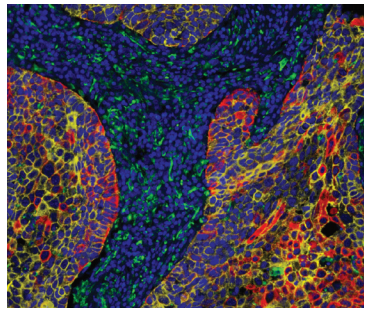
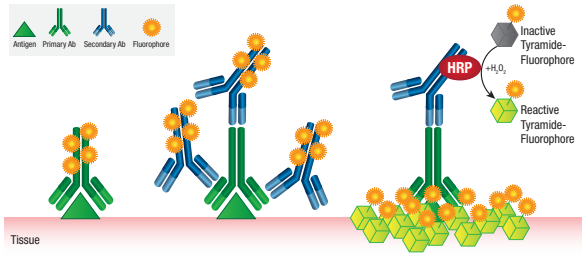
Fluorescent Multiplex Immunohistochemistry



Benefits of multiplexing:

- Maximal information garnered per tissue section - critical when tissue samples are limited
- Enables analysis of spatial organization and co-expression of multiple targets in the context of preseved tissue architecture

Multiplexing options:



Antigen Detection	Direct immunofluorescence with primary antibody conjugated to a fluorophore	Indirect immunofluorescence with secondary antibody conjugated to a fluorophore	Indirect immunofluorescence involving primary/ secondary antibody pairs and HRP- catalyzed deposition of fluorophore-conjugated tyramide at the site of the antigen
Protocol	Parallel staining	Parallel staining	Serial staining
Primary Antibody	Same host species can be used for multiple targets	Different host species or isotype for each target	Same host species can be used for multiple targets
Seconday Antibody	No	Yes	Yes
Signal Amplification	None	Moderate	High

Multiplexing with tyramide:

Signal amplification:

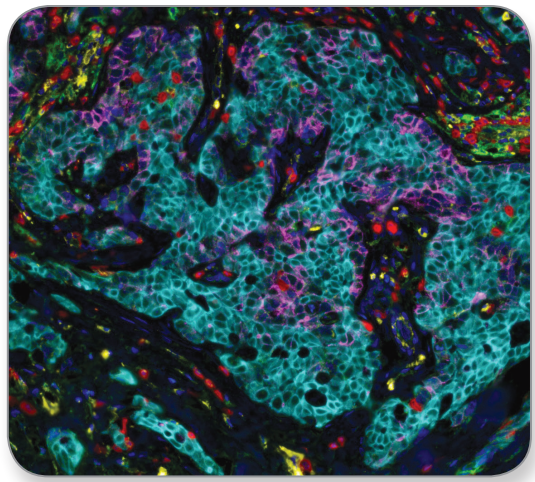
- Provides marked enhancement of antigen-associated fluorescence signal
- Enables detection of low abundance targets

Simplified panel design:

- Compatible with **any** IHC-Paraffin (IHC-P) validated antibody regardless of the host species or isotype

Multiplex detection:

- As many as six or more targets can be detected within the same tissue sample, contingent upon analysis with a multi-spectral imaging platform



5-Plex fluorescent mIHC analysis of paraffin-embedded human invasive ductal carcinoma of the breast using:

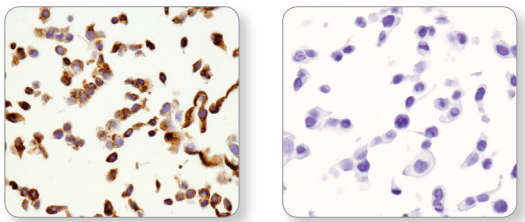
PD-L1 (E1L3N®) XP® Rabbit mAb #13684 (green)
VISTA (D1L2G™) XP® Rabbit mAb #64953 (yellow)
CD8α (C8/144B) Mouse mAb (IHC Specific) #70306 (red)
B7-H4 (D1M8I) XP® Rabbit mAb #14572 (magenta)
Pan-Keratin (C11) Mouse mAb #4545 (cyan)
Blue pseudocolor = DAPI #8961 (fluorescent DNA dye).

Validated antibodies: the key to successful multiplexing

Well-validated antibodies that exhibit exceptional specificity to the target of interest are critical to the success and reliability of multiplex IHC analysis

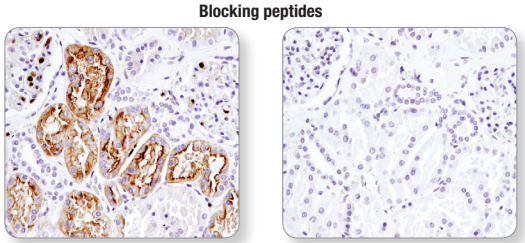
- Cell Signaling Technology® (CST) offers >700 primary antibodies stringently validated for IHC on formalin-fixed paraffin-embedded (FFPE) tissue sections
- CST criteria for IHC validation include:
  - Western blot analysis: to demonstrate specific bands of the appropriate molecular weight, with minimal cross-reacting bands
  - Positive and negative controls: cell pellets known to express or be devoid of the target of interest, respectively
  - Blocking peptides: to verify antigen specificity and rule out Fc-receptor mediated binding as well as other non-specific staining artifacts
  - Cancer tissue arrays: to survey Ab performance over a broad spectrum of tissue samples
  - Validation example:
    VISTA (D1L2G™) XP® Rabbit mAb #64953

Control cell pellets



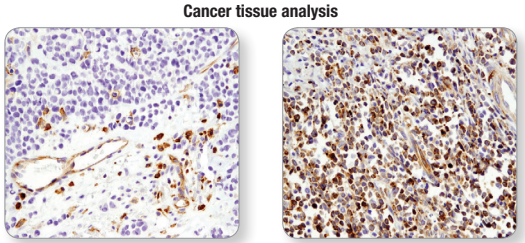
IHC anlaysis of paraffin-embedded control cell pellets using #64953 reveals appropriate staining in VISTA-expressing cells (OVKATE, left) and lack of staining in cells devoid of VISTA (MCF7 cells, right).

Blocking peptides



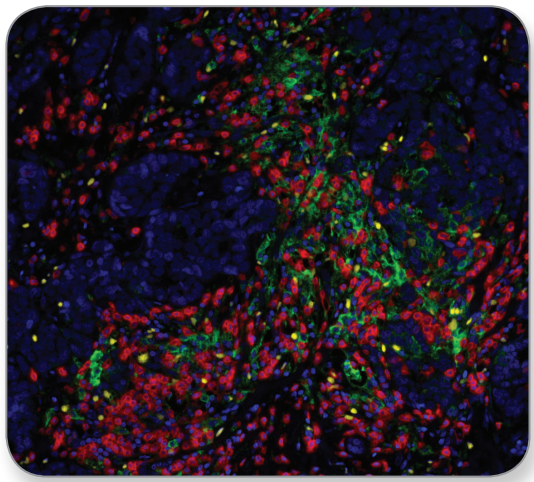
IHC analysis of paraffin-embedded human kidney in the presence of control peptide (left) or antigen-specific peptide (right) #64953.

Cancer tissue analysis



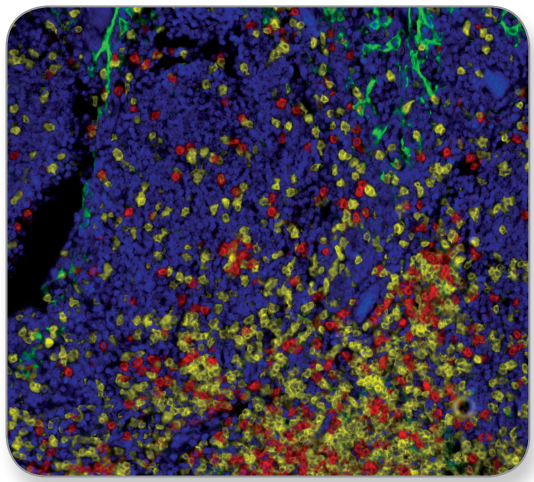
IHC analysis of paraffin-embedded human breast carcinoma (left) and non-Hodgkin's B-Cell Lymphoma (right) using #64953.

- **All CST monoclonal antibodies recommended for IHC-P are compatible with fluorescent multiplex IHC (mIHC)**
- CST offers Multiplex IHC Antibody Panels:
  - Thoughtfully pre-selected sets of monoclonal antibodies
  - Validated for fluorescent multiplex IHC
  - Relevant to the field of tumor immunology
  - Include detailed and highly optimized protocols



PD-L1, FoxP3, CD8α Multiplex IHC Panel #78701: Fluorescent mIHC analysis of paraffin-embedded human breast cancer using PD-L1 (E1L3N®) XP® Rabbit mAb #13684 (green), FoxP3 (D2W8E™) Rabbit mAb (IHC Specific) #98377 (yellow), and CD8α (C8/144B) Mouse mAb (IHC Specific) #70306 (Red). Blue pseudocolor = DAPI #8961 (fluorescent DNA dye).

Enables assessment of the ratio of effector to regulatory T cells in the tumor



PD-L1, CD3ε, CD8α Multiplex IHC Panel #65713: Fluorescent mIHC analysis of paraffin-embedded human tonsil using PD-L1 (E1L3N®) XP® Rabbit mAb #13684 (green), CD3ε (D7A6E™) Rabbit mAb #85061 (yellow), and CD8α (C8/144b) Mouse mAb (IHC Specific) #70306 (Red). Blue pseudocolor = DAPI #8961 (fluorescent DNA dye).

Enables assessment of tumor infiltrating lymphocytes (TILs).

For more information about our expansive portfolio of IHC-P antibodies, CST validation principles, and multiplex IHC please visit:
www.cellsignal.com/mIHC

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