# Multiplex Staining Protocol — Chromogenic
## with UltraTag™ Kit Labelled Antibodies

This protocol allows for the detection of three UltraTag Kit labeled primary antibodies on a single tissue with one antigen retrieval step, using different chromogen colors to distinguish each biomarker.

The primary antibody cocktail containing all three primary antibodies each conjugated to a different UltraTag will be added to the slide. This will be followed by the addition of enzyme-labeled anti-Tag antibodies provided in the UltraTag kits; first, a secondary antibody solution containing a cocktail of anti-Tag-AP1 and anti-Tag-HRP1. An AP Chromogen substrate and first HRP Chromogen substrate will then be added to develop the anti-Tag-AP1 and anti-Tag-HRP1 (chromogen order to be determined by user). An HRP-arrest step will then arrest the anti-Tag-HRP1, allowing for the addition of the 2nd secondary antibody solution containing anti-Tag-HRP2, which will then be developed with the second HRP Chromogen. A suitable counterstain should be used last, followed by mounting medium that is compatible with chromogens, since many chromogens are soluble in xylene and alcohol and therefore require aqueous mounting medium.

**Note**: We recommend using CPAR-050 Cell Palette AP Red Chromogen, CPHB-050 Cell Palette HRP Blue Chromogen, and CPHY-050 Cell Palette HRP Yellow Chromogen available from Cell IDx. A combination kit (cat# CPK-015) of all three chromogens is available. For this chromogen combination, we have determined the optimal order to be Red, Blue, then Yellow. We recommend the use of a purple hematoxylin counterstain (cat# HP-050) followed by aqueous mounting medium or Cell IDx ChromoSealant (cat# CS-015) to seal stain followed by resin-based mounting medium.

## Recommended User-Supplied Material/Equipment

| Description |
|---|
| Cover Glass 24 x 50mm |
| 1 AP and 2 HRP Chromogen substrates |
| Tris-based Antibody Diluent *(recipe provided below)* |
| Rabbit Blocking Solution *(recipe provided below)* |
| HRP-Arrest Solution *(recipe provided below)* |
| Hydrophobic "PAP" slide pen |
| Slide staining tray with water reservoir for humidification and light-blocking lid |
| Heat-resistant plastic "Coplin" style slide jars |
| TBS-T wash buffer *(recipe provided below)* |
| Distilled Water |
| Citrate Antigen Retrieval Buffer, pH 6 *(see recommended formulation in recipe section)* |
| Tris-based Wash Buffer *(see recommended formulation in recipe section)* |
| Xylene ACS grade, ≥ 98%. |
| Reagent-grade Alcohol, 95% and 100% |
| Chromogen-compatible mounting medium |
| Pressure Cooker that can reach up to 120°C |
| Hematoxylin or other appropriate counterstain |

## 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 labeled primary antibodies is 5–10 µg/mL, however, performing a titration may suggest a different optimal concentration. You can combine all UltraTag labeled primary antibodies in a cocktail for multiplex, if desired.

Recommended concentration for staining with enzyme-labeled anti-Tag detector antibody is 5 µg/mL (200X dilution from stock). It is recommended to have two secondary antibody steps, with the first step containing a mixture of anti-Tag-AP1 and anti-Tag-HRP1 and the second step containing anti-Tag-HRP2. Do not combine two anti-Tag-HRPs together.

### 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 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 $dH_2O$. Confirm pH of 6.0 before each HIER.
2. **Tris-Based Wash Buffer**: 1X TBS-Tween: 0.05 M Tris HCl, 150mM NaCl pH 8.0 with 0.1% Tween 20
3. **Rabbit Block**: 3% Rabbit Serum in TBS-Tween 20
4. **Antibody Diluent**: 1% BSA in TBS-Tween 20
5. **HRP-Arrest Solution**: 100mM sodium azide, 0.3% $H_2O_2$ in MES buffer pH 5.8. The $H_2O_2$ should be added fresh.

## Manual Staining Protocol

This protocol assumes use of a combination of CPAR-050 Cell Palette AP Red Chromogen, CPHB-050 Cell Palette HRP Blue Chromogen, and CPHY-050 Cell Palette HRP Yellow Chromogen available from Cell IDx. A combination kit (cat# CPK-015) of all three chromogens is available.

1. **Dewax** slides as typically performed in your lab, or as follows:
   a. Cell IDx Manual De-waxing

| | | |
|---|---|---|
| i. | Xylene | 5 min |
| ii. | Xylene | 5 min |
| iii. | 100% Reagent Alcohol | 2 min |
| iv. | 100% Reagent Alcohol | 2 min |
| v. | 95% Reagent Alcohol | 2 min |

2. **Wash** slides with distilled and/or tap water
3. Perform **antigen retrieval** by placing slides in a staining container and steaming in a pressure cooker on high pressure (approximately 120°C) with 200mL of 10mM citrate buffer, 0.05% Tween 20, pH 6 for 15 minutes.
   a. Fill a clean glass slide container with citrate buffer.
   b. Fill an additional clean glass slide container with distilled water and place next to citrate buffer container inside the pressure cooker.
   c. Steam for 15 minutes.
   d. Power off the apparatus and cool slides within for 10 minutes.
   e. After 10-minute cool down, release pressure from apparatus, if using a pressure cooker.
   f. Using heat-resistant gloves, move slides to the hot distilled water for 2 minutes.
   g. To cool, place slides under cool running tap water for 5 minutes.
4. After antigen retrieval, **block sections** with **3% $H_2O_2$** for 10 minutes
5. **Wash** 3 times briefly with tris-based wash buffer *(see buffer formulation, below)*
6. **Block section** with **Rabbit Block** *(see formulation above)* for 15 minutes.
7. **Remove Rabbit Block** and add 150 µL of primary antibody cocktail (containing all three UltraTag labeled primary antibodies in antibody diluent) per slide and incubate for 30 minutes at room temperature.
8. **Wash** slide 3 times with tris-based wash buffer for 3 minutes each.
9. Add 150–200 µL of first secondary antibody cocktail, (usually containing anti-Tag-AP1 and the first anti-Tag-HRP1) per section and incubate for 30 minutes at room temperature in a humidified chamber.

10. **Wash** slide 3 times with tris-based wash buffer for 3 minutes each.
11. Prepare **Red Chromogen (AP) Solution** (1 to 50 ratio) by adding 40 µL of **Red Chromogen** to 1,960 µL **Red Chromogen Buffer**. **This solution is only stable for 20 minutes.**
12. Add 150 µL of **Red Chromogen (AP) Solution (freshly mixed 1 to 50 ratio)** to each slide and incubate for 4 minutes.
13. **Wash** 1–3 times with tris-based buffer briefly to wash off chromogen solution
14. Prepare **Blue Chromogen (HRP) Solution** (1 to 50 ratio) by adding 40 µL of **Blue Chromogen** to 1,960 µL **Blue Chromogen Buffer**. This solution is stable for up to 24 hours.
15. Add 150 µL of **Blue Chromogen (HRP) Solution** (1 to 50 ratio) to each slide and incubate for 2.5 minutes.
16. **Wash** 1–3 times with tris-based buffer briefly to wash off chromogen solution.
17. Add 150 µL of **HRP Arrest Solution** (see buffer formulation above) to each slide and incubate for 5 minutes at room temperature. Do not leave on tissue for more than 15 minutes.
18. **Wash** slides 3 times with tris-based wash buffer for 3 minutes each.
19. Add 150 µL of second secondary antibody cocktail (containing the last anti-Tag-HRP2) per slide and incubate for 30 minutes at room temperature.
20. **Wash** slides with tris-based wash buffer for 3 changes for 3 minutes each.
21. Prepare **Yellow Chromogen (HRP) Solution** (1 to 50 ratio) by adding 40 µL of **Yellow Chromogen** to 1,960 µL **Yellow Chromogen Buffer**. This solution is stable for up to 24 hours.
22. Add 150 µL of **Yellow Chromogen (HRP) Solution (1 to 50 ratio)** to each slide and incubate for 2.5 minutes.
23. **Wash** 1–3 times with tris-based buffer briefly to wash off chromogen solution.
24. **Wash** briefly with distilled water.
25. Add 150 µL of **Purple Hematoxylin** or **Light Blue Hematoxylin** per slide, incubating for 30 seconds to 1 minute. Counterstain should be light. It is recommended that the customer optimize this prior to multiplex staining.
26. **Wash** with distilled water.
27. Mount with aqueous mount and coverslip or **Cell IDx ChromoSealant** (cat# CS-015) to seal stain followed by resin-based mounting medium and coverslip. Do **not** use xylene or alcohols.

## 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.

## Disclaimer

The products offered here are for research use only. Any commercial application will require a license from Cell IDx. Cell IDx UltraPlex technology is patented and has multiple patents pending. Please contact Cell IDx for information regarding licensing information. Information in this manual is subject to change without notice and does not constitute a commitment on the part of Cell IDx. It is supplied on an "as is" basis without any warranty of any kind, either explicit or implied. This documentation is proprietary information and protected by the copyright laws of the United States and international treaties. The manufacturer of this documentation is Cell IDx.