Multiplex Staining Protocol — Fluorescent
with UltraTag™ Kit Labelled Antibodies

This protocol allows for the detection of 1-4 UltraTag kit labelled primary antibodies on a single tissue with one antigen retrieval step and a simple two-step antibody incubation protocol.

Recommended User-Supplied Material/Equipment

Description
Antibody Diluent Buffer *(recipe provided below)*
Citrate Antigen Retrieval Buffer, pH 6 *(see recommended formulation in recipe section)*
Cover Glass 24 x 50mm
Distilled Water
Heat-resistant plastic "Coplin" style slide jars
Hydrophobic "PAP" slide pen
Pressure Cooker that can reach up to 120°C
Rabbit Blocking Solution *(recipe provided below)*
Reagent-grade Alcohol, 95% and 100%
Slide staining tray with water reservoir for humidification and light-blocking lid
Wash buffer *(recipe provided below)*
Xylene ACS grade, ≥ 98%.
Mounting Medium, suggested: Fluoroshield plus DAPI (ImmunoBioSciences, Inc., cat# AR- 6501-01). DO NOT USE Vector VectraShield Mounting Reagent (cat# H-1500) as mounting medium.

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 per your routine IHC processes.

Pre-Staining Preparation

Recommended concentration for staining with UltraTag labelled primary antibodies is 5–10 µg/mL, however, optimization may be necessary and performing a titration may suggest a different optimal concentration. You can prepare a primary antibody cocktail containing a mixture of different primary antibodies tagged with different UltraTags if a cocktail for multiplex is desired.

Recommended concentration for staining with fluor-labeled anti-Tag detector antibody is 5 µg/mL. You can prepare a secondary antibody cocktail containing a mixture of different anti-Tag secondary antibodies.

Recipes for Buffers

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.

   10X Solution: 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 dH_2O. 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. Confirm pH of 6.0 before each HIER.
2. **Wash Buffer**: A suitable 1X wash buffer can be prepared as 0.2% Tween 20 in PBS pH 6.8–7.2 [137 mM sodium chloride (NaCl), 2.68 mM potassium chloride (KCl), 10 mM sodium phosphate dibasic (Na_2HPO_4), 1.763 mM potassium phosphate monobasic (KH_2PO_4), 0.2% Tween 20]. A 10X wash buffer [1.37 M sodium chloride (NaCl), 26.8 mM potassium chloride (KCl), 100 mM sodium phosphate dibasic (Na_2HPO_4), 17.63 mM potassium phosphate monobasic (KH_2PO_4), 2% Tween 20 pH 6.8–7.2] should be diluted to 1X using dH_2O prior to use.
3. **Rabbit Blocking Solution**: 3% normal rabbit serum 0.1% Triton X-100 with 15mM sodium azide in PBS.
4. **Antibody Diluent**: PBS, 1% BSA, 0.2% Tween 20, 15 mM sodium azide.

Manual Staining Protocol

Before conducting slide staining, visually verify that the slide is free from fingerprints, dust, and excess wax and that the tissue section is not substantially fragmented.

1. **Dewax** slides in the following order by dunking up and down a few times in each specified solution before incubating for the listed times:
   a. Xylene 5 min
   b. Xylene 5 min
   c. 100% Ethanol 2 min
   d. 100% Ethanol 2 min
   e. 95% Ethanol 2 min
2. Fill a clean glass slide staining container with tap H_2O. Immerse slides for 2 X 2 minutes, using fresh tap H_2O for the second immersion step.
3. Fill a clean glass slide staining container with distilled H_2O and immerse slides for 2 minutes.
4. Fill a clean glass slide container with desired HIER buffer (Citrate, EDTA, or other commercial buffer). Fill an additional clean glass slide container with distilled H_2O and place next to HIER buffer container inside the pressure cooker. Steam for 15 minutes. Power off the apparatus and cool slides within for 10 minutes. After 10-minute cool down, release pressure from apparatus, if using a pressure cooker. Using heat-resistant gloves, move slides to the hot distilled H_2O for 2 minutes.
5. **Wash** slides under cool running tap water for 5 minutes.
6. Place slides in wash buffer for 5 minutes.
7. Remove slides from wash buffer. Remove excess slide moisture by blotting the edge of glass with absorbant lens paper (*e.g.*, "Kimwipes") or lint-free paper towels. Use caution to avoid contact with tissues.
8. Transfer slides to elevated supports within humified staining tray and encircle or draw lines around the tissue with the hydrophobic pen.
9. Pipet 150 µL of **Rabbit Blocking Solution**, or enough volume to cover tissue, per section. Cover humidified tray. Block slides 20 minutes.
10. Prepare the **1° antibody staining solution**. Into antibody diluent buffer, pipet correct amount of each 1° antibody concentrate to achieve specified dilution. Mix by vortex or pipetting.

11. Remove **Rabbit Blocking Solution** by tilting each slide to a 90° angle on a paper towel. Place slides flat on elevated supports in humidity tray.
12. Pipet enough volume to cover tissue, typically 150 µL, of the **diluted 1° antibody staining solution** onto each section. Place lid on tray. Incubate for 60 minutes, or specified time, at ambient temperature.
13. Fill a clean glass slide staining container with 200 mL of **wash buffer**. Load slides into container insert, slowly immerse in container, and incubate for 3 minutes. Repeat 2 more times, adding fresh wash buffer each time.
14. Prepare the **2° antibody staining solution**. Into antibody diluent buffer, pipet correct amount of each **2° antibody concentrate** to achieve specified dilution. Mix by vortex or pipetting.
15. After Step 14 is concluded, tip off excess wash buffer onto paper towels. Place slides flat in staining tray.
16. Pipet enough volume to cover tissue, typically 150 µL, of the **diluted 2° antibody staining solutio**n onto each section. Place lid on tray. Incubate for 60 minutes, or specified time, at ambient temperature.
17. **Wash** slides as described in Step 14.
18. Add 200 mL of dH_2O to a clean glass slide staining container. Incubate slides 2 minutes. Align slides in staining tray and apply 2 drops of Fluoroshield™ plus DAPI to each slide, ensuring tissue is covered by media. Incubate for 3–5 minutes in the staining tray with the light-blocking lid in place.
    DO NOT USE Vector VectraShield Mounting Reagent (cat# H-1500).
19. Apply cover glass to each slide, avoiding the introduction of air bubbles to the specimen under the glass.
20. Allow mounting media to harden by letting slides sit for at least 1 hour at room temperature before imaging. Use light-blocking tray lid or foil to protect from ambient light. Additionally, clear nail polish may also be applied to the borders of the cover glass for immobilization.
21. When slides are dry, load into slide box for transport to imaging and long-term storage.

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 minutes	Incubate in antigen retrieval buffer no longer than 30 minutes 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; do not adjust during drying.

Precautions

- **For Research Use Only**. Not for diagnostic or therapeutic use.
- Consult Federal, State, and local regulations for disposal of any potentially toxic components.

Safety Information

- WARNING – CHEMICAL HAZARD. Some chemicals used can be potentially hazardous, and can cause injury or illness.
- Read and understand the Material Safety Data Sheets (MSDS) available at www.cellidx.com before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with and inhalation of chemicals. Wear appropriate personal protective equipment when handling chemicals (*e.g.*, safety glasses, gloves, or clothing). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's clean-up procedures as recommended in the MSDS.
- Comply with all local, state/provincial, and/or national laws and regulations related to chemical storage, handling, and disposal.

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