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## **UltraPlex mxIF Multiplex Immunofluorescence Staining Protocol S kits**

“S” kits are single stains labeled with 490, 550, 594 and 650 nm fluors. Please ensure that your imaging scanner or fluorescent microscope can detect these wavelengths.

“S” kits are single stains commonly used to set up a spectral library for use with a “B” kit composed of an equivalent multiplex panel. Depending on your imaging scanner or fluorescent microscope you may want to use an “A” Kit labeled with 490, 550, 650 and 750 nm fluors instead of a “B” Kit. Please contact us if you have any questions about which kits to select.

“S” kits are optimized for use on FFPE tissue sections.

### **Materials Provided**

1. Vial 1: Antibody Diluent Solution
2. Vial 2: Primary Antibody Solution (20X concentrate)
3. Vial 3: Fluorescent Secondary Antibody Solution (20X concentrate)

### **Materials Not Provided**

Antigen Retrieval Buffer: Citrate Buffer or EDTA Buffer (see notes)  
Wash Buffer: PBS/0.2% Tween 20, pH 7.2 (see notes)  
Blocking Solution: PBS/3% normal rabbit serum/0.1% TritonX (see notes)  
Fluoroshield plus DAPI (Immunobiosciences, Inc, Cat # AR-6501-01)

### **Protocol**

- 1) Deparaffinize slides and perform antigen retrieval (download from the web site either Antigen Retrieval – Citrate Buffer or Antigen Retrieval – EDTA Buffer protocols as specified in the appropriate kit PDS).
- 2) Wash slides with wash buffer for 5 min.
- 3) Dry slides around tissue sample and draw a ring with hydrophobic pen.
- 4) Block section with blocking solution for 20 minutes.
- 5) Prepare diluted primary antibodies by diluting concentrate 20X with supplied antibody diluent. Aspirate blocking solution from the slide and then add 150 to 200ul per section and incubate for 1 hour at room temperature in a humidified chamber.
- 6) Wash slide with wash buffer, 3 changes for 5 min each.
- 7) Prepare diluted secondary antibodies by diluting concentrate 20X with supplied antibody diluent. Add 150 to 200ul per section and incubate for 1 hour at room temperature in a humidified chamber.
- 8) Wash slide with wash buffer, 3 changes for 5 min each.
- 9) Rinse slides with distilled water for 2 min.
- 10) Apply 1-3 drops of Fluoroshield 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.



- 11) Allow slides to dry.
- 12) Image slides. Optimal exposure times and gain settings should be determined by the user. For further imaging time considerations, see "Notes" section.

## Notes

1. Citrate antigen retrieval buffer (10mM citrate, 0.05% Tween 20, pH 6.0) may be prepared from solid trisodium citrate dihydrate and stored as a concentrated 10X solution (100mM citrate, 0.5% Tween 20) in dH<sub>2</sub>O, pH 6.0.  
Dissolve 29.4g of trisodium citrate dihydrate in 950ml distilled water then bring pH to 6.0 using 1N NaOH. Add 5ml Tween 20 and mix well before bringing final volume to 1000ml with distilled water. This solution can be stored for 3 months at room temperature or longer if stored at 4°C. From the concentrated 10X solution, 1X working solution can then be prepared as needed by further dilution of the 10X stock in dH<sub>2</sub>O. Buffer pH = 6.0 should be confirmed prior to each use, as pH levels may change during storage. Download the Antigen Retrieval - Citrate Buffer protocol from the web site for antigen retrieval instructions.
2. EDTA antigen retrieval buffer (1mM EDTA, 0.05% Tween 20, pH 8.0) may be made from solid EDTA disodium salt dihydrate and stored as a concentrated 10X solution (10mM EDTA, 0.5% Tween 20) in dH<sub>2</sub>O, pH 8.0.  
Dissolve 3.7g of disodium EDTA dihydrate in 950ml distilled water then bring pH to 8.0 using 1N NaOH. Add 5ml Tween 20 and mix well before bringing final volume to 1000ml with distilled water. This solution can be stored for 3 months at room temperature or longer if stored at 4°C. From the concentrated 10X solution, 1X working solution can then be prepared as needed by further dilution of the 10X stock in dH<sub>2</sub>O. Buffer pH = 8.0 should be confirmed prior to each use, as pH levels may change during storage. Download the Antigen Retrieval - EDTA Buffer protocol from the web site for antigen retrieval instructions.
3. Antigen retrieval methods have been successfully validated for this kit using pressure cookers and steamers. Microwave antigen retrieval has not been tested. If a pressure cooker or steamer is not available, a 100°C boiling water bath may be used, but results may vary. Do not immerse pyrex container in boiling water bath.
4. Wash Buffer: PBS/0.2% Tween 20 pH 7.2. Wash buffer should be freshly prepared and stored for no longer than a week to avoid contamination.
5. Blocking Buffer: PBS/3% normal rabbit serum/0.1% TritonX. Blocking buffer should be freshly prepared.
6. Mounting Medium: we recommend using Fluoroshield plus DAPI (Immunobiosciences, Inc, Cat # AR-6501-01) as mounting medium. DO NOT USE Vector VectraShield Mounting Reagent – cat # H-1500



## Troubleshooting

Issue	Possible Cause(s)	Solution
<b>No antigen signal</b>	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
<b>High background</b>	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.
<b>Tissue damaged</b>	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**

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