00 Temperature: Ambient Dispense type: 150 µL

Step Reagent
2 *Bond ER Solution 2
Supplier: Leica Microsystems
Step type: Reagent Inc. (min): 0:00 Temperature: Ambient Dispense type: 150 µL

Step Reagent
3 *Bond ER Solution 2
Supplier: Leica Microsystems
Step type: Reagent Inc. (min): 20:00 Temperature: 100 Dispense type: Intermediate

Step Reagent
4 *Bond ER Solution 2
Supplier: Leica Microsystems
Step type: Reagent Inc. (min): 0:00 Temperature: Ambient Dispense type: 150 µL

Step Reagent
5 *Bond Wash Solution
Supplier: Leica Microsystems
Step type: Wash Inc. (min): 0:00 Temperature: Ambient Dispense type: 150 µL

Step Reagent
6 *Bond Wash Solution
Supplier: Leica Microsystems
Step type: Wash Inc. (min): 0:00 Temperature: Ambient Dispense type: 150 µL

Step Reagent
7 *Bond Wash Solution

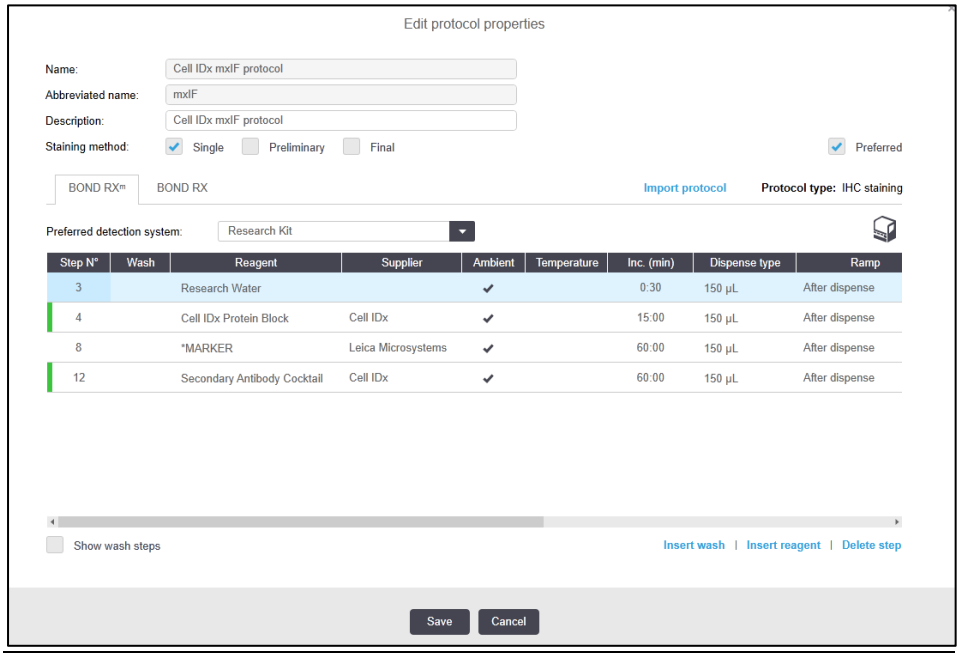
Supplier: Leica Microsystems
Step type: Wash Inc. (min): 0:00 Temperature: Ambient Dispense type: 150 µL

Step Reagent
8 *Bond Wash Solution
Supplier: Leica Microsystems
Step type: Wash Inc. (min): 0:00 Temperature: Ambient Dispense type: 150 µL

Step Reagent
9 *Bond Wash Solution
Supplier: Leica Microsystems
Step type: Wash Inc. (min): 3:00 Temperature: Ambient Dispense type: 150 µL

Custom Staining Protocol:

Use a Research Detection Kit as the Preferred Detection System and use water or buffer that you assigned to the kit as the one reagent used from that kit ("Research Water" in example below).



Step Reagent
1. Research Kit Water or Buffer
Supplier: Not applicable
Step type: Reagent Inc. (min): 0:00 Temperature: Ambient Dispense type: 150 µL
Step Reagent
2. *Bond Wash Solution
Supplier: Leica Microsystems
Step type: Wash Inc. (min): 0:10 Temperature: Ambient Dispense type: 150 µL
Step Reagent
3. *Bond Wash Solution
Supplier: Leica Microsystems
Step type: Wash Inc. (min): 0:10 Temperature: Ambient Dispense type: 150 µL
Step Reagent
4. Cell IDx Protein Block Solution
Supplier: Cell IDx
Step type: Reagent Inc. (min): 20:00 Temperature: Ambient Dispense type: 150 µL

Step Reagent
5. Primary Antibody Cocktail
Supplier: Cell IDx
Step type: Reagent Inc. (min): 60:00 Temperature: Ambient Dispense type: 150 µL
Step Reagent
6. *Bond Wash Solution
Supplier: Leica Microsystems
Step type: Wash Inc. (min): 3:00 Temperature: Ambient Dispense type: 150 µL
Step Reagent
7. *Bond Wash Solution
Supplier: Leica Microsystems
Step type: Wash Inc. (min): 3:00 Temperature: Ambient Dispense type: 150 µL
Step Reagent
8. *Bond Wash Solution
Supplier: Leica Microsystems
Step type: Wash Inc. (min): 3:00 Temperature: Ambient Dispense type: 150 µL
Step Reagent
9. Secondary Antibody Cocktail
Supplier: Cell IDx
Step type: Reagent Inc. (min): 60:00 Temperature: Ambient Dispense type: 150 µL
Step Reagent
10. *Bond Wash Solution
Supplier: Leica Microsystems
Step type: Wash Inc. (min): 3:00 Temperature: Ambient Dispense type: 150 µL
Step Reagent
11. *Bond Wash Solution
Supplier: Leica Microsystems
Step type: Wash Inc. (min): 0:00 Temperature: Ambient Dispense type: Open
Step Reagent
12. *Bond Wash Solution
Supplier: Leica Microsystems
Step type: Wash Inc. (min): 3:00 Temperature: Ambient Dispense type: 150 µL
Step Reagent
13. *Bond Wash Solution
Supplier: Leica Microsystems
Step type: Wash Inc. (min): 0:00 Temperature: Ambient Dispense type: 150 µL
Step Reagent
14. *Bond Wash Solution
Supplier: Leica Microsystems
Step type: Wash Inc. (min): 0:00 Temperature: Ambient Dispense type: 150 µL
Step Reagent
15. *Bond Wash Solution
Supplier: Leica Microsystems
Step type: Wash Inc. (min): 0:30 Temperature: Ambient Dispense type: 150 µL
Step Reagent
16. *Bond Wash Solution
Supplier: Leica Microsystems
Step type: Wash Inc. (min): 0:30 Temperature: Ambient Dispense type: 150 µL
Step Reagent
17. *Bond Wash Solution
Supplier: Leica Microsystems
Step type: Wash Inc. (min): 0:30 Temperature: Ambient Dispense type: 150 µL

Mounting:

:

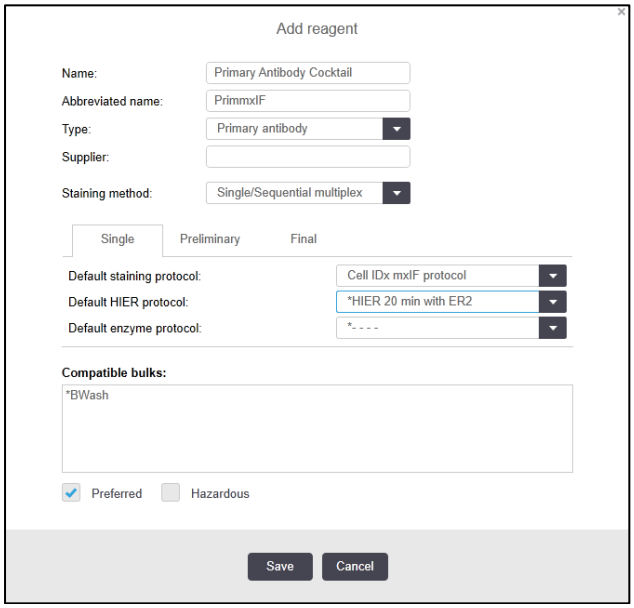
1) Apply 1-3 drops of mounting medium, suggest using Fluroshield with DAPI (ImmunoBioSciences, Inc, Cat # AR-6501-01) to each slide and then apply coverslip after incubating 3-5 minutes in the dark at room temperature. **DO NOT USE** Vector VectaShield Mounting Reagent – cat # H-1500
2) Allow slides to dry.
3) Image slides. Optimal exposure times and gain settings should be determined by the user.

Register Reagents and Assign to BOND Container:

For the first time they are used, the primary antibody cocktail, Cell IDx Protein Block, and Secondary Antibody Cocktail need to be added to the database. Alternatively, you can use reagent names you already have containers assigned to and make the custom IF protocol with those reagent names accordingly.

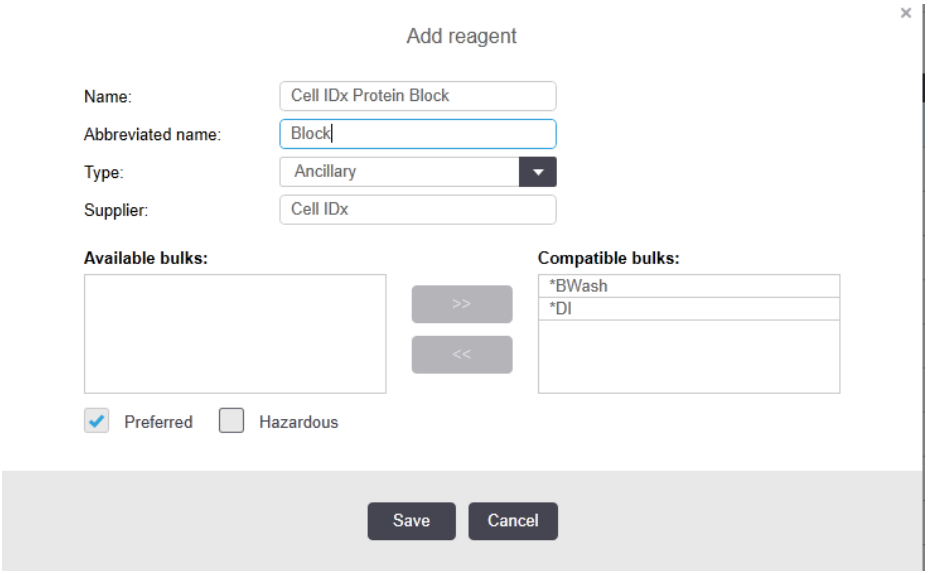
For Primary Antibody Cocktail:

- Go to Reagent setup screen and click on Add.
- When the Add reagent window opens, give the reagent a unique name and abbreviated name. For example, Primary antibody Cocktail as the name and PrimmxIF as the abbreviated name.
- Assign type as Primary Antibody
- Assign Staining method as Single/Sequential multiplex
- Assign Default Staining Protocol as the Cell IDx mxIF protocol you end up creating
- Assign Default HIER protocol as *HIER 20 min with ER2
- Make sure to checkmark Preferred
- Click on Save.



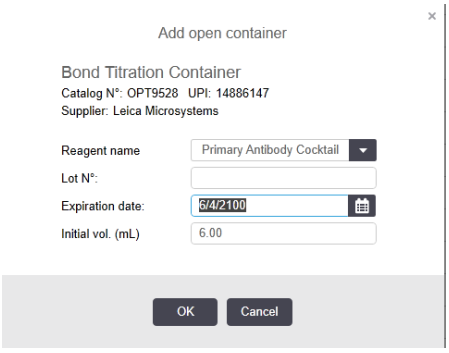
For Protein Block and Secondary Antibody Cocktail:

- Go to Reagent setup screen and click on Add.
- When the Add reagent window opens, give the reagent a unique name and abbreviated name.
- Assign type as Ancillary
- Make sure to checkmark Preferred
- Click on Save.



To assign to container:

- Select one of the reagents from the drop-down menu
- If required, type in a Lot No.
- Select an Expiration Date (note: the instrument will not allow you to use container once expiration date has passed)
- Click on OK
- Ensure the Titration Container is clearly labelled
- Repeat for each of the reagents.
- Add the BOND Containers to a reagent tray



Preparation of Reagents

Protein Block Solution is Ready to Use.

Dilute antibodies in appropriate amount of Cell IDx antibody diluent. Suggested dilution is 1/100 dilution of concentrate. For example, to make 200 µl of primary antibody solution add 2 ul of each primary x 4 antibodies = 8 µl total primary Abs + 192 µl Antibody Diluent. If individual stains are required, dilute 2 µl of primary antibody with 198 µl Antibody Diluent. For example, to make 200 µl of secondary antibody solution add 2 µl of each secondary x 4 antibodies = 8 µl total primary Abs + 192 µl Antibody Diluent. If individual stains are required, dilute 2 µl of secondary antibody with 198 µl Antibody Diluent. Make sure to prepare enough antibody solution to account for the dead volume on the BOND RX.

Troubleshooting

Issue	Possible Cause(s)	Solution
No antigen signal	Tissue is negative for antigen	Include known positive control tissue in experimental design
	Imaging settings are not optimal	Adjust settings using positive control tissue
	Antibody did not bind	Always use freshly diluted antibody cocktails
High background	Blocking incomplete	Always use freshly prepared blocking buffer and IgG-free BSA
	Tissue autofluorescence	Autofluorescence is caused by formaldehyde used for fixation of FFPE tissue and is a common artifact in FFPE based experiments. If autofluorescent background is a significant concern, please contact Cell IDx.
Tissue damaged	Antigen retrieval pH < 6.0	Check pH of antigen retrieval solution
	Antigen retrieval time > 30 min	Incubate in antigen retrieval buffer no longer than 30 min total
	Tissue poorly affixed to slide	Use positively charged glass slides (e.g. SuperFrost Plus)
	Tissue damaged by handling	Gently wash and rinse slides. If using rotator, use low speed
	Tissue damaged by handling	Do not allow slides to come in contact with each other
		Use caution applying coverslip and do not adjust during drying.

Disclaimer

For Research Use Only. Not for Diagnostic or Therapeutic use.

Version 2.0 Revised: 03.28.22