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UltraPlex mxIF
Multiplex Immunofluorescence Staining Protocol
A kits

For use with ***UltraPlex mxIF*** "A" Kits. "A" kits 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. "B" kits are commonly used with spectral imaging scanners together with an "S" kit composed of single stain controls used to set up a spectral library. Please contact us if you have any questions about which kits to select.

"A" kits 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. Vial 3: Primary Cocktail in Antibody Diluent (25X concentrate)
4. Vial 4: Secondary Conjugate Cocktail in Antibody Diluent (25X 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)
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 antibody cocktail by diluting primary cocktail 25X with supplied antibody diluent. For a 200 uL working cocktail, you would add 8 uL of primary cocktail + 192 uL 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 antibody cocktail by diluting fluor-labeled secondary anti-tag antibodies 25X with supplied antibody diluent. For a 200 uL working cocktail, you would add 8 uL of secondary cocktail + 192 uL 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.
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.

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