Store at -20°C

#78701

PD-L1, FoxP3, CD8α Multiplex IHC Antibody Panel

1 Kit (3 x 20 µl)

Support: +1-978-867-2388 (U.S.)
www.cellsignal.com/support

Orders: 877-616-2355 (U.S.)
orders@cellsignal.com

rev. 10/19/16

For Research Use Only. Not For Use In Diagnostic Procedures.

Products Included	Item #	Quantity	Isotype
PD-L1 (E1L3N®) XP® Rabbit mAb	77563	20 µl	Rabbit IgG
CD8α (C8/144B) Mouse mAb (IHC Specific)	38553	20 µl	Mouse IgG1
FoxP3 (D2W8E™) Rabbit mAb (IHC Specific)	62072	20 µl	Rabbit IgG

See www.cellsignal.com for individual component applications, species cross-reactivity, dilutions and additional application protocols.

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA, 50% glycerol and less than 0.02% sodium azide. Store at –20°C. *Do not aliquot the antibodies.*

Unmasking buffer: SignalStain® EDTA Unmasking Solution (10X) #14747

Description: The PD-L1, FoxP3, CD8α Multiplex IHC Antibody Panel enables researchers to simultaneously detect these targets in paraffin-embedded tissues using tyramide signal amplification. Each antibody in the panel has been validated for this approach. For recommended staining conditions optimized specifically for this antibody panel please refer to Table 1.

Background: The field of cancer immunotherapy is focused on empowering the immune system to fight cancer. This approach has seen recent success in the clinic with targeting immune checkpoint control proteins, such as PD-1 (1,2). Despite this success, clinical biomarkers that predict response to therapeutic strategies involving PD-1 receptor blockade are still under investigation (3-5). While PD-L1 expression has been linked with an increased likelihood of response to anti-PD-1 therapy, research studies have shown that additional factors, such as tumor-immune infiltration and the ratio of effector to regulatory T cells within the tumor, could play a significant role in predicting treatment outcome (6-9).

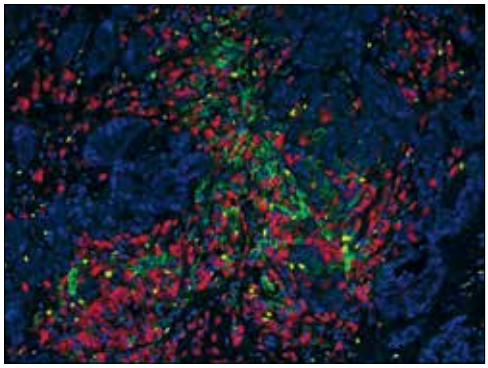
Programmed cell death 1 ligand 1 (PD-L1) is a member of the B7 family of cell surface ligands that regulate T cell activation and immune responses. The PD-L1 ligand binds the PD-1 transmembrane receptor and inhibits T cell activation. PD-L1 is expressed in several tumor types, including melanoma, ovary, colon, lung, breast, and renal cell carcinomas (10-12).

FoxP3 is a transcription factor that is crucial for the development of T cells with regulatory properties (Treg) (13). Mutations in FoxP3 are associated with immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome (IPEX) (15), while overexpression in mice causes severe immunodeficiency (15). Research studies have shown that FoxP3 functions as a tumor suppressor in several types of cancer (16-18).

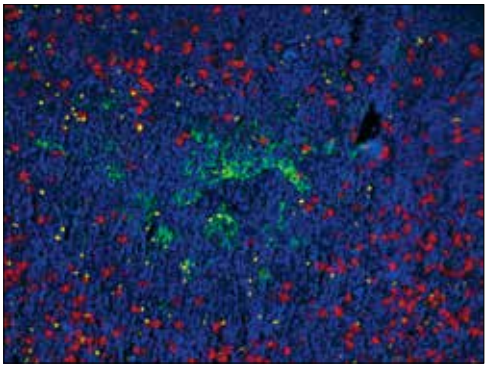
CD8 (Cluster of Differentiation 8) is a disulphide-linked heterodimer consisting of α and β subunits. On T cells, CD8 is the coreceptor for the TCR, and these two distinct structures recognize the Antigen–Major Histocompatibility Complex (MHC). CD8 ensures specificity of the TCR–antigen interaction, prolongs the contact between the T cell and the antigen presenting cell, and the α chain recruits the tyrosine kinase Lck, which is essential for T cell activation (19).

Item #	Target	Dilution	Staining Order	Fluorophore Pairing	Pseudocolor
38553	CD8	1:250	1st	Cy5®	Red
62072	FoxP3	1:250	2nd	Cy3®	Yellow
77563	PD-L1	1:250	3rd	FITC	Green

Table 1: Recommended staining conditions to generate the composite fluorescent immunohistochemistry images shown below using PD-L1, FoxP3, CD8α Multiplex IHC Antibody Panel.



Fluorescent multiplex immunohistochemical analysis of paraffin-embedded human breast cancer using PD-L1 (E1L3N®) XP® Rabbit mAb (green), FoxP3 (D2W8E™) Rabbit mAb (IHC Specific) (yellow), and CD8α (C8/144B) Mouse mAb (IHC Specific) (Red). Blue pseudocolor = DAPI #8961 (fluorescent DNA dye). Image acquisition was performed with a multispectral camera.



Fluorescent multiplex immunohistochemical analysis of paraffin-embedded human tonsil using PD-L1 (E1L3N®) XP® Rabbit mAb (green), FoxP3 (D2W8E™) Rabbit mAb (IHC Specific) (yellow), and CD8α (C8/144B) Mouse mAb (IHC Specific) (Red). Blue pseudocolor = DAPI #8961 (fluorescent DNA dye). Image acquisition was performed with a multispectral camera.

Specificity/Sensitivity: Each antibody in this panel recognizes endogenous levels of its specific target protein.

Source/Purification: Monoclonal antibodies are produced by immunizing animals with a synthetic peptide corresponding to residues near the carboxy terminus of human PD-L1 protein, recombinant protein specific to human FoxP3, or a synthetic peptide corresponding to residues near the carboxy terminus of human CD8α protein.

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Applications: **W**—Western **IP**—Immunoprecipitation **IHC**—Immunohistochemistry **ChIP**—Chromatin Immunoprecipitation **IF**—Immunofluorescence **F**—Flow cytometry **E-P**—ELISA-Peptide **Species Cross-Reactivity:** **H**—human **M**—mouse **R**—rat **Hm**—hamster **Mk**—monkey **Mi**—mink **C**—chicken **Dm**—D. melanogaster **X**—Xenopus **Z**—zebrafish **B**—bovine **Dg**—dog **Pg**—pig **Sc**—S. cerevisiae **Ce**—C. elegans **Hr**—Horse **All**—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.

Background References:

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#78701

Protocol for Fluorescent Multiplex Immunohistochemistry (mIHC) with Tyramide Signal Amplification

A Solutions, Reagents, and Kits

1. Xylene
2. Ethanol, anhydrous denatured, histological grade (100% and 95%)
3. Deionized water (dH_2O)
4. Antigen unmasking solutions:
   - **a.** Citrate: 10 mM Sodium Citrate, pH 6.0
     - **i.** To prepare 500 ml 1X Citrate Unmasking Solution: add 50 ml SignalStain® Citrate Unmasking Solution (10X) #14746 to 450 ml dH_2O.
   - **b.** EDTA: 1mM EDTA, pH 8.0
     - **i.** To prepare 500 ml 1X EDTA Unmasking Solution: add 50 ml SignalStain® EDTA Unmasking Solution (10X) #14747 to 450 ml dH_2O.
5. 3% Hydrogen Peroxide
   - **a.** To prepare 500 ml 3% H_2O_2: add 50 ml of 30% H_2O_2 to 450 ml of dH_2O.
6. SignalStain® Antibody Diluent #8112.
7. Wash Buffer: 1X Tris Buffered Saline with Tween® 20 (TBST)
   - **a**. To prepare 1L 1X TBST: add 100 ml 10X TBST #9997 to 900 ml dH_2O.
8. SignalStain® Boost IHC Detection Reagent (HRP rabbit, #8114; HRP mouse, #8125).
9. TSA® Plus Fluorescein kit (PerkinElmer, NEL741001KT).
10. TSA® Plus Cyanine 5 kit (PerkinElmer, NEL745001KT).
11. TSA® Plus Cyanine 3 kit (PerkinElmer, NEL744001KT).
12. Stripping solution: 10 mM Sodium Citrate, pH 6.0
    - **a.** To prepare 500 ml 10 mM Citrate solution: add 50 ml SignalStain® Citrate Unmasking Solution (10X) #14746 to 450 ml dH_2O.
13. ProLong® Gold Antifade Reagent with DAPI #8961.

B Protocol overview

Fluorescent mIHC involving tyramide signal amplification (TSA®) is a methodology that enables simultaneous detection of multiple proteins of interest in a given tissue section in a stepwise fashion. It is based on detection via indirect immunofluorescence involving primary and secondary antibodies to facilitate signal amplification. In the protocol described below, an HRP-conjugated secondary antibody binds to an unconjugated primary antibody specific to the target/antigen of interest. Detection is ultimately achieved with a fluorophore-conjugated tyramide molecule that serves as the substrate for HRP. Activated tyramide forms covalent bonds with tyrosine residues on or neighboring the protein of interest and is permanently deposited upon the site of the antigen. This allows for serial stripping of the primary/secondary antibody pairs, while preserving the antigen-associated fluorescence signal, making this process amenable to multiple rounds of staining in a sequential fashion. Importantly, one of the key advantages of this method is that multiple primary antibodies of the same species can be used without the concern for crosstalk. This greatly simplifies and enables the process of a multiplex panel design.

C Important tips

There are a number of considerations that can impact the success of a fluorescent multiplex IHC experiment involving tyramide.

Concentration of primary antibody: An optimal dilution of each primary antibody within a multiplex panel must be determined empirically and often can differ dramatically from the dilution recommended by the manufacturer due to the amplification of fluorescence signal afforded by tyramide deposition. We highly recommend optimizing the individual components of the multiplex panel by performing titrations for each component using a fluorophore of moderate intensity.

Order optimization: It is critical to optimize the order in which the antibodies in a multiplex panel are applied to the tissue section to ensure that multiple rounds of heating do not compromise target-specific epitopes. We recommend testing each optimized primary antibody within each slot of the multiplex panel using a fluorophore of moderate intensity to ensure that the fluorescence signal is not affected by the relative position within the panel.

Antibody-fluorophore pairing: Generally, it is good practice to pair antibodies detecting the lowest expressing targets with the brightest fluorophores. We recommend testing a matrix comprised of optimized primary antibodies and each available fluorophore in order to acheive the best possible signal intensity and signal to noise ratio for each target of a panel.

D Protocol

1. **Slide Baking (Optional)**
   - **a.** Incubate slides positioned horizontally for 1.5 hr at 60°C.
   - **b.** Re-position the slides from a horizontal to an upright position and incubate for an additional 30 min at 60°C.

   NOTE: This step allows for the paraffin wax to melt.
2. **Deparaffinization/Hydration**
   - **a.** Incubate sections in three washes of Xylene for 5 min each.
   - **b.** Incubate sections in two washes of 100% ethanol for 10 min each.
   - **c.** Incubate sections in two washes of 95% ethanol for 10 min each.
   - **d.** Wash sections two times in dH_2O for 5 min each.

   NOTE: All washes are to be done with gentle agitation at room temperature.
3. **Antigen Unmasking**

   NOTE: Consult product data sheet for a recommendation on the optimal unmasking solution to use for each primary antibody in a multiplex panel. If the multiplex panel includes one antibody that is recommended for use with EDTA retrieval, use EDTA as the unmasking solution.

   For Citrate:
   - **a.** Using a microwave, bring slides to a boil in 10 mM sodium citrate buffer, pH 6.0.
   - **b.** Maintain at a sub-boiling temperature for 10 min.
   - **c.** Cool slides to room temperature on bench top for 30 min.

   For EDTA:
   - **a.** Using a microwave, bring slides to a boil in 1 mM EDTA, pH 8.0.
   - **b.** Maintain at a sub-boiling temperature for 15 min. No cooling is necessary.
4. **Quenching**
   - **a.** Wash sections in dH_2O three times for 5 min each.
   - **b.** Incubate sections in 3 % hydrogen peroxide for 10 min.
   - **c.** Wash sections in dH_2O two times for 5 min each.
   - **d.** Wash sections in 1X TBST for 5 min.
5. **Staining/Detection**

   NOTE: A separate pre-blocking of tissue sections may be performed but is not necessary. Optimal dilutions of the primary antibody must be determined empirically.
   - **a.** Dilute primary antibody in SignalStain® Antibody Diluent #8112.
   - **b.** Add 100-400 µl to each section and incubate in a humidified chamber at room temperature for 60 min.
   - **c.** During incubation with the primary antibody, equilibrate SignalStain® Boost Detection Reagent (HRP rabbit, #8114 or HRP mouse, #8125) to room temperature.
   - **d.** Remove antibody solution and wash sections with 1X TBST two times for 3 min each.
   - **e.** Cover tissue sections in several drops (100-400 µl) of SignalStain® Boost IHC detection Reagent (HRP rabbit, #8114 or HRP mouse, #8125) specific to the species of the primary antibody.
   - **f.** Incubate in a humidified chamber at room temperature for 30 min, protected from light.
   - **g.** Wash slides with 1X TBST two times for 3 min per wash, with gentle agitation and protected from light.

6. **Tyramide Signal Amplification (TSA®)**
   NOTE: When choosing the appropriate fluorophore-conjugated TSA® Plus amplification reagent, it is important to consider target expression levels and fluorophore intensity. Optimal pairing of primary antibody and fluorophore should be established in advance (see Important tips above).
   a. Dilute fluorophore-conjugated TSA® Plus amplification reagent as per manufacturer's recommendation.
   b. Apply 100-400 µl per slide and incubate for 10 min at room temperature in a humidified chamber, protected from light.
   c. Wash slides with 1X TBST two times for 3 min per wash, with gentle agitation and protected from light.
7. **Serial staining**
   a. If performing multiplex staining whereby more than one target of interest is to be detected:
      i. Proceed to stripping **(Step 8)**.
   b. If you have completed your multiplex panel staining OR if you are performing a singleplex assay whereby a single target of interest is detected:
      i. Proceed to mounting **(Step 9)**.
8. **Stripping**
   a. Using a microwave, bring slides to a boil in 10 mM sodium citrate buffer, pH 6.0.
   b. Maintain at a sub-boiling temperature for 10 min.
   c. Cool slides to room temperature on bench top for 30 min.
   Proceed with **Staining/Detection (Step 5)** using a different tyramide-fluorophore conjugate.
9. **Mounting**
   Mount sections with coverslips using ProLong® Gold Antifade Reagent with DAPI #8961.
   NOTE: If slides are being used for the purpose of constructing a spectral library, ProLong® Gold Antifade Reagent #9071 should be used.