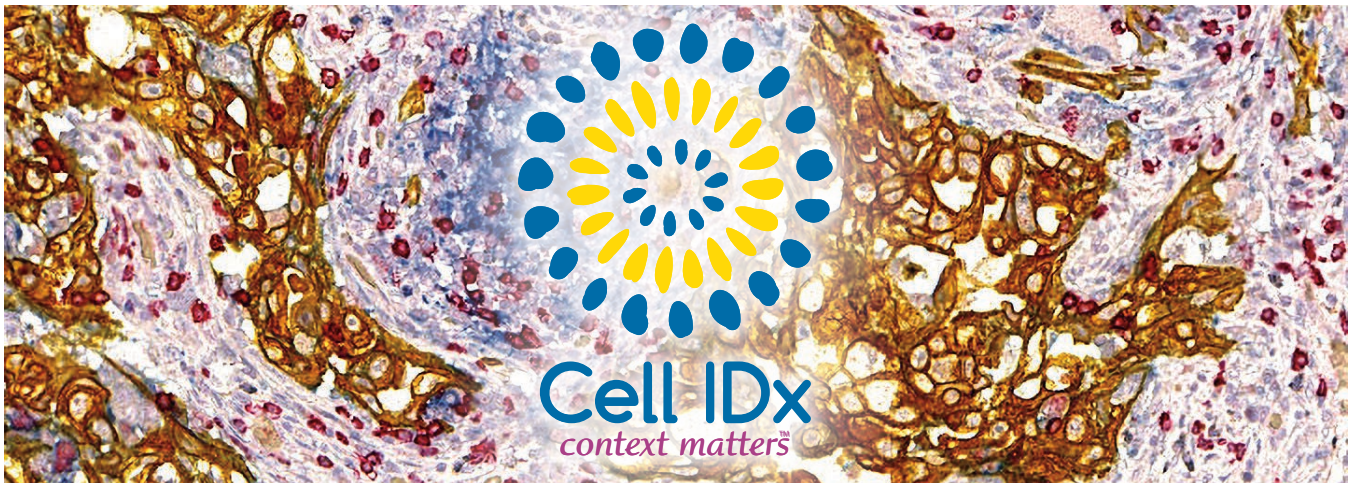

UltraPlex™ Chromogenic Multiplex IHC CD4, CD8, PanCK Protocol CP02M Kit for Manual Staining

Cat #: **CP02M-010** | Protocol Version 2022.01.26A

Store Entire Kit 2-8°C

Do NOT use Sodium Azide or a phosphate-based buffer in this protocol

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Contents

Intended Use

This UltraPlex Chromogenic Multiplex IHC kit allows for the detection of CD4, CD8, and PanCK on a single tissue with one antigen retrieval step, using different chromogen colors to distinguish each biomarker.

How the Protocol Works

The primary antibody cocktail of CD4, CD8, and PanCK antibodies, each labeled with a different Tag, will be added to the slide. This will be followed by the addition of secondary antibody solution #1 (Cell IDx Polymer 1, containing anti-UT015-AP and anti-UT014-HRP). Red Alkaline Phosphatase (AP) Chromogen will be added to develop the anti-UT015-AP followed by Blue Chromogen addition to develop anti-UT014-HRP. An HRP-arrest step will then arrest the UT014-HRP, allowing for the addition of the secondary antibody solution #2 (Cell IDx Polymer 2, containing anti UT016-HRP), which will then be developed with a Yellow Chromogen. Both Purple and Blue Hematoxylin options are provided to generate a light purple or light blue counterstain at the end of the protocol. We recommend that the user determine their preferred color of hematoxylin and the optimal concentration (neat or diluted) and incubation time for hematoxylin staining in order to provide optimal results for their specific use prior to multiplex staining.

Precautions

- **For Research Use Only**. Not for diagnostic or therapeutic use.
- Consult Federal, State, and local regulations for disposal of any potentially toxic components
- Chromogen order and combinations have been selected to provide optimal staining, and Cell IDx is developing additional panels and chromogen combinations. Consult with Cell IDx before substituting or adding any markers or chromogens.
- Mount with aqueous mounting medium or or Cell IDx ChromoSealant (cat# CS-015) to seal stain followed by resin-based mounting medium. Do **not** use xylene or alcohols.

Staining Protocol

UltraPlex Chromogenic Multiplex Reagents for CP02M-010

Cell IDx Cat#	Description	Amount Provided
SL-038/010	Peroxidase Block (Ready to Use)	2 mL
SL-049/010	Concentrated primary antibody cocktail (CD4-UT014, CD8-UT015, PanCK-UT016)	49 µL
SL-050/010	Concentrated Cell IDx Polymer #1 (anti-UT015-AP, anti-UT014 HRP)	19 µL
SL-051/010	Concentrated Cell IDx Polymer #2 (anti-UT016 HRP)	9.5 µL
SL-027/010	Cell IDx Rabbit Block (Ready to Use)	2 mL
SL-028/010	Antibody Diluent	5 mL
SL-029/010	Cell IDx HRP arrest solution part A (Azide in MES)	2.0 mL
SL-030/010	Cell IDx HRP arrest solution part B (30% H_2O_2)	20 µL
SL-035/010	Yellow HRP Chromogen	0.05 mL
SL-036/010	Yellow HRP Chromogen Buffer	2 mL
SL-031/010	Blue HRP Chromogen	0.05 mL
SL-032/010	Blue HRP Chromogen Buffer	2 mL
SL-033/010	Red AP Chromogen	0.05 mL
SL-034/010	Red AP Chromogen Buffer	2 mL
SL-037/010	Purple Hematoxylin	2 mL
SL-047/010	Blue Hematoxylin	2 mL

Recommended User-Supplied Material/Equipment for Manual Staining

Description
Cover Glass 24 x 50mm
Distilled Water
Oven that can reach up to 60°C
Citrate Antigen Retrieval Buffer, pH 6 (see recommended formulation in recipe section)
Tris-based Wash Buffer (see recommended formulation in recipe section)
Hydrophobic "PAP" slide pen
Heat-resistant plastic "Coplin"-style slide jars
Xylene ACS grade, ≥ 98%.
Reagent-grade Alcohol, 95% and 100%
Aqueous mounting medium or Cell IDx ChromoSealant (cat# CS-015) to seal stain followed by resin-based mounting medium
Pressure Cooker that can reach up to 120°C

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 as per your routine IHC processes.

Preparation of Components Prior to Staining

Reagents provided are sufficient for 10 test slides and 1 slide of secondary antibody alone (no primary) control, assuming 150 µL per slide.

Working Primary Antibody Cocktail (1 to ~33 dilution)

Add 45 µL of **Concentrated Primary Antibody Cocktail** (**SL-049/010**) to 1,455 µL of **Antibody Diluent** (**SL-028/010**). This solution should be made fresh for each use.

Cell IDx Polymer 1 Solution (anti-UT014 HRP/anti-UT015 AP) (1 to 100 dilution)

Add 17 µL of concentrated ***Cell IDx Polymer 1** (**SL-050/010**) to 1,683 µL of **Antibody Diluent** (**SL-028/010**). This solution should be made fresh for each use.

Cell IDx Polymer 2 Solution (anti-UT016 HRP) (1 to 200 dilution)

Add 8.5 µL of concentrated ***Cell IDx Polymer 2** (**SL-051/010**) to 1,691.5 µL of **Antibody Diluent** (**SL-028/010**). This solution should be made fresh for each use.

Cell IDx HRP-Arrest Solution (1 to 100 dilution)

Add 17 µL of ***Cell IDx HRP-Arrest Solution Part B** (**SL-030/010**) to 1,977 µL of **HRP Arrest Solution Part A** (**SL-029/010**). This solution should be made fresh for each use.

Manual Staining Protocol

1. Prior to starting experiment, **set an oven to 60°C** for use in Step 18.
2. **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
3. **Wash** slides with distilled and/or tap water
4. Perform **antigen retrieval** by placing slides in a staining container and steaming in a pressure cooker on high pressure (approximately 120°C) with 200 mL 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.

5. After antigen retrieval, block sections with **Peroxidase Block** (**SL-038/010**) for 10 minutes
6. **Wash** 3 times briefly with tris-based wash buffer (see buffer formulation, below)
7. Block section with **Cell IDx Rabbit Block** (**SL-026/010**) for 15 minutes.
8. **Remove Cell IDx Rabbit Block** and add 150 µL of working primary antibody cocktail (containing parts **SL-049/010** in **SL-028/010**) per slide and incubate for 30 minutes at room temperature.
9. **Wash** slide 3 times with tris-based wash buffer for 3 minutes each.
10. Add 150 µL of working **Cell IDx Polymer 1 Solution** (containing parts **SL-050/010** + **SL-028/010**) per slide and incubate for 30 minutes at room temperature in a humidified chamber.
11. **Wash** slide 3 times with tris-based wash buffer for 3 minutes each.
12. Prepare **Red Chromogen (AP) Solution (1 to 50 ratio)** by adding 40 µL of **Red Chromogen** (**SL-033/010**) to 1,960 µL **Red Chromogen Buffer** (**SL-034/010**). **This solution is only stable for 20 minutes.**
13. Add 150 µL of **Red Chromogen (AP) Solution (freshly mixed 1 to 50 ratio)** to each slide and incubate for 4 minutes.
14. **Wash** 1–3 times with tris-based buffer briefly to wash off chromogen solution.
15. Prepare **Blue Chromogen (HRP) Solution (1 to 50 ratio)** by adding 40 µL of **Blue Chromogen** (**SL-031/010**) to 1,960 µL **Blue Chromogen Buffer** (**SL-032/010**). This solution is stable for up to 24 hours.
16. Add 150 µL of **Blue Chromogen (HRP) Solution (1 to 50 ratio)** to each slide and incubate for 2.5 minutes.
17. **Wash** 1–3 times with tris-based buffer briefly to wash off chromogen solution.
18. Add 150 µL of previously prepared **Cell IDx HRP-Arrest Solution** (containing parts **SL-030/010** + **SL-029/010**) to each slide and incubate for **5 minutes at 60°C**.
19. **Wash** slides 3 times with tris-based wash buffer for 3 minutes each.
20. Add 150 µL of **Cell IDx Polymer 2 Solution** (containing parts **SL-051/010** + **SL-028/010**) per slide and incubate for 30 minutes at room temperature.
21. **Wash** slides with tris-based wash buffer for 3 changes for 3 minutes each.
22. Prepare **Yellow Chromogen (HRP) Solution (1 to 50 ratio)** by adding 40 µL of **Yellow Chromogen** (**SL-035/010**) to 1,960 µL **Yellow Chromogen Buffer** (**SL-036/010**). This solution is stable for up to 24 hours.
23. Add 150 µL of **Yellow Chromogen (HRP) Solution (1 to 50 ratio)** to each slide and incubate for 2.5 minutes.
24. **Wash** 1–3 times with tris-based buffer briefly to wash off chromogen solution.
25. **Wash** briefly with distilled water.
26. Add 150 µL of **Purple Hematoxylin** (**SL-037/010**) or **Blue Hematoxylin** (**SL-047/010**) per slide, incubating for 30 seconds to 1 minute. We recommend that the user determine their preferred color of hematoxylin and the optimal concentration (neat or diluted) and incubation time for hematoxylin staining in order to provide optimal results for their specific use prior to multiplex staining.
27. **Wash** with distilled water.
28. Mount with aqueous mount and coverslip or **Cell IDx ChromoSealant** (**cat# CS-015**) to seal stain followed by resin-based mounting medium. Do **not** use xylene or alcohols.

Recipes

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 950 mL distilled water then bring pH to 6.0 using 1N NaOH. Add 5 mL Tween 20 and mix well before bringing final volume to 1000 mL 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, 150 mM NaCl pH 8.0 with 0.1% Tween 20
3. **Cell IDx Rabbit Block**: 3% Rabbit Serum in TBS-Tween 20
4. **Cell IDx Antibody Diluent**: 1% BSA in TBS-Tween 20
5. **Cell IDx HRP Arrest Solution**: 100mM sodium azide, 0.3% H_2O_2 in MES buffer pH 5.8

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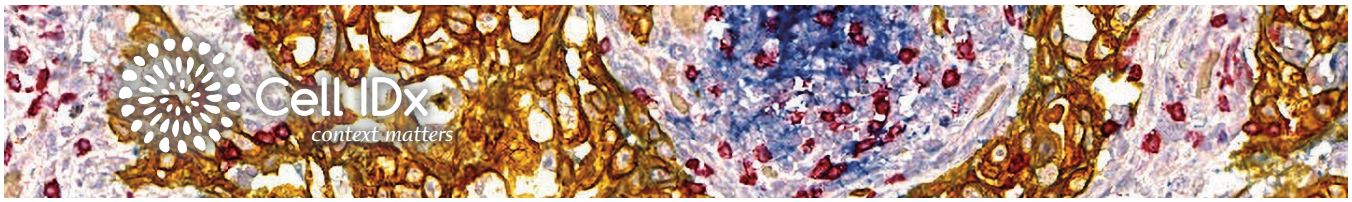
Safety Information

- 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.



Our Vision

Cell IDx is a technology leader in multiplexed tissue profiling, developing highly sensitive and specific chromogenic and fluorescent multiplex immunohistochemistry reagents to meet the needs of precision medicine.

Our UltraPlex platform barcoding technology has enabled the generation of UltraPlex fluorescent and chromogenic multiplex immunohistochemistry panels, providing simultaneous detection of multiple markers in tissue sections and allowing analysis of sub-populations of cells *in situ* in the context of tissue morphology. Multiplex staining is achieved in virtually the same time it takes to perform a single marker stain, enabling truly rapid tissue phenotyping on a large scale.

Our vision is the widespread application of this technology to address both the present and future needs of the research and clinical markets in oncology, immuno-oncology and other disease states. We offer an ever-expanding range of multiplex staining panels as well as rapid development of custom panels, tissue staining, imaging, and analysis services.

We invite you to learn more about our multiplex biomarker technology and see how it can further your tissue profiling and biomarker discovery research. Please contact us to discuss potential collaborations, services, or licensing opportunities.

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context matters®

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