# *UltraPlex mxIF*
# Multiplex Immunofluorescence Staining Protocol - Manual Staining

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 (see notes for composition)
2. Vial 2: Protein Block Solution (see notes for composition)
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)

## *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)
Suggested mounting medium, Fluoroshield plus DAPI (ImmunoBioSciences, Inc, Cat # AR-6501-01)

## ***MANUAL 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 antibody cocktail by diluting each individual primary conjugate 100X (suggested) with supplied Antibody Diluent. For a 200 ul cocktail, you would add 2 ul of each primary x 4 antibodies = 8 ul total primary Abs + 192 ul Antibody Diluent. If individual stains are required, dilute 2ul of primary antibody with 198ul Antibody Diluent. Aspirate blocking solution from the slide and then add 150 to 200 ul 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 antibody cocktail by diluting each individual fluor-labeled secondary anti-hapten antibody 100X (suggested) with supplied Antibody Diluent. For a 200 ul cocktail, you would add 2 ul of each secondary x 4 secondaries = 8 ul total secondary Abs + 192 ul Antibody Diluent. If individual stains are required, dilute 2ul of secondary antibody with 198ul 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 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 VectraShield Mounting Reagent – cat # H-1500
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 $dH_2O$, 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 $dH_2O$. 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 $dH_2O$, 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 $dH_2O$. 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. Antibody Diluent Solution: PBS, 1% BSA, 0.2% Tween 20, 15 mM sodium azide.

5. 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.

6. Blocking Buffer: PBS, 3% normal rabbit serum, 0.1% TritonX, 15 mM sodium azide.

7. 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 |
|---|---|---|
| **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. |
| | | |

## ***SUGGESTED BOND RX PROTOCOL***

*See suggested Bond Protocol.*

### *Disclaimer*

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